MEDWIN PUBLISHERS
Committed to create value for Researchers

International Journal of Lipid Research & Advances

Study of the Distribution of the Apolipoproteins A-I and A-II, Among Lipoprotein Subclasses in Plasma of Normo- and Hyperlipidemic Subjects

M Abu Odeh[1,2]*, Nima Ebadat[2] and Atmeh RF†[3]

[1]Department of Medical Laboratory Sciences, Jordan University of Science and Technology, Jordan

[2]Division of Metabolism and Endocrinology, Department of Medicine, University of California, USA

[3]Department of Chemical Sciences, USA

Research Article

Volume 1 Issue 1

Received Date: February 14, 2019

Published Date: March 11, 2019

***Corresponding author:** Mohammad Abu Odeh, Department of Medical Laboratory Sciences, Jordan University of Science and Technology, Irbid, Jordan, Tel: 858 366 2273; Email: aomohammad@ucsd.edu

Abstract

Depending on epidemiological study, high density lipoprotein Cholesterol (HDL-C) was considered as a negative risk factor for Coronary artery disease (CAD). However, this inverse relationship is not proved in individual subjects, where there are individuals with high HDL-C but suffer from CAD, and others with low HDL-C but do not have CAD. Moreover, this inverse relationship is now questionable. HDL is a heterogeneous complex of particles that differ in the size, chemical composition, and function, where some of them may have a role in the protection from atherosclerosis; therefore, deeper understanding of the identification and quantitation of its subclasses is badly needed. Here we use an improved immunoblotting method that can directly detect and qauntitate discrete Lipoprotein subclass containing apoA-I (AI-Lp) and Lp subclass containing apoA-II (AII-Lp) subclasses from fresh plasma of normo- and hyperlipidemic subjects. Fresh plasma sample from 167 of normo- and hyperlipidemic males and females were analyzed. At least thirteen AI-Lp subclasses were detected with relative molecular mass ranging from 42,000 to >354,000, these subclasses were given the numbers 1- 13. Seventeen AII-Lp subclasses were detected with relative molecular mass from 50,000 to 900,000; these subclasses were given the roman numbers I-XVII. The smallest subclasses (42,000– 50,000) were not detected in all normoglycemic-normolipidemic subjects. A Large significant increase (P<0.05) in subclass 4 (70,000) was noticed in hypercholesterolimic subjects. AII-Lp subclass IV (105,000-150,000) in hyperlipidemic subjects were significantly correlated with plasma total apo-AI (r=0.32, P<0.05) and total apoA-II (r = 0.72, P<0.01), and a similar correlation was also seen in the normoglycemic group (r = 0.34, P<0.01; r = 0.71, P<0.01, respectively). This may indicate that the

function of this main AII-Lp subclass is impaired in different pathologies, and this is more significant than its plasma level. The immunoblotting method used is the method of choice, at present, to study the quantitative distribution of AI-Lp and AII-Lp subclasses in fresh plasma because it's superior to the other method available in terms of the quantitative aspects and its performance.

Keywords: Apolipoproteins; Normoglycemic-Normolipidemic; Immunoblotting; Apolipoprotein

Abbreviations: Pl: Phospholipids; TAGs: Triacylglycerols; FC: Free Cholesterol; EC: Esterified Cholesterol; CVD: Cardiovascular Diseases; HDL-C: High-Density Lipoprotein Cholesterol; CAD: Coronary Artery Disease; CETP: Cholesterol Ester Transfer Protein; FDA: Food and Drug Administration.

Introduction

Lipoproteins are lipid protein complexes composed of lipids [phospholipids, (PL), triacylglycerols, (TAGs), free cholesterol, (FC), and esterfid cholesterol, (EC)] and proteins known as apolipoproteins (such as apoAI, A-II, B, C-I, C-II, C-III, and apoE). The interest in measuring the lipoproteins level in plasma was resulted from epidemiological studies where high plasma level of low-density lipoprotein cholesterol (LDL-C) was considered as a positive risk factor in developing atherosclerosis and cardiovascular diseases (CVD), whereas high plasma level of high-density lipoprotein cholesterol (HDL-C) was considered as a negative risk factor. Therefore, it was assumed that the plasma level of HDL-C is inversely related to the incidence of Coronary Artery Disease (CAD) [1]. However, this inverse relationship is not proved in individual subjects, where there are individuals with high HDL-C but suffer from CAD and others with low HDL-C but do not have CAD [2,3]. Moreover, Van Acker, et al. reported that HDL-C does not protect against CAD when associated with combined cholesterol ester transfer protein (CETP) and hepatic lipase gene variants [4]. Extending the inverse relationship in population led to the assumption that raising HDL-C by drugs is beneficial in subjects with high risk and that will lower the risk and give them adequate protection from cardiovascular events [5].

This dogma received a dramatic blow up when the food and drug administration (FDA) stopped a large clinical trial for raising HDL-C by the drug Torcetrapib [6]. In that trial, the HDL-C level in a group of high risk was raised 61% with 20% decrease in LDL-C, but there was no significant decrease in the progression of coronary atherosclerosis, rather, there was an increase in systolic blood pressure and progression of atherosclerosis in the common segment of the carotid artery associated with excess of death, myocardial infarctions, angina and heart failure [7,8]. At present, the significance of measuring HDL-C is questionable, where it has limited utility in assessing an individual's risk of CAD [9]. Moreover, the term HDL is an operational one which indicates a lipoprotein subclass that is separated from plasma by ultracentrifugation in the density range of 1.063-1.210 g/mL. Therefore, it has no direct physiological relevance. On the other hand, HDL is heterogeneous and comprises up to12 different subclasses [10-12]. Since the apolipoprotein A-I (apoA-I) is the major protein of HDL, and it is the main active component of HDL in term of stabilizing the particles structure and interaction with peripheral cells, it will be more convenient to consider the measurement of aopA-I containing lipoproteins (AI-Lp) [11] which are more physiologically relevant [13].

In order for the cholesterol acceptor to remove excess cholesterol from the cells, it has to be of small size, has high negative charge, and high diffusion coefficient and highly hydrated. The discrete particles identified by Atmeh group verify these criteria, and are candidates as the initiators of the **Promoting Reverse Cholesterol Transport** (RCT) process [12]. To enable the measurement of the plasma concentration of these LpAI subclasses, Atmeh and Robenek adapted the method of immunoblotting against agarose gel containing anti-apoA-I, which was previously developed by Atmeh and Abu Harfeil [14]. At present, there is intense search for new biomarkers that better assess the risk of CAD. As part of that search, we here present a contribution that offers candidate biomarkers for CAD. We will adopt a direct method for the detection and quantitation of the distribution of apolipoproteins in various lipoprotein subclasses within the HDL range [11]. Therefore, fresh plasma from normo- and hyperlipoproteinemic subjects will be analyzed to, first establish the normal distribution of the apolipoproteins A-I, A-II, C-I, C-II, C-III, and apoE among various lipoprotein subclasses. This will be done on each sample simultaneously where no other known method can do that efficiently. Second, detect the

presence of any lipoprotein subclass that contains one apolipoprotein, such as LpA-II, and estimate its molecular mass and concentration in plasma. Finally, detect any statistically significant changes in the distribution of the lipoprotein subclasses in hyperlipoproteinemic subject, and to detect the presence or absence of particular subclasses in these subjects.

Materials and Methods

Materials

Chemicals used were of either electrophoresis or biochemical grade: glycine and acrylamide were purchased from Scharlau (France), bisacrylamide from Fluka Chimica AG (USA), TEMED from Acros (USA), Tris(hydroxymethyl)-aminomethane and polyethylenglycol 6000 from Merck (Germany), agarose from Janssen Chimica (Belgium), all other chemicals and solvents were of analytical grade. Goat-anti-apolipoprotein A-I, A-II, C-I, C-II, C-III, apoE, pure apoA-I, pure apoA-II, and apoA-II standard serum, were purchased from Meridian Life Science (USA), polyester gel plates (Gel-Fix™) were purchased from Serva (Behring, Germany).

Methods

Subjects: Plasma from 167 male and female subjects were collected after 12h fasting, and processed to study AI-Lp subclasses, 108 sample of them for AII-Lp subclasses distribution, 23 samples were processed for CI, CII, CIII, and apoE. Three samples were used to study the stability of AI-Lp subclasses on storage without and with LCAT inhibitor (5, 5-dithiobis-2- nitrobenzoic acid) (DNBA).

Blood sample preparation: Blood samples were collected into tubes containing $EDTAK_2$, plasma were isolated by low speed centrifugation (3,000 rpm) at 4°C for 15 min, then 40 µL of the LCAT inhibitor (5,5-dithiobis-2-nitrobenzoic acid) (DNBA, 37.5 mM)) were added immediately to each mL of plasma in addition to the following preservatives that have a final concentration of: gentamycin (0.08 mg/mL), sodium azide (0.1 mg/mL), and chloramphenicol (0.08 mg/mL).

Biochemical tests: Plasma level of glucose was measured by enzymatic reference method with hexokinase (COBAS® INTEGRA Glucose HK), plasma total cholesterol was measured by enzymatic, colorimetric (CHOD/PAP) method (COBAS® INTEGRA Cholesterol), HDL-C and LDL-C were measured by enzymatic, colorimetric (CHOD/PAP) method (COBAS© INTEGRA HDL-Cholesterol Direct; COBAS® INTEGRA LDL-Cholesterol direct), respectively, TAGs were measured by enzymatic, colorimetric (GPO/PAP) method ((COBAS® INTEGRA Triglecerides (TRIG)). All tests were purchased from Roche and runs on COBAS INTEGRA 400+ of Roche series at Al KHALDI MEDICAL CENTER. VLDL-C was calculated from the equation: VLDL-C (mg/dL) = TAG (mg/dL) / 5.

Assay of plasma apoA-I and apoB: COBAS® INTEGRA Apolipoprotein A-I, and COBAS® INTEGRA ApolipoproteinB,were purchased from Roche

- **Method:** Immunoturbidimetric
- **Principle:** Human apoA-I and apoB forms precipitate with specifice antiserum which is determined turbidimetrically at 340 nm.

Assay of plasma apoA-II: Total apoA-II in plasma samples was assayed in duplicate by the Laurell electroimmunoassay method using the specific anti-apoA-II antibody. Pure apoA-II was used as a standard and calibration serum was used as a control.

Preparation of agarose gel matrix: *Solution A* (4 % PEG): polyethylenglycol was dissolved in the buffer of double concentration (tris, 28 mM and glycine, 220 mM, pH 8.3). *Solution B* (2 % agarose): agarose was dissolved in warm water until a clear solution was obtained. Agarose gel matrix (1% agarose): Equal volumes of solution A and hot solution B were mixed gently to avoid air bubble formation. When the temperature of the mixture reached about 55 °C, the antibody was added and mixed quickly and gently (as described in Table A). The area of the gel matrix required is calculated and appropriate glass plate is prepared and cleaned. The volume of the solution required is calculated to make a gel of 1.5 mm thickness. When the gel solidified, it was used for electrotransfer of the antigen, or kept in a moist chamber at 8 °C until used.

Apolipoprotein class	Percentage of Anti-Apolipoprotein (%)	Sample Volume (µL)
apoA-I	1.0	3.0
apoA-II	1.0	3.0
apoC-I	1.5	3.0
apoC-II	1.5	10.0
apoC-III	1.5	3.0
apoE	1.5	10.0

Table A: Plasma sample volume used on 4-25 % gPAG and the percentage of the antibody specific to the required apolipoprotein used for immunoblotting.

The Improved Immunoblotting Technique

Method description: A 4-25 % nondenaturing gPAG was prepared and casted in 11.1 x 7.3 cm glass plates and run in Bio-Rad vertical electrophoresis system (model No. Mini-PROTEAN® 3 Cell).

Component	25 %	4%	4 % staking
Sucrose (gm)	0.30	____	____
Acrylamide Solution (40%) (µL)	1310	210	210
Tris-glycine Buffer (x8) (µL)	260	263	263
Distilled water (µL)	350	1620	1620
Ammonium Persulfate (10%w/v) freshly prepared (µL)	7	7	10
TEMED (µL)	7	7	10

Table B: 4-25 % nondenaturing gPAG preparation.

Appropriate amounts of plasma were applied to the gel wells depending on the apolipoprotein to be tested (Table A), and 12 µL of protein markers solution containing 4 µg each of ova albumin, bovine serum albumin (BSA), and urease were applied to one well. The gPAG was run at 70 V for 30 min, then at 120 V for 4.5 h in 14mM tris and 110 mM glycine buffer with pH = 8.30. After the end of the run the part containing the molecular mass markers was cut and fixed in 10% sulfosalicylic acid for 1 h, then stained in commassie brilliant blue R0250 (CBB) staining solution (0.04 % CBB-R250 in 3.5 % perchloric acid), and destained in 5 % acetic acid solution until clear background was obtained.

The rest of the gPAG was subjected to immunoblotting as follows:

1. Agarose gel matrix was prepared as described in section 2.2.5.
2. Immediately after the end of the electrophoresis, the gPAG was placed on a wet filter paper supported by one piece of wet sponge (Scot Brite®) in the transfer cassete.
3. The solidified agarose gel was slipped down from its support slowly over the gPAG, covered by a sheet of wet filter paper and a second piece of wet sponge.
4. Both gels, agarose and gPAG, that were already sandwiched in the cassette between two layers of filter paper and sponge, were immersed in the transfer cell (Trans-Blot Cell, Bio-Rad, USA) filled with the buffer, where the agarose gel layer facing the anode.
5. The transfer was done overnight at 100 mA constant current.
6. After completion of the transfer the agarose gel was carefully removed and placed on a Gel-Bond film, wrapped with a piece of soft medical gauze and washed overnight in 0.15M normal saline to remove the unreacted antibody.
7. The agarose gel was dried and stained with 1% CBB R-250 in 50% methanol and 7% acetic acid, then de-stained with fresh solution of 10% methanol and 7% acetic acid.

Measurement of apoA-I distribution: The stained bands on the agarose gel were computer scanned the area in pixels under the peaks was used for the calculation of the percentage of each fraction. The amount of apoA- I in each fraction was calculated from the percentage of the individual fraction multiplied by the plasma concentration of apoA-I.

Measurement of relative molecular mass of lpai fraction: Molecular mass markers were run in each 4-25 % gPAG; and at the end of each run the gPAG region containing the markers was cut, fixed, stained with 0.04 % CBB in 3.5 % perchloric acid, and de-stained with 5 % acetic acid The logarithm of migration distance of each marker was plotted against the logarithm of its molecular mass forming linear standard curve .The logarithms of migrating distance of each fraction were measured and the corresponding relative molecular masses were obtained from the standard curve.

Linearity of the gradient polyacrylamid gel: We check the linearity of the gradient gel by preparing gel with concentrated stain (CBB- G250) added to the 25 % solution, then the gel was cast; this give us gel with gradual dilution of stain. After polymerization the gel was dried between two layers of cellophane impregnated with 5% gelatin as was described elsewhere[14]. The dried gel was scanned by a densitometer where the change of a layer banc with distance was linear.

Statistical analysis: The statistical package SPSS was used to calculate the "*t*" values and their significance level, and the correlation between different parameters. The coefficient of variation (CV) was calculated from duplicate measurement of the subclasses as follows:

The difference between the two measurements were calculated (d), the mean value of each measurement was calculated (x_1, x_2), the grand mean was calculated from the two means:

$$\text{grand mean} = (x_1, x_2)/2.$$

The standard deviation (SD) of the measurement was calculated the relation:

$SD = SQRT(d^2/n)$. The CV% was calculated from the relation:

$CV\% = (SD/\text{grand mean}) * 100\%$.

Results

Subjects Group and Lipid Profiling

The participants were classified into groups according to the fasting blood glucose (FBG), and to the fasting lipid profile results. The groups are shown in Table 1.

Group	Group type	Abbreviation
1	Normolipidemic	Nl
2	All hyperlipidemia	Hl
3	Hyperlipidemia Mixed	Hl.mixed
4	Hypercholesterolemia	Hl.chol
5	Hypertriacylglycerol	Hl.TAG
6	Normoglycemic	Ng
7	Hyperglycemic	Hg

Table 1: Group types and abbreviation.

Group: the number of subjects in each group will be depicted in tables of comparison.

Characterization of the Subclasses Detected

The molecular mass and Stokes' radius of the AI-Lp subclasses detected are shown in Tables 2 and 3 [15-18] for AII-Lp subclasses, where the smallest subclasses (42,000, 50,000, 60,000-65,000) of AI-Lp and AII-Lp were not detected in all subjects, whereas the large subclasses were detected in all normoglycemic– normolipidemic subjects. Other subjects showed variable number of subclasses. The coefficient of variation (CV) of measurement of subclass was determined from duplicate and was less than 5% for most AI-Lp subclasses and AII-Lp subclasses, Tables 4 and 5, respectively. Figure 1 and sub .s1, shows plasma run in duplicates.

Subclass Number	Relative Molecular Mass (Mr)	Stokes' Radius (nm)	Asztalos et al. [18]	Atmeh et al. [11]
1	42,000	2.96	Pre-β_{1a}-HDL	$SLpAI_4$
2	50,000	3.22	α3-HDL,	$SLpAI_2$
3	60,000 - 65,000		preα4-HDL	
4	70,000	3.56	preα3-HDL	$SLpAI_1$
5	80,000	3.77	α2-HDL,	
6	89,000 - 105,000	3.80 – 4.00	preα2HDL	
7	105,000 - 126,000	4.00 – 4.23	α1-HDL,	
8	126,000 - 158,000	4.23 – 4.53	preα1HDL	
9	158,000 - 200,000	4.53 – 4.87	preβ2-HDL,	
10	200,000 - 240,000	4.87 – 5.15	preβ2c-HDL,	
11	240,000 - 282,000	5.15 – 5.41	preβ2b-HDL,	
12	282,000 - 354,000	5.41 – 5.89	preβ2a-HDL	
13	>354,000	> 5.89		

Table 2: Relative molecular mass and Stokes radius of AI-Lp subclasses detected and compared with similar subclasses reported in the literature.

AII-Lp subclass	Relative Molecular Mass(*Mr*)	Stokes Radius (nm)	Cheung, et al. [15]	Atmeh, et al. [16]	Leroy, et al. [17]	Ohta, et al. [18]
I	50,000					
II	60,000-65,000					
III	70,000			(AI + AII)HDL		
IV	105,000-150,000	4.0 – 4.53	Lp (AI +AII)$_3$		Lp (AI +AII)	Lp (AI +AII)
V	150,000-200,000	4.53 – 4.87	Lp (AI +AII)$_2$		Lp (AI +AII)	Lp (AI +AII)
VI	200,000-282,000	4.87 – 5.41	Lp (AI +AII)$_1$			Lp (AI +AII)
VII	282,000-354,000	5.41 – 5.89	Lp (AI +AII)			
VIII	4,00,000					
IX	4,50,000					
X	5,00,000					
XII	5,50,000					
XII	6,00,000					
XIII	6,50,000					

XIV	7,00,000					
XV	7,50,000					
XVI	8,00,000					
XVII	9,00,000					

Table 3: Relative molecular mass and Stokes' radius of AII-Lp Subclasses detected compared with similar subclasses reported in the literature.

AI-Lp subclass	*Mr*	Number of samples	% CV
6	89,000 – 105,000	101	3.1
7	105,000 – 126,000	81	3.6
8	126,000 – 158,000	54	4.3
9	158,000 – 200,000	39	3.2
11	240,000 – 282,000	28	3.6
12	282,000 – 354,000	83	4.6
13	> 354,000	74	5.3

Table 4: Coefficient of variation of AI-Lp subclasses. Samples were run in duplicates and the difference between the two values was used to calculate the cv as mentioned in section 2.5. (n = 101).

AII-Lp subclass	*Mr*	n	CV %
II	65,000	18	8.2
IV	105,000-150,000	51	3.5
V	150,000-200,000	41	3.6
VI	200,000-282,000	35	3.2
VII	282,000-353,000	53	3
	> 350,000	44	5.5

Table 5: Coefficient of variation of AII-Lp subclasses. Samples were run in duplicates and the difference between the two values was used to calculate the cv as mentioned in section 2.5. (n = 53).

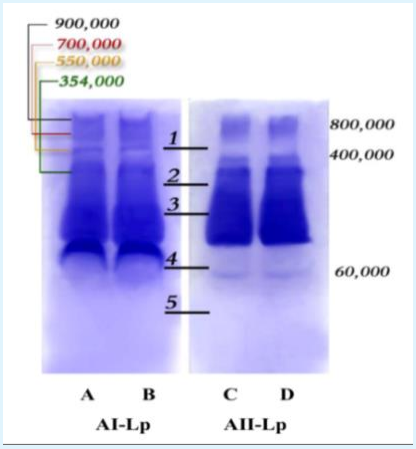

Figure 1: The distribution of AI-Lp and AII-Lp subclasses in a female subject characterized by high hypertriacylglycerols (TAGs = 365 mg/dL), hyperglycemia (FBG = 147 mg/dL), Total-C = 169 mg/dL, HDL-C = 31 mg/dL. The plasma was run in 4-25% gPAGE, followed by immunoblotting as follows: **Lanes A & B**: the plasma was immunoblotted against anti-apoA-I. **Lanes C & D**: the plasma was immunoblotted against anti-apoA-II.**Thenumbers1-5** represent molecular mass standard:
1 = urease hexamer (545,000), 2 = urease trimer (272,000), 3 = bovine serum albumin (BSA) dimer (133,000), 4 = BSA monomer (66,500), 5 = ova albumin (45,000).

Variation of Lipid Profiles of the Subjects

The lipid profiles and the difference between the different groups are calculated in (Tables 7a, 8a, and 9a) all the groups were compared with the normoglycemic and normolipidemic group of similar age where there was no statistically significant difference between the ages of the groups. Marked differences were seen in the plasma levels of total cholesterol, HDL-C, LDL- C, VLDL-C, TAGs, apo-AI, apo-B, and the ration of apoB/apo- AI, with highly significant difference in some of them, Table 9a and appendix A (Tables A1,3,5,7,9,11,13). Moreover, some AI-Lp subclasses (supplementary figures 2) showed statistically significant differences in the mass of apo-AI in the different groups, (tables 7b,8b & 9b). Detailed information about the distribution of the thirteen AI-Lp subclasses in the groups that are compared in Table 9b are shown in appendix A (Tables A2,4,6,8,10,12,14).

Total Plasma apoA-II Measurement

Total apoA-II was measured by immunoelectrophoresis (Rocket) for 108 participants. After separating the participant into groups, the mean values of total apoA-II in mg/dL were not differs significantly between different groups. For normolipidemic group (n = 57) total apoA-II was 55.6±13 mg/dL (±SD), and for hyperlipidemic group (n = 51) it was 56.4±11.5 mg/dL. Whereas, for normoglycemic group (n = 72), total apoA-II was 56.1±12.2 mg/dL, and for hyperglycemic group (n = 36) it was 55.9±12.6 mg/dL (supplementary figures 3). The apoA-II content of AII-Lp subclasses, that are differ significantly, of this groups and other aged matched group are shown in Table 8. Detailed information about the distribution of the seventeen AII-Lp subclasses, are shown in appendix B, (Tables B1,2,3,4). Correlations between mass of AI-Lp subclasses and AII-Lp subclasses with Lipid Profile parameters were summarized in Tables 10 and 12.

Qualitative Distribution of apoC-I, apoC-II, apoC-III, and apoE

ApoC-I lipoprotein was detected in the high molecular weight subclasses of AI-Lp, in the molecular mass range of 126,000-282,000. Whereas apoC-II was detected in the molecular mass range 105,000-272,000 (Figure 2). While ApoC-III was detected in all samples and it spans the molecular mass range ~ 40,000- 545,000, however, in many samples no defined bands were detected (Figure 2).

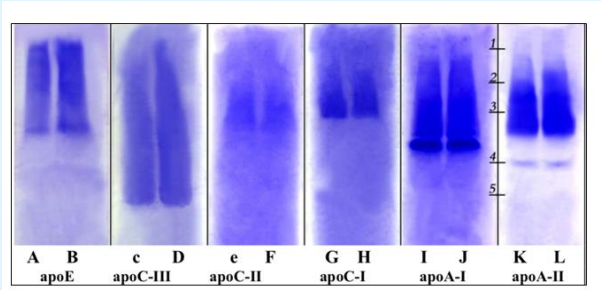

Figure 2: Distribution of apoA-I, apoA-II, apoC-I, apoC-II, apoC-III, and apoE subclasses in a subject with hyperglycemia and mixed hyperlipidemia. The plasma was run in 4-25% gPAGE, followed by immunobloting as follows:
Lanes A & B: the plasma was immunoblotted against anti-apoE.
Lanes c & D: the plasma was immunobloted against anti-apoC-III.
Lanes e & F: the plasma was immunoblotted against anti-apoC-II.
Lanes G & H: the plasma was immunoblotted against anti-C-I.
Lanes I & J: the plasma was immunoblotted against anti-apoA-I.
Lanes K & L: the plasma was immunoblotted against anti-apoA-II.
The numbers 1-5 represent the molecular mass standared: (1 = urease hexamer (545,000), 2 = urease trimer (272,000), 3 = BSA dimer (133,000), 4 = BSA monomer (66,500), 5 = ovalbumin (45,000).

Interestingly, ApoE was detected in all samples in the high molecular mass region ~ 272,000-545,000, whereas, in some samples it appears in lower molecular mass of ~ 89,000-105,000 (Figures 2 and 3) with varying proportions. Similar subclasses were reported in the literature as shown in Tables 2 and 3, respectively.

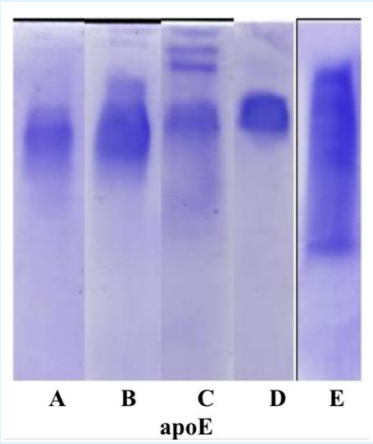

Figure 3: Distribution of apoE in different samples. The plasma was run in 4-25% gPAGE followed by immunoblotting against anti-apoE Lanes **A, B, & D:** normoglycemic- normolipidemic subject. Lane **C:** normoglycemic-hyperlipidemic. Lane **E:** hyperglycemic- hyperlipidemic subject.

Discussion

The use of HDL–C as a predictive risk factor for CAD is now questionable, and other risk factors are sought. Nevertheless, HDL still has an important role in the RCT process. Moreover, HDL includes a diverse group of particles with differences in apolipoprotein and lipid composition, size, charge, and function. Therefore, more sophisticated methods to subclassify HDL are expected to lead to tests that provide better clinical utility [11,19]. Measurement of the subclasses HDL_2 and HDL_2 did not provide any additional predictive value beyond that of HDL-C [20]. Measurement of apoA-I, the major apoprotein in HDL, has been proposed as a better method to assess protective levels of HDL, but the results were controversial [21].

Distribution of apoA-I in various subclasses of HDL is a promising approach and two methods are available at present to study this distribution [11,21]. The method of Asztalos, et al. [22] is semiquantitative due to the use of nitrocellulose membrane, as discussed in detail elsewhere [11] and has other disadvantages where: it requires one gradient gel for each sample, involves several reagents and steps, and the subclasses appear as blurred spots. The method of choice for detection and quantitation of apoA-I distribution among lipoprotein subclasses, is the method that was developed by Atmeh, et al. [11-14] where the method was proved to be quantitative [14] the subclasses appear as bands, and up to 8 samples can be run on the same gel.

In this work, the developed immunoblotting method was used to study the distribution of apoA-I among lipoprotein subclasses from fresh plasma, and was further extended to study the distribution of apoA-II, apoC-I, apoC-II, apoC-III, and apoE, in both normolipidemic and hyperlipidemic subjects. At least 13 AI-Lp subclasses and 17 AII-Lp subclasses were detected (Tables 2 and 3, respectively). The CV for AI-Lp subclasses was found to be less than 5% (Table 4) which is better than that reported by Asztalos et al. as less than 10% [23] and the CV of AII-Lp subclasses was in the range 3.0 – 5.5% except for the small subclass II (60,000-65,000) where it was 8.2% (Table 5). This small CV is an advantage for this method.

The proportion of the subclasses in plasma samples changed on storage at 8 °C, where the AI-Lp subclasses 8 and 9 showed moderate changes in the presence or absence of the LCAT inhibitor, DNBA (Table 6). LCAT catalyse the esterification of free cholesterol into CE in the HDL subclasses, this process occurs during the maturation of small HDL particles and their conversion into larger particles, as a result of RCT and interaction between the small and large HDL particles. Therefore, inhibiting the activity of the enzyme inhibits the esterification of free cholesterol in the small particles and expected to stop the increase in particle size. This result is in accordance with the observation reported by Atmeh and Issa [12] where purely isolated small HDL subclasses in the presence and absence of DNBA underwent marked fusion and rearrangement and the appearance of large number of larger particles. From these results, it seems that such rearrangement is not related to the action of LCAT, but it might occur by a different mechanism. The

smallest subclasses (42,000 – 50,000) were not detected in Ng–Nl subjects (Table 9b and appendix A: Table A2). Whereas the 42,000 subclasses (9%) was detected in a female subject with severe hyperglycemia (FBG = 418 mg/dL) and hyperlipidemia (TAGs = 370 mg/dL, total cholesterol = 250 mg/dL). The subclass 50,000 was detected in 7 hyperlipidemic subjects (Table 7b). 5 subjects were Ng; and 2 were Hg (Tables 8b). The other subclasses were detected in variable proportions in normo- and hyperlipidemic subjects and some subclasses were not detected in some individuals (Tables 7b,8b,9b and appendix A: Tables A2,4,6,8,10,12,14).

Subclass	Day 1	Day 2 with DNBA	% Diff[a]	Day 2 without DNB[A]	% Diff[b]	% Diff[c]	Day 3	% Diff[d]	Day 4	% Diff[e]	Day 5	% Diff[f]	Day 6	% Diff[g]	Day 7	% Diff[h]
3											5.6(n=1)					
4									14.6±7.8				5.6(n=1)		22.6±6.3	
5	14.6(n=1)			16.9±0.8					16.2±2.8		19.1±5.4		7.1(n=1)			
6	14.3±2.6[i]	14.8±2.1	3.2	15.5 ± 3.9	8.4	4.7	19.1±5.4	33.7	16.7±5.4	16.8	15.7±0.5	9.8	22.1±4	54.5	19.8±8.4	38.5
7	12.9±0.14	11.95±2.2	-7.4	19.0(n=1)			14.3±6.4	10.9			20.5±3.2	58.9	12.5±5	-3.1	14.2±4.7	10.1
8	19.6±2.6	12.7±0.1	-	13.6±3.2	-	7.1	20.2±3.4	3.1	18.2±2.8	-7.1			13.9±1.7	-		
			35.2[j]		30.6									**29.1[J]**		
9	18.4±10.4	19.4±2.1	5.4	17.5±8.8	-4.9	-9.8			23.2±7.4	26.1	24.9±7.8	35.3			11.9±9.5	-
																35.3
10	19.6±8.1	19.9±7.4	0	10.7±2.1	-	-	11±2.3	-	16.9±1.6	-	10.0(n=1)		21.2±8.9	8.2	19.6(n=1)	0
					45.4	45.4		43.9		13.8						
11	13.7(n=1)	7.4(n=1)		10.9±0.1							12.9±2.4		16.9±6.4	23.4	17.9±3.6	
12	14.6±0.14	10.3 ± 4.2	-	10.8±4.7	-	4.9	15.2±6.1	4.1	13.7±0.7	-6.2	12.1±3.1	-	13.6±3.4	-6.8	13.2±5.9	-9.6
			29.5		26							17.1				
13	13.2±2.7	11.0±1.7	-	10.5±2.9	-	-4.5	19.5±6	47.7	6.7±5.1	-	5.9±2.5	-	8.5±1.5	-	9.7±5.3	-
			16.7		20.5					49.2		**55.3[k]**		**35.6[J]**		26.5

Table 6: The stability of the AI-Lp subclasses on storage at 8°C. Each sample was divided into two portions, one was kept as it is, and to the other portion the LCAT inhibitor (DNBA) was added a percent difference= [(day2with–day1)/day1]*100%, b {day2 without DNBA–day1/[day1]}*11%,c {(day2 without) –(day2with)/day2with}*100%, IMean±SD, [j]P<0.05.

AI-LP subclassess											
Group type	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
All Normolipidemic n = 56				VLDL-C (0.40 [b]) TAGs (0.40 [b]) Subc.11 (0.58 [a])			Subc. 13 (-0.47 [a])	T-C (-0.35 a) VLDL-C (- 0.35 [a]) TAGs (-0.35 [a])			
All Normolipidemic n = 56	Total-AI (-0.44 [a])	Subc.3 (0.52 [a])		Subc.8 (-0.29 [a])			Subc. 11 (0.34 [a])		Subc.13 (0.35 [a])		
All Normoglycemic n = 92					T-C (0.27 [a]) VLDL-C (0.42 [b]) TAGs (0.42 [b])	Subc. 9 (-0.30 [a])	Total-AI (-0.30 [a]) HDL-C (-0.31 [a])		Total-C (0.42 [a]) LDL-C (0.53 [a])		

All Normolipidemic n = 56	Subc.8 (0.69[a]) Subc.9 (0.7 [a])		Subc.6 (0.74 [b]) Subc.13 (0.77 [b])		Subc. 12 (0.44 [b]) Subc. 13 (0.37 [a])					Subc.13 (0.42[a])	
Ng – Nl n = 42	VLDL-C (- 0.72 [a]) TAGs (-0.72 [a])	T-C (0.66 [a])					T-C (-0.57 [b]) LDL-C (-0.57 [b])				
Ng – Hl n = 20	Total-AI (0.90[b])			HDL-C (0.51 [a])		Total-AI (0.72 [b]) Subc. 12 (0.71 [b]) Subc. 13 (0.65 [a])			T-C (0.86[b])	Total-AI (0.64 [a]) VLDL-C (0.54[a]) TAGs(0.54 [a])	Subc.12 (0.70 [b])
Ng – Hl.chol n = 17											Total-AI (0.62 [b]) HDL-C (0.57 [a])
Ng – Hl.TAG n = 14	Subc.4 (-0.91 [a])	Subc.7 (-0.73[a])			T-C (-0.64 [a]) LDL-C (-0.67 [a])			Subc.13 (0.73 [a])		Total-AI (0.61 a) Subc.13 (0.61[a])	
Ng – (Nl + Hl.chol + Hl.TAG + Hl) (male) n = 38		Subc.5 (- 0.66 [a])	T-C (0.65 [a]) LDL-C (0.69 [a])		VLDL-C (-0.48 [b]) TAGs (-0.49 [b])	Subc. 5 (0.7[b])					
Ng – (Nl + Hl.chol + Hl.TAG + Hl) (female) n =	Subc.4 (-0.87 [a])			Total-AI (0.48 [b]) Subc.7 (0.43 [a])		Total-AI (-0.5 [a])		HDL-C (0.47 [a]) VLDL-C (-0.47 [a])			Total-AI (0.41 [a])

30								TAGs (-0.51 [a])			
Ng – Nl (male) n = 31			Subc.10 (-0.77 [a])				VLDL-C (-0.64 [a]) TAGs (-0.63 [a])		Total-AI (- 0.84 [a])		
Ng – Nl (female) n = 11					Total-AI (0.67 [a])					Subc.13 (0.69[a])	
Ng – (Hl.chol + Hl.TAG + Hl) n = 43	Subc.9 (-0.66 [a])	Subc.7 (0.43 [a])		Subc.11 (-0.68 [a])	HDL-C (0.49 [b]) VLDL-C (-0.39 [a]) TAGs (-0.39 [a])	Subc. 7 (0.44 [a])					
Hg – (Hl.chol + Hl.TAG + Hl) n = 28		LDL-C (0.57 [a]) Subc.12 (- 0.56 [a])		Total-AI (0.42 [a]) Subc.12 (0.66 [b])	Subc. 12 (0.59 [a])	Subc. 13 (0.54 [a])		T-C (0.55 [a])	Total-AI (0.71 [a])		
Hg – (Nl + Hl.chol + Hl.TAG + Hl) (Male) n = 25		VLDL-C (0.62 [a]) TAGs (0.63 [a]) Subc.9 (0.6 [a])		Total-AI (0.42 [a]) HDL-C (0.50 [a]) LDL-C (-0.42 [a]) Subc.12 (0.74 [b])		VLDL-C (-0.54 [a]) TAGs (-0.55[a])	VLDL-C (0.56 [a]) TAGs (0.58 [a]) Subc. 13 (0.62 [a])	Subc.11 (-0.71 [a])		Total-C (0.51[a]) HDL-C (0.62[b]) Subc.12 (-0.45[a])	
Hg – (Nl + Hl.chol +Hl.TAG + Hl) (female) n = 27		T-C (0.77 [b]) VLDL-C (0.70 [a])TAGs (0.70 [a]) Subc.7 (0.72 [a])		Subc.12 (0.61 [b])					LDL-C (0.66 [a])	Total-AI (0.50[a])	
Hg – Nl n = 13				Total-AI (0.66 [a]) VLDL-C (-						Total-AI (0.77[b]) T-C (- 0.57[a])	

				0.55 [a]) TAGs (-0.55 [a]) Subc.12 (0.67 [a])						HDL-C (0.65[a]) VLDL-C (-0.57[a]) TAGs (-0.56[a])	

Table 7: Statistically significant correlations between different parameters of the subjects studied (n = 141). The correlation coefficient and its sign are shown between two brackets with a superscript denoting the level of significance.
[a]. P < 0.05 , [b]. P , 0.01

AII-Lp subclasses					
Subjects	**V**	**VII**	**IX**	**X**	**XII**
All nomolipidemic (n = 57)	19.5 ± 9.3[a,b] (n=30)[c]	8.1 ± 2.8 (n=24)			
All hyperlipidemic (n = 51)	15.3 ± 5.2 (n =30)	12.3 ± 9.5 (n=9.5)			
% Diff.[d]	**-22[e]**	**52[e]**			
All normoglycemic (n = 72)	19.2 ± 9 (n=35)		2.5 ± 0.6 (n=7)	5.2 ± 0.9 (n=2)	
All hyperglycemic (n = 36)	14.9 ± 4.9 (n=25)		4.7 ±2 (n=3)	2 ± 0.9 (n=3)	
% Diff.	**- 22[e]**		**88[e]**	**- 62[e]**	
Ng-Nl n = 44		8.2 ± 3 (n=19)			
Ng-(Hl.chol + Hl.TAG) (n = 23)		13.9 ± 10.4 (n=12)			
% Diff.		**70[e]**			
Ng-(Nl + Hl.mixed) female (n=23)	20.9 ± 7 (n=9)				3.3 (n=3)
Hg-(Nl + Hl.mixed) male (n=17)	12.3 ± 2.3 (n=7)				2.01 (n=3)
% Diff.	**- 34[f]**				**- 39[e]**
Ng-(Nl +Hl.mixed) male (n=26)	18 ± 6.6 (n=13)				
Hg-(Nl + Hl.mixed) male (n=17)	12.3 ± 2.3 (n=7)				
% Diff.	**- 32[e]**				
Ng-(Nl + Hl.mixed) female (n=23)	20.9 ± 7 (n=9)				
Hg-(Nl + Hl.mixed) female (n=22)	15.9 ± 5.3 (n=19)				
% Diff.	**- 24[e]**				

Table 8: The apoA-II content of AII-Lp subclasses, of the groups subjects which are compared with the corresponding age-matched group. The subclasses that are differing significantly are shown below in the table. The detailed information of the compared groups is present in appendix B (Tables B1,2,3,4). In that group apoA-I and apoA-II were measured.
c mean ± SD, values are in mg/dL., number of subjects who have this subclass.
percent difference of the mean = [(second group – first group) / first group] * 100% .
[f]P<0.05, P<01.

AII-Lp subclasses					
Group type	**II**	**IV**	**V**	**VI**	**VII**
All the 108 AII sample	VLDL-C (0.48[a]) TAGs (0.47[a])	Total-AII (0.72[b]) Subc.9 (0.37[a])	Subc.4 (0.39[a]) Subc.10 (-0.28[a])	Subc.12 (0.22[a])	Subc.10 (-0.34[a])
All normolipidemic (n = 57)		Total-AI (0.72[b])			
All hyperlipidemic (n = 51)		Total-AI (0.32[a]) Total-AII (0.72 b) Subc.VII (-0.48[a])			
All normoglycemic (n = 72)	Total-AII (-0.58[a]) Subc.IV (-0.6[a])	Total-AI (0.34[b]) Total-AII (0.71[b])	Subc.VII (0.52[b])		
All hyperglycemic n = 36		Total-AII (0.74[b])			
Ng-Nl (n = 44)	Subc.8 (0.75[a]) Subc.11 (0.85[b])	Total-AII (0.69[b])	Total-AII (0.62[b]) Subc.8 (0.52[a])		

Ng-(Hl.chol + Hl.TAGs) (n = 23)		Total-AII (0.78[b])	Subc.4 (0.78[a])		
Hg-Nl (n = 13)	Subc.12 (-0.9[a])	Total-AII (0.79[b]) subc.8 (0.9[a])	LDL-C (-0.72[a]) Subc.6 (0.77[a])	Total-AII (0.68[a])	Total-AII (0.96[b])
Ng- (Nl+Hl.mixed) (female) (n = 23)		Total-AI (0.51[a]) Total-AII (0.8[b]) Subc.6 (0.47[a])	Subc.8 (-0.87[b])		Total-AI (-0.68[a]) Subc.4 (0.8[a]) Subc.8 (-0.78[b])
Hg-(Nl+Hl.mixed) (female) (n = 22)		Total-AII (0.81[b])	VLDL-C (-0.47[a]) TAGs (-0.47[a]) Subc.8 (0.65[a])	Subc.4 (-0.73[a])	LDL-C (-0.71[a]) Subc.3 (0.89[a])
Ng- (Nl+Hl.mixed) (female) (n = 23) (male) n = 26		Total-AII (0.7[b])	Subc.7 (0.67[a])		
Hg- (Nl+Hl.mixed) (male) n = 17	Total-AI (-0.9[a]) Subc.7 (-0.9[a])	Total-AII (0.7[b]) T-C (0.56[a])			Subc.11 (0.89[a])

Table 9: Statistically significant correlations between AII-Lp Subclasses and different parameters including AI-Lp subclasses, of the subjects studied (n = 108) for AI and AII distribution. The correlation coefficient and its sign are shown between two brackets with a superscript denoting the level of significance. Both apoA-I, and apoA-II were measured for each subject in this group (n = 108).
[a]P<0.05, [b]P<0.01

Regarding the lipid profiles, AI-Lp, and AII-Lp of the subjects, there was statistically significant differences between the different groups. All the hyperlipidemic subjects (n = 85) showed statistically significant differences from all normolipidemic subjects (n = 56), where an increase in all the lipid profile was observed except in the HDL-C (Table 7a); moreover, the AI-Lp subclass 4 and AII-Lp subclass VII were higher than the normolipidemic group (98 and 52%, respectively) and AII-Lp subclass V was lower (- 22%) (Tables 7b, 11). All the hyperglycemic subjects (n = 49) showed statistically significant increase in TAGs and VLDL-C, when compared with normoglycemic subjects (n = 92) (Table 7a) moreover, significant increase (19%) in AI-Lp subclass 7 was observed, and a non-significant increase (15%) in AI-Lp subclass 4 (Table 7b) and a significant decrease (-22%) and (-62%) in AII-Lp subclasses V and X, respectively, were observed (Table 11), and appendix B (Table B1) for detailed information about AII-Lp subclasses distributions in these groups.

Separating the normo- and hyperglycemic group into males and females, there was no significant difference in the lipid profile between the hyperglycemic males vs normoglycemic males, hyperglycemic females vs normoglycemic females, normoglycemic males vs normoglycemic females, and between hyperglycemic males vs hyperglycemic females (Table 9a Detailed information about these groups are found in appendix A: Table A7 and A9). However, the AI-Lp subclass 13 was decreased significantly in both hyperglycemic males and females (-21 and -22%, respectively), and AI-Lp subclass 3 was lower in normoglycemic females than normoglycemic males (-38%, Table 9b and appendix A: Table A10).

All the normoglycemic-hyperlipidemic [Ng – (Hl.chol + Hl.TAG + Hl.mixed) (n = 43) subjects showed statistically significant increase in total apoA-I, total-C, LDL-C, VLDL-C, TAGs, apoB, and apoB/apoA-I ratio, and no change in HDL-C and apoA-II, when compared with the normoglycemic-normolipidemic (Ng-Nl) (n = 42) subjects (Table 9a, and appendix A: Table A3). Moreover, there was a statistically significant increase in the apoA-I content of subclasses 4 and 6 (P<0.05), and 13 (P<0.01), and a significant decrease (P<0.05) in subclass 12, Table .9.b. Similar results were reported in patients with CHD, were the subclasses α_3–HDL (corresponding to AI-Lp subclass 6) and pre β_2–HDL (corresponding to AI-Lp subclass 13) increased in the patients, and the subclasses α_1-HDL and pre α_1–HDL (both subclasses corresponding to AI-Lp subclass 12) were decreased [24] Subclass 1 was not detected in both groups, and subclass 2 was not detected in normoglycemic- normolipidemic group, but was detected in five subjects of the Ng– (Hl.chol + Hl.TAG + Hl.mixed) group (Table 9b, and appendix A: Table A4).The hyperglycemic–hyperlipidemic [Hg-(Hl.chol + Hl.TAG + Hl.mixed)] subjects showed a significant increase in total-C, LDL-C, VLDL-C, TAGs, apoB, and apoB/apoA-I, while total apoA-I was non–significantly increased, and HDL-C was significantly decreased (Table 9a, and appendix A: Table A5). Similar to the [Ng – (Hl.chol + Hl.TAG + Hl.mixed) group, the AI-Lp subclasses 4 and 6 were increased, in addition to an increase in subclass 7; on the contrast, subclasses 12 and 13 were significantly decreased, Table 9b and appendix A: Table

A6. However, no significant differences were detected in the lipid profile between the Hg–Nl group and the Ng-Nl group; but there was a significant increase in subclasses 7 and 13 (31 and 29%, respectively) (Table 9b and appendix A: Table A6). An interesting observation was the highly significant (P<0.001) increase in both HDL-C (42%) and total apoA-I (29%) in the Ng–Hl.chol group (Table 9a and appendix A: Table A1) in addition to the highly significant (P<0.001) increase in total cholesterol (52%), LDL-C (66%), apoB (60%), and apoB/apoA-I ratio (27%, P<0.01), with non-significant increase in apoA-II, VLDL-C, and TAGs. This large increase in HDL-C and apoA-I was accompanied by large significant increase in the apoA-I content of subclasses 4 (238%, P<0.05), 6(44%, P<0.01), 7(49%, P<0.01), 8(40%, P<0.05), and 13(40%, P<0.05) as shown in Table 9b and appendix A: Table A2. These increases in the hypercholesterolemic group are not unexpected, were there are several situations of hypercholesterolemia and high HDL-C as well as patients with CAD that have high HDL- C [70]. Since apoA-I has increased but not apoA-II, and the molar ratio of apoA-I/apoA-II is almost constant 1:1 [25] then most of the increase in these subjects was in the LpA-I particles that do not contain apoA-II. On the other hand, the Ng–Hl.TAG subjects showed a statistically significant decrease in HDL-C (-23%, P<0.01) with significant increase in total-C (14%, P<0.05), VLDL-C (160%, P<0.001), TAGs (160%, P<0.001), apoB (40%, P<0.001), and apoB/apoA-I ratio (42%, P<0.001), with minor non–significant change in LDL-C (-5%) (Table 9a and appendix A: Table A3). In this group, although HDL-C was significantly decreased and total apoA-I was slightly decreased (-2%), the apoA-I content of the subclass 4 was significantly increased (136%, P<0.05) with concomitant significant decrease in the subclasses 8 (-35%, P<0.05), 9 (-27%, P<0.05), and 12 (-34%, P<0.01), and the appearance of subclass 2 (50,000) in one subject; whereas the other subclasses did not change significantly as shown in Table .9.b, and appendix A: Table A4. This can be explained by a redistribution of apoA-I among the subclasses, i.e., the disturbance in the lipid profile is associated with disturbance in the AI-Lp subclass distribution. In the normoglycemic subjects with mixed hyperlipidemia (Ng-Hl.mixed), HDL-C was non–significantly decreased (-12%), while LDL-C was significantly increased (72%, P<0.001) as well as total-C (69%, P<0.001), VLDL-C (220%, P<0.001), TAGs (213%, P<0.001), apoB (106%, P<0.001), apoB/apoA-I ratio (83%, P<0.001), with non– significant increase in total–apoA-I (10%) (Table 9a and appendix A: Table A1).

Moreover, there was a significant increase in subclass 4 (134%, P<0.01) and subclass 13 (23%, P<0.05), with the appearance of subclass 2 (50,000) in four subjects (Table 9b and appendix A: Table A2). Comparing the Ng-Hl.chol group with Ng-Hl.TAGs group, significant difference were detected where the Ng-Hl.chol group had higher total-C (24%, P<0.001), HDL-C (46%, P<0.001), LDL-C (42%, P<0.01), apoA-I (24%, P<0.001) and apoB (11%, P<0.01), and lower VLDL-C (-124%, P<0.001) and TAGs (-129%, P<0.001) (Table 9a and appendix A: Table A11). Moreover, the AI-Lp subclasses 7, 8, 9, 12, and 13 were significantly higher in the Ng-Hl.chol group (40%, P<0.001); (54%, P<0.01); (39%, P<0.05); (42%, P<0.01); (31% P<0.05), respectively (Table 7 and appendix A: Table A12). This increase can be interpreted as the increase in both total apoA-I and total-C in those subjects was reflected in the increase of their subclasses. On the other hand, comparing the Ng- Hl.chol group with the Ng-Hl.mixed, the Ng-Hl.chol group had higher HDL-C (68%, P<0.001), and total apoA-I (20%, P<0.05), and lower VLDL-C (-61%, P<0.001), TAGs (- 61%, P<0.001), apoB (-23%, P<0.001), and apoB/apoAI (-33%, P<0.001); whereas no significant differences were seen in the total-C and LDL-C (Table 9a and appendix A: Table A11). Moreover, the AI-Lp subclasses 7 and 8 were significantly higher in the Ng- Hl.chol group (38% P<0.05); (39%, P<0.01), respectively (Table 9b and appendix A: Table A12). Again, the increase in the total apoA-I in this group was mainly appeared in the increase in the apoA-I content of subclasses 7 and 8. The Ng-Hl.mixed group showed higher total-C (30%, P<0.001), LDL-C (44%, P<0.001), and apoB (31%, P<0.001) when compared with Ng-Hl.TAGs group (Table 9a and appendix A: Table A13); whereas, there were no significant differences in the AI-Lp subclasses (Table 9b and appendix A: Table A14).

In a similar manner, Hg–(Hl.chol +Hl.TAGs + Hl.mixed) subjects showed significant increases in subclasses 4 (122%, P<0.05), 6 (25%, P<0.05), and 7 (27%, P<0.05) and significant decrease in subclasses 12 (-26%, P<0.05) and 13 (-22%, P<0.05), with the appearance of the smallest subclass 1 (42,000) in one subject and subclass 2 (50,000), in two subjects (Table 9b and appendix A: Table A6). The common feature between the normoglycemic-hyperlipidemic and hyperglycemic– hyperlipidemic subjects is the significant increase in the subclass 4 (70,000) in several subjects, and the appearance of the small subclass 2 (50,000) in some subjects. This is in accordance with the findings of Miida, et al. [26,27] where they reported that the size distribution of LpA-I particles may change in various disorders. These observations are of important clinical application, were such subclasses were reported to increase in hyperlipidemia and in subjects who drink alcohol moderately [11] and in patients with CHD [28]. Moreover, very recently Tashiro, et al. [29] reported that the preβ_1-HDL level (a subclass of molecular mass~70,000) was significantly increased in CAD patients, and the increase was significantly higher in patients with unstable angina pectoris subgroup than in

the stable CAD subgroup. These data suggest that HDL-C is no longer "good cholesterol" and its protective value is weak, were "both human and animal studies strongly suggested that levels of HDL-C alone do not necessarily predict the cardio-protective effects of the lipoprotein [30,31].

The variable association between AI-Lp and AII-Lp subclasses with different lipoprotein parameters of the groups may indicate variability in the structure and/or function of these subclasses in different pathological conditions. An interesting significant correlation is that between AII-Lp subclass IV (105-150,000) and plasma total apoA-I (r = 0.32, $P<0.05$) and plasma total apoA-II (r = 0.72, $P<0.01$) in the hyperlipidemic group; moreover, similar correlation was also seen in the normoglycemic group (r = 0.34, $P<0.01$; r = 0.71, $P<0.01$, respectively) and normoglycemic females (r = 0.51, $P<0.05$; r = 0.80, $P<0.01$), Table 9. This subclass IV comprises about one third of the plasma apoA-II in all the groups, and strongly and significantly correlated (r = 0.72, $P<0.01$) with plasma total apoA-II in all the subjects, whereas the other AII-Lp subclasses did not correlate significantly (Table 9). Moreover, the proportion of these subclasses did not vary significantly in all subjects. These data show that AII-Lp subclass IV is the main Lp(AI+AII) subclass in plasma and we propose that this subclass undergo variable compositional changes in different pathologies, and its function rather than its plasma level is affected. In accordance with our proposition is the reported positive correlation, in control subjects, between the molar ratio of apoA-I/apoA-II and the anti-apoptotic activity of HDL sub-fractions including the small dense HDL_{3C} (estimated molecular mass: 163,000), whereas this correlation was lost in subjects with metabolic syndrome [32,33].

Moreover, in support of this proposition, Gomaraschi, et al. [30] reported that elevation of plasma Lp(AI+AII) particles in some patients with myocardial infarction is an independent predictor of a more severe inflammatory response, and these particles are more sensitive to inflammation-induced modifications, although the reasons for this sensitivity are presently unknown. These authors suggest that these particles are of lower anti-inflammatory, or even pro- inflammatory activity; and expressed the need for large prospective studies to investigate the prognostic significance of Lp (A-I+AII) levels and their utility for risk stratification in patients suffering from acute coronary syndrome. Comparing the AI-Lp and AII-Lp subclasses showed the presence of AII-Lp subclass without corresponding AI-Lp subclass, such as subclasses II, VIII – XVI. This suggests the presence of LpA-II subclass without apoA-I. This is in accordance with reported LpA-II subclass by Bekaert et [34]. Moreover, the largest subclass of AII-Lp (~ 900,000) has an AI-Lp subclass counterpart in some subjects (Figures 1, supplementary s3-s6). The smallest AII-Lp subclasses (I and II Table 3) and the large LpA-II subclasses that we detected (VIII – XVII, Table 3), for the best of our knowledge, were reported for the first time.

Conclusion

In conclusion, the distribution of AI-Lp and AII-Lp subclasses was variable in different groups of hyperlipidemia or hyperglycemia and studying these subclasses is expected to be more relevant and more predictive than HDL-Cholesterol. This has important clinical applications. The results showed the presence of lipoprotein particles that contain apoA-II but not apoA-I (LpA-II), and the smaller particles (I and II) and the larger particles (VIII – XVII) of LpA-II are reported for the first time. The data presented in this work can be considered exploratory and hypothesis- generating, and more detailed studies are required to establish the predictive and protective values of these lipoprotein subclasses.

Acknowledgements

This work was supported by the Research Council of Jordan University of Science and Technology, grant 211/2007.

References

1. Birjmohun RS, Stroes ESG, Kastelein JJP (2006) Raising high-density lipoprotein cholesterol for better prevention of cardiovascular disease. Future Lipidology 1(1): 47-54.

2. Zhong S, Sharp DS, Grove JS, Bruce C, Yano K, et al. (1996) Increased coronary heart disease in Japanese-American men with mutation in the cholesteryl ester transfer protein gene despite increased HDL levels. J Clin Invest 97(12): 2917-2923.

3. Bekaert ED, Alaupovic P, Gibson CK, Norum RA, Laux MJ, et al. (1992) Isolation and partial characterization of lipoprotein A-II (LP-A-II) particles of human plasma. Biochim Biophys Acta 1126(1): 105-113.

4. Acker BAV, Botma GJ, Zwinderman AH, Kuivenhoven JA, Dallinga-Thie GM, et al. (2008) High HDL cholesterol does not protect against coronary artery disease when associated with combined cholesteryl ester transfer protein and hepatic lipase gene variants. Atherosclerosis 200(1): 161-167.

5. Tall AR (2007) CETP inhibitors to increase HDL cholesterol levels. N Engl J Med 356(13): 1364-1366.

6. Tall AR, Charvet LY, Wang N (2007) The failure of torcetrapib: was it the molecule or the mechanism? Arterioscler Thromb Vasc Biol 27(2): 257-260.

7. Nissen SE, Tardif JC, Nicholls SJ, Revkin JH, Shear CL, et al. (2007) Effect of torcetrapib on the progression of coronary atherosclerosis. N Engl J Med 356(13): 1304-1316.

8. Kastelein JJ, Leuven SIV, Burgess L, Evans GW, Kuivenhoven JA, et al. (2007) Effect of torcetrapib on carotid atherosclerosis in familial hypercholesterolemia. N Engl J Med 356(16): 1620-1630.

9. Ansell BJ, Watson KE, Fogelman AM, Navab M, Fonarow GC (2005) High-density lipoprotein function recent advances. J Am Coll Cardiol 46(10): 1792-1798.

10. Trinder M, Boyd JH, Brunham LR (2019) Molecular regulation of plasma lipid levels during systemic inflammation and sepsis. Curr Opin Lipidol

11. Atmeh, RG, Robenek H (1996) Measurement of small high density lipoprotein subclass by an improved immunoblotting technique. J Lipid Res 37(11): 2461-2469.

12. Atmeh RF, Elrazeq IOA (2005) Small high density lipoprotein subclasses: some of their physico-chemical properties and stability in solution. Acta Biochim Pol 52(2): 515-525.

13. Tian L, Long S, Fu M, Liu Y, Xu Y, et al. (2011) Characteristics of high-density lipoprotein subclasses distribution for subjects with desirable total cholesterol levels. Lipids Health Dis 10: 10-64.

14. Atmeh RF, Abuharfeil NM (1993) Electroblotting of proteins on agarose gel containing specific antibodies. Electrophoresis 14(1-2): 69-72.

15. Cheung MC, Albers JJ (1984) Characterization of lipoprotein particles isolated by immunoaffinity chromatography. Particles containing A-I and A-II and particles containing A-I but no A-II. J Biol Chem 259(19): 12201-12219.

16. Atmeh RF (1990) Isolation and identification of HDL particles of low molecular weight. J Lipid Res 31(10): 1771-1780.

17. Leroy A, Toohill KL, Fruchart JC, Jonas A (1993) Structural properties of high density lipoprotein subclasses homogeneous in protein composition and size. J Biol Chem 268(7): 4798-4805.

18. Ohta T, Hattori S, Nishiyama S, Matsuda (1988) Studies on the lipid and apolipoprotein compositions of two species of apoA-I-containing lipoproteins in normolipidemic males and females. J Lipid Res 29(6): 721-728.

19. Otvos JD, Collins D, Freedman DS, Shalaurova I, Schaefer EJ, et al. (2006) Low-density lipoprotein and high-density lipoprotein particle subclasses predict coronary events and are favorably changed by gemfibrozil therapy in the Veterans Affairs High-Density Lipoprotein Intervention Trial. Circulation 113(12): 1556-1563.

20. Sharrett AR, Ballantyne CM, Coady SA, Heiss G, Sorlie PD, et al. (2001) Coronary heart disease prediction from lipoprotein cholesterol levels, triglycerides, lipoprotein(a), apolipoproteins A-I and B, and HDL density subfractions: The Atherosclerosis Risk in Communities (ARIC) Study. Circulation 104(10): 1108-1113.

21. Nambi V, Ballantyne CM (2005) Fat, fit, and leading the charge: the evolution of measuring high-density lipoprotein subpopulations. Arterioscler Thromb Vasc Biol 25(10): 2013-205.

22. Asztalos BF, Sloop CH, Wong L, Roheim PS (1993) Two-dimensional electrophoresis of plasma lipoproteins: recognition of new apo A-I-containing subpopulations. Biochim Biophys Acta 1169(3): 291-300.

23. Asztalos BF, Collins D, Cupples LA, Demissie S, Horvath KV, et al. (2005) Value of high-density lipoprotein (HDL) subpopulations in predicting recurrent cardiovascular events in the Veterans Affairs HDL Intervention Trial. Arterioscler Thromb Vasc Biol 25(10): 2185-2191.

24. Asztalos BF, Roheim PS, Milani RL, Lefevre M, McNamara JR, et al. (2000) Distribution of ApoA-I-containing HDL subpopulations in patients with coronary heart disease. Arterioscler Thromb Vasc Biol 20(12): 2670-2676.

25. Furukawa T, Hinou H, Takeda S, Chiba H, Nishimura SI, et al. (2017) An Efficient Glycoblotting-Based Analysis of Oxidized Lipids in Liposomes and a Lipoprotein. Chembiochem 18(19): 1903-1909.

26. Miida T, Inano K, Yamaguchi T, Tsuda T, Okada M (1997) LpA-I levels do not reflect pre beta1-HDL levels in human plasma. Atherosclerosis 133(2): 221-226.

27. Krimbou L, Tremblay M, Davignon J, Cohn JS (1997) Characterization of human plasma apolipoprotein E-containing lipoproteins in the high density lipoprotein size range: focus on pre-beta1-LpE, pre-beta2-LpE, and alpha-LpE. J Lipid Res 38(1): 35-48.

28. Hattori H, Kujiraoka T, Egashira T, Saito E, Fujioka T, et al. (2004) Association of coronary heart disease with pre-beta-HDL concentrations in Japanese men. Clin Chem 50(3): 589-595.

29. Tashiro, Miyazaki O, Nakamura Y, Miyazaki A, Fukamachi I, et al. (2009) Plasma pre beta1-HDL level is elevated in unstable angina pectoris. Atherosclerosis 204(2): 595-600.

30. Cavigiolio G, Shao B, Geie EG, Ren G, Jay W Heinecke, et al. (2008) The interplay between size, morphology, stability, and functionality of high-density lipoprotein subclasses. Biochemistry 47(16): 4770-4779.

31. Rye KA (2014) Biomarkers associated with high-density lipoproteins in atherosclerotic kidney disease. Clinical and Experimental Nephrology 18(2): 247-250.

32. Souza DJA, Vindis C, Hansel B, Salvayre AN, Therond P, et al. (2008) Metabolic syndrome features small, apolipoprotein A-I-poor, triglyceride-rich HDL3 particles with defective anti-apoptotic activity. Atherosclerosis 197(1): 84-94.

33. Hussein H, Saheb S, Couturier M, Atassi M, Orsoni A, et al. (2016) Small dense high-density lipoprotein 3 particles exhibit defective antioxidative and anti-inflammatory function in familial hypercholesterolemia: Partial correction by low-density lipoprotein apheresis. J Clin Lipidol 10(1): 124-33.

34. Bekaert ED, Alaupovic P, Gibson CSK, Laux MJ, Pelachyk JM, et al. (1991) Characterization of apoA- and apoB-containing lipoprotein particles in a variant of familial apoA-I deficiency with planar xanthoma: the metabolic significance of LP-A-II particles. J Lipid Res 32(10): 1587-1599.