Postprandial determination of Apo B-48 levels in whole plasma of healthy young individuals by a double-sandwich ELISA*

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Abstract

High postprandial concentrations of chylomicrons and its remnants are correlated with an atherosclerosis progression. Apolipoprotein B-48 is an essential component of these lipoproteins and appears to be a suitable marker for clinical studies of postprandial lipid metabolism and its relationship to cardiovascular risk. A double-sandwich ELISA was developed for routine Apo B-48 quantification in untreated human plasma. The postprandial behavior of Apo B-48 in healthy young individuals was described employing the method, and correlations between plasma Apo B-48 and lipid and clinical parameters. A polyclonal antibody directed against the carboxy-terminal extreme of Apo B-48 was developed, and appropriate calibrators were prepared using an affinity column to which the developed antibody was attached. A mouse monoclonal antibody able to recognize both Apo B-48 and Apo B-100 was used as second antibody, and an enzyme-coupled anti-mouse IgG was used as third antibody. The plasma Apo B-48 levels were determined in 42 healthy young individuals of both sexes in the fasting state and hourly after the consumption of a breakfast with 40 g fat. The technique showed an intra-assay variation of 3,0-3,8%. Plasma Apo B-48 fluctuated between 0,5 and 0,8 mg/L in the fasting state, with an hourly increase to reach a maximum between 4,6 and 8,4 mg/L at the third postprandial hour. The postprandial Apo B-48 area under curve and third hour Apo B-48 showed a strong correlation with body mass index (r=0,58 and 0,8 respectively). This paper presents a novel assay that makes Apo B-48 quantification easier and faster with adequate precision and without requirements for sample processing. Plasma Apo B-48 in healthy young individuals showed postprandial kinetics characterized by low fasting concentrations that increase to a peak about 3 hours after a meal and return to low values after 6 hours. *(Mantilla G, Sierra ID, Mendivil CO, Pérez CE. Postprandial determination of Apo B-48 levels in whole plasma of healthy young individuals by a double-sandwich ELISA.* **Med**UNAB *2003; 6:130-6).*

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Introduction

Apo B-48, a lipoprotein synthesized at the human gut from the same gene as Apo B-100 employing a mRNA edition mechanism, is an essential component of chylomicrons and its remnants, with one only copy of the protein per lipoprotein particle, and therefore it is an excellent marker of postprandial lipid metabolism.[1]

Apo B-48 and Apo B 100 share an important part of their aminoacids chain and biochemical properties, this fact has difficulted the development of suitable techniques for Apo B-48 quantification. Besides, the fasting concentrations of Apo B-100 are much higher than those of Apo B-48, making excellent specificity an indispensable requirement for any Apo B-48 quantification method.

Several studies have shown the utility of Apo B-48 as a marker and predictor of atherosclerotic disease. In a case-control study,[2] Simon et al showed that the Apo B-48/Apo B 100 ratio was higher among individuals with angiographic coronary artery disease than among those without it. The association persisted after correcting for potential confounders and showed a dose-response fashion. In another case-control study,[3] Karpe et al found that the Apo B-48 level in the Sf 20-60; lipoprotein subfractions was related to the rate of progression of angiographic coronary

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atherosclerotic disease in men, raising a great interest on the study of triglyceride-rich lipoproteins (TRL) remnants. The same group studied the correlation between remnant-like particles composition and atherosclerosis in a cohort of middle aged men, evidencing a strong correlation between postprandial TRL Apo B-48 and remnant-like lipoproteins cholesterol which, in turn, correlated with carotid atherosclerosis.[4]

Currently for Apo B-48 quantification it is necessary to perform a series of complicated and tiresome procedures including ultracentrifugation, delipidation, separation and visualization in SDS-PAGE gels, gel staining and densitometry.[5-7] Some investigators have used affinity chromatography,[8] and methods combining SDS-PAGE and western blot with anti-Apo B-48 antibodies based recognition followed by enhanced chemiluminescence.[9] However, in all the reports the results obtained are very different, and it isn't not possible to determine if such variability among the results is owing to technical imprecisions.

Two previous reports of ELISA procedures for Apo B-48 quantification exist. In 1998, Uchida et al, using a monoclonal antibody directed against Apo B-48 developed a simple-sandwich ELISA that allows easy quantification, however, the authors only recommend its use for monitoring of Apo B-48 levels in isolated lipoproteins, not whole plasma.[10] In 2,000, Lorec et al described a competitive ELISA suitable for routine Apo B-48 determination.[11] The technique showed to be specific and precise, but the Apo B-48 concentration in the primary calibrator was determined by the SDS-PAGE method, raising concern about its accuracy.

We developed a double-sandwich ELISA using polyclonal antibodies raised against the carboxy-terminal extreme of Apo B-48. The calibrator used was an extract of pure Apo B-48 prepared with an affinity column with the anti Apo B-48 antibody (aAB-48 Ab) attached to it. This allowed us to guarantee adequate accuracy and sensitivity. The assay can be performed in untreated plasma samples.

Materials and methods

Antibody production. 3 adult rabbits New Zealand with 3-5 Kg weight and 12-15 weeks of age were selected, and 10 ml of blood from each rabbit was obtained for pre-immune serum preparation. Each rabbit was immunized with a peptide representing the 6 last aminoacids of the Apo B-48 sequence (LQTYMI). This peptide was chosen according to previous structural studies,[12] and produced at the immunology institute of the University by solid-phase synthesis. The peptide was coupled to bovine serum albumin (BSA) following the one-step protocol.[13] The immunogen concentration was measured employing the Lowry method with BSA as standard.

The animals received an injection of an emulsion containing 300 µg of antigen (peptide-BSA) in 500 µL of Freund's adjuvant. Four immunizations were made with 3-weeks intervals. 10 days after each immunization a blood sample was drawn from the rabbits and its serum analyzed by dot-blot techniques. 10 days after the fourth immunization one of the rabbits was completely bled obtaining approximately 150 mL of blood. The separated serum was inactivated at 56° C for 30 minutes. Then, the antibodies were purified by precipitation with a saturated ammonium salt solution. The antibodies extract was passed through a DEAE affinity column according to the protocol proposed by Cooper.[14] The fractions positive to the peptide were determined coupling the peptide to an ELISA plate and making aliquots of the fractions react with the antigen. Subsequently we revealed them with an anti-rabbit IgG antibody coupled to alkaline phosphatase employing para-nitrophenyl phosphate (PNPP) as substrate. Apo B-48 and Apo B-100 were visualized in a 6% PAGE gel in denaturing and non-denaturing conditions. The separated proteins were transferred to a 0,2 µm PVDF membrane for the immunoblotting.[7]

The electro-transfer was made in a semi-dry transfer device (sigma semi-dry system). The gel, the membrane and the filters were moistened in the transfer buffer (Boric acid 50 mM, Sodium Tethraborate 10 mM)[15] and preserved for later analyses of aAB-48 Ab specificity.

Preparation of an Apo B-48 extract. 250 µL of the purified antibody were mixed with 1 mL of Sepharose-Protein A for 3 hours at 4° C. The proteic extract was added and left reacting overnight at 4° C. Later the affinity column was set up and the protein was eluted and visualized in a 6% SDS-PAGE gel, stained with silver nitrate and preserved to posterior analyses.

Determination of the antibody title. The anti-peptide IgG obtained after the fourth challenge (third boost) were separated by precipitation with a saturated ammonium salt solution. This IgG extract was pre-absorbed with BSA and its title was determined by dot-blot, showing a positive reaction up to a dilution of 1:3,200.

Definitive purification of the antibody. After title determination, a rabbit was completely bled and the IgG precipitated and purified in a DEAE ionic interchange column. The eluted was pre-absorbed with BSA to avoid masking of the response against the peptide.

Determination of antibody specificity. The above-mentioned western-blot membranes with separated Apo B-48 and Apo B-100 were incubated with the purified IgG and the pre-immune serum in a 1:150 dilution, and later confronted to anti-rabbit IgG and revealed with BCIP/NBT. The same procedure was made in non-denaturing conditions, to confirm the conformational specificity of the aAB-48 Ab (figures 1 and 2).

Calibrators preparing. An affinity column was made employing sepharose-protein A as matrix with the aAB-48 Ab coupled to it. New proteic extract isolation was performed following the protocol described above and passed through the column. The eluted was analyzed in a 6% SDS-PAGE and stained with silver nitrate. The same extract underwent a non-denaturing conditions electrophoresis (figure 3). From this extract, calibrators with concentrations of 0.5, 1.0, 2.0, 4.0, 8.0 and 12 mg/L were prepared.

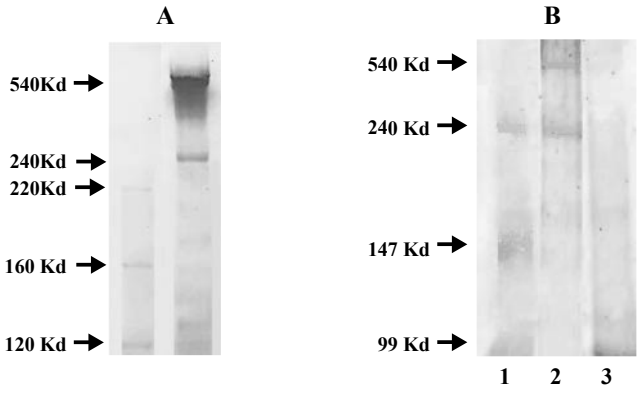

Figure 1. First Western Blot (separation in denaturing conditions). WM = weight marker, SPE = serum proteic extract, P IgG = purified IgG, PIS = pre-immune serum. The molecular weights included in the marker are shown with a black triangle, those obtained with computer software are shown with a white triangle. The anti-peptide IgG recognizes both Apo B-48 and Apo B-100 in denaturting conditions.

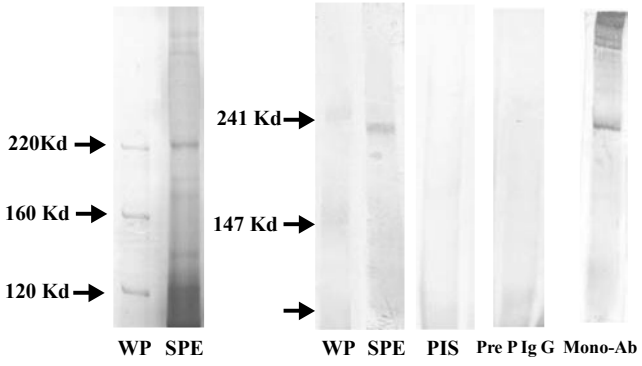

Figure 2. Western Blot in non-denaturing conditions. WM = weight marker, SPE= serum proteic extract, P IgG = purified IgG, PIS= pre-immune serum, Pre P IgG = IgG extract pre-incubated with 1,0 µg of the peptide, Mono-Ab = Commercial monoclonal antibody able to recognize both Apo B-48 and Apo B-100. The produced antibody was able to recognize Apo B-48 but not Apo B-100 , this ability is abolished by the pre-incubation of the IgG extract with the peptide indicating a high specificity of the antibody for the epitope only exposed in Apo B-48.

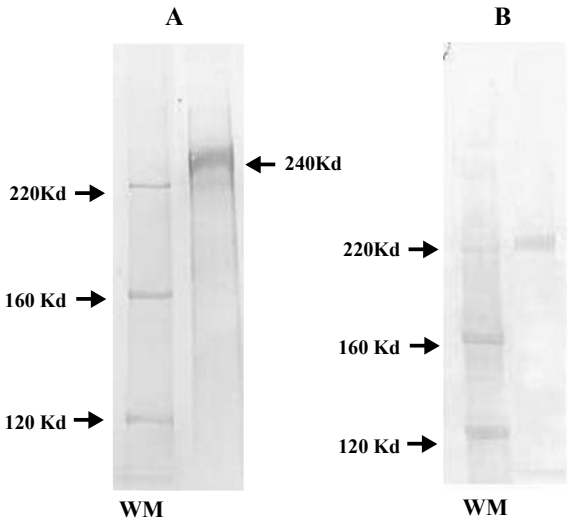

Figure 3. Verification of the Apo B-48 extract purity. WM = weight marker. Panel A: Denaturing conditions (SDS-PAGE 6%), panel B: Non-denaturing conditions (PAGE 6%).

ELISA Protocol. Apo B-48 was coupled to the ELISA plates and fronted to dilutions of the aAB-48 Ab ranging from 1:50 to 1:6,400. The title that generated the absorbance closest to 0,500 was chosen.

The second antibody (commercial monoclonal antibody directed against N-terminal extreme of Apo B-48 and Apo B-100 (ICN 59410) was employed in a dilution of 1:200 according to manufacturer's recommendations.

For the determination of the third antibody dilution, the aAB-48 Ab was coupled to the microELISA wells as follows: a 1:200 dilution of the antibody in covering buffer (sodium carbonate 0,16%, sodium carbonate 0,3%, sodium azide 0,02%) was prepared, and 100 µL of the solution were incubated overnight at 4° C, then washed with PBS and incubated 2 more hours in blocking buffer (gelatin 0,15% in PBS). The aAB-48 Ab covered wells were incubated with Apo B-48 7mg/L. The second antibody was added in the chosen title and then alkaline phosphatase-coupled anti-mouse IgG was added in several dilutions and revealed with para-nitro phenyl phosphate (PNPP). Again, the title producing the absorbance closest to 0,500 was chosen.

Identified the optimal dilutions for the three antibodies, a calibration curve was drawn with the calibrators. Simultaneously a calibration curve was drawn for Apo B-100 (SIGMA) and albumin as controls (figure 4).

Subjects. The Subjects were taken of the protocol "Evaluation of the behavior of the lipidic profile after intake of the test breakfast in young healthy students of the National University of Colombia, 1999" carried out by the Unit of Lipids and Diabetes of the Medical School of the National University of Colombia, in the second semester of the 1.999.[16]

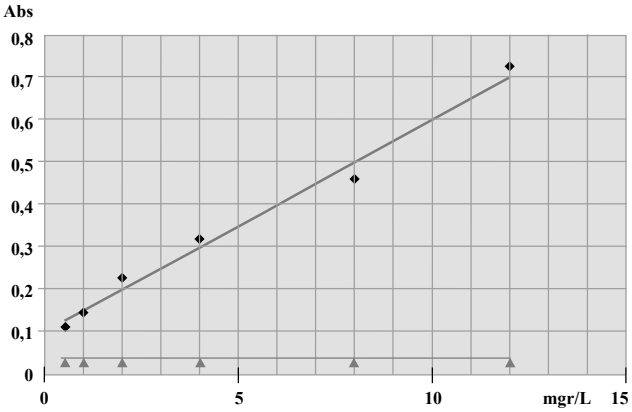

Figure 4. Calibration curve for Apo B-48 and Apo B-100. The Apo B-48 calibration curve was lineal in a range from 0,5 to 12 mg/L (r = 0,987). A flat behavior was observed for Apo B-100, with absorbances lesser than 0,035.

50 non drinkers, non smokers individuals of both sexes (25 men and 25 women) aged 18-24 years, with no medical antecedents were studied. Patients with body mass index (BMI) >25, lipid alterations (triglycerides > 150 mg/dL, cholesterol > 200 mg/dL, HDL < 40 mg/dL) in preliminary analyses, taking any medication able to modify plasma lipids or pregnant were excluded. The participants underwent a regular medical examination including height, weight, waist and hip circumferences, blood pressure and interview about personal and familial antecedents. They ate a breakfast with 40 g fat consistent in 200 mL of milk, 2 eggs fried in 5 mL safflower oil, 30 g of margarine and 40 g or bread.

Venous blood samples were drawn in precooled sterile EDTA tubes (Vacutainer, Beckton Dickinson) in the fasting state and hourly after the meal. The sample was divided in 2 tubes, one of which was used for preliminary analyses and triglycerides while the other was added with 5 mg/L aprotinin and 1 mg/L phenyl methyl sulphonyl fluoride (PMSF) and stored quickly at -70° C for later Apo B-48 quantification. Eight subjects were excluded, because of fasting total cholesterol > 200 mg/dL, HDL cholesterol <40 mg/dL or triglycerides > 200 mg/dL.

Blood plasma was separated by ultracentrifugation. 100 µL of each plasma sample were added to the aAB-48 Ab coated wells and then incubated at 37° C for 2 hours. After the plate was washed 5 times with PBS, 100 µL of the monoclonal antibody diluted 1:400 in PBS were added and incubated 2hours at 37° C. After 5 additional washes with PBS, 100 µL of the conjugated third antibody diluted 1:5,000 in PBS were added. The samples ELISA was revealed with 100 µL PNPP 1mg/mL, incubated at room temperature for 45 minutes and the reaction was stopped by the addition of 50 µL NaOH 3N.

The plates for Apo B-48 quantification included 2 PBS controls (Blank), 1 albumin 20 mg/L and 2 Apo B-100 (4 and 8 mg/L) negative controls and 2 Apo B-48 (4 and 8 mg/L) internal controls. The absorbances were measured with the Labsystems Uniskan II ELISA reader. Variations in absorbencies between repetitions were accepted if lesser than 10%.

Statistic analyses. Mean and standard deviations were calculated for physical examination data, plasma Apo B-48 concentrations and calibrators Apo B-48 concentrations. Variation percentages were calculated. Differences in Apo B-48 concentrations according to sex were compared employing Student's t test.

Pearson's correlation coefficients between plasma Apo B-48 and age, BMI, waist circumference, total cholesterol, HDL cholesterol, LDL cholesterol, tryglicerides and Apo B-48 were calculated.

Results

The characteristics of the participants are shown at the table 1. We found a great variability in the postprandial behavior of triglycerides among individuals. The greater percentage of individuals showed the greatest triglycerides levels in the third postprandial hour. The HDL cholesterol and total cholesterol had only slight variations along the study, and they were not statistically significant (table 2).[16]

Table 1. Characteristics of the study group

	Sample	Male	Female
n=	42	19	23
Age	19,7	20,2	19,2
Family History			
Diabetes	40%	44%	42%
Hypertension	70%	72%	68%
Dyslipidemia	43%	40%	46%
Weight (Kg)	59,8	64,9	54,8
Height (cm)	164,7	170,5	159
BMI	22.8	22,2	23,4
WHR	0,77	0,81	0,73
SBP	102,4	106	98,8
DBP	68,85	69,2	68,5

Data are mean. Family history of a given condition was considered positive if present in up to a second degree relative. BMI= Body Mass Index, WHR= Waist-to-Hip Ratio, SBP= Systolic Blood Pressure, DBP= Diastolic Blood Pressure

Table 2. TAG and C-DHL characteristics of the study group

SAMPLE	TAG (mg/dL)	C-HDL (mg/dL)
Basal	84,2 ± 31,.4	48.0 ± 9.6
1	106 ± 41,2	47,4 ± 10.7
2	135,9 ± 61,3	47,4 ± 9.6
3	139,2± 64,5	46,1 ± 12.9
4	131,4 ± 66,8	46,8 ± 10
5	110,8 ± 60,9	46,7 ± 10.4
6	101,1 ± 48,5	48,9 ± 12.4

Postprandial triglycerides and HDL- cholesterol average levels in the whole sample TAG: triglycerides, C-HDL: HDL-cholesterol (mgr/dL ± SD)

Appropriate calibration curve were obtained with Apo B-48 extract. The curve was linear from 0,5 to 12 mg/L (r=0,987; figure 4). Simultaneous a calibration curve was drawn for Apo B-100 and no detection was observed.

The lowest limit of Apo B-48 detection was 0,150 mg/L. The calibrators concentrations of Apo B-48 was determined by the ELISA were 7,94 mg/L (IC 95%= 7,7-8,18) for the 8 mg/L calibrator and 3,94 mg/l (IC 95%= 3,81-4,05) for the 4 mg/L calibrator. Figure 5 shows the postprandial Apo B-48 curves. Apo B-48 concentrations in the fasting state ranged between 0,5 and 0,8 mg/L, raising to a concentration up to 10 fold the basal concentration (4,6-8,4 mg/L) at the third hour, and returning to a sixth hour value close to the basal concentration (1,14 mg/L higher, IC 95%= 0,75-1,52).

No statistically significant difference was found in fasting or postprandial Apo B-48 concentrations between sexes at any hour. (p>0,4). The BMI showed an important correlation with Apo B-48 area under curve (AUC) (r=0,58) and third hour Apo B-48 (r=0,8). Fasting total cholesterol, HDL cholesterol, and LDL cholesterol didn't show correlation to fasting or postprandial Apo B-48 at any hour. A strong correlation was found between plasma Apo B-48 and plasma triglycerides, especially at the third hour (figure 6).

Discussion

Non cross-reactive rabbit antibodies can be raised against human apoliproteins, using synthetic peptide as immunogens.[10, 12] The specificity of anti Apo B-48 antibodies obtained was demonstrated by inmunoblotting.

Apo B-48 quantification is one of the biggest difficulties that lipid investigators have fronted. Its low stability and solubility, short half-life and low plasma concentration are some of the characteristics that make it a hard protein-to-quantify. We developed a precise, accurate and easy to use method for Apo B-48 determination in whole untreated human plasma that enables lipid investigators to explore postprandial lipoprotein metabolism with special emphasis in gut-derived lipoproteins allowing to determine relative contributions of chylomicrons and its remnants to postprandial lipemia phenomena.

The Apo B-48 had a behavior postprandial like to the triglycerides in the group of young healthy, (r = 0.957).[17] The baseline, mean levels of Apo B-48 were 0.59 g/ml, to reach a maximum value at the third hour (value average 5.3 g/ml). Later on the Apo B-48 concentration begins to descend until reaching a near value to the basal one around the sixth hour. These results coincide with previous works in those that you evaluates the kinetics of the Apo B-48 in different fractions separated by ultracentrifugation.[6, 17-19] The fact that the Apo B-48 has a similar kinetics to the observed one for the triglycerides in this group it was of being expected if one keeps in mind that the chylomicron is the lipoprotein, in percentage, richer in triglycerides and the responsible for the transport of the lipids coming from the diet.

The use of the controls in each one of the boards used in our study allows to guarantee the specificity of the antibody in the first place, since so much in the calibration curve like during the whole procedure, the readings of the Apo patterns B-100 they didn't go superior at 0.036, what assures that in none of the utilized boards during the quantification, there was crossed reaction with this apoproteín. On the other hand the use of two negative controls of PBS allowed to verify the homogeneity of the utilized boards and, finally when placing the Apo patterns B-48 you could verify reproducibility of the rehearsal.

The concentration average of the controls of 4 and 8 g/ml in the realized measures along the rehearsal was of 7.94 and 3.94 g/ml respectively, with a standard deviation 0.24 and 0.15 and a coefficient of variation of 3.0% and 3.8% respectively, that that statistically doesn't have meaning (p=0.20) and it demonstrates the reproducibility of the rehearsal.

When carrying out the histogram of the distribution of frequencies in absorbencies and concentrations of the patterns, they were a similar behavior of the normal curve, in and of itself the test of Student was used to compare the absorbance data and concentration with those wanted according to that settled down in the calibration curve, tracing a level of significance of 0.05 (95% of probability of certainty in the summations) what contributes a good grade of dependability in the obtained data with this procedure.

In the consulted literature we were not some report with respect to the levels Apo B-48 in total serum, all make reference to the Apo B-48 concentrations in the different

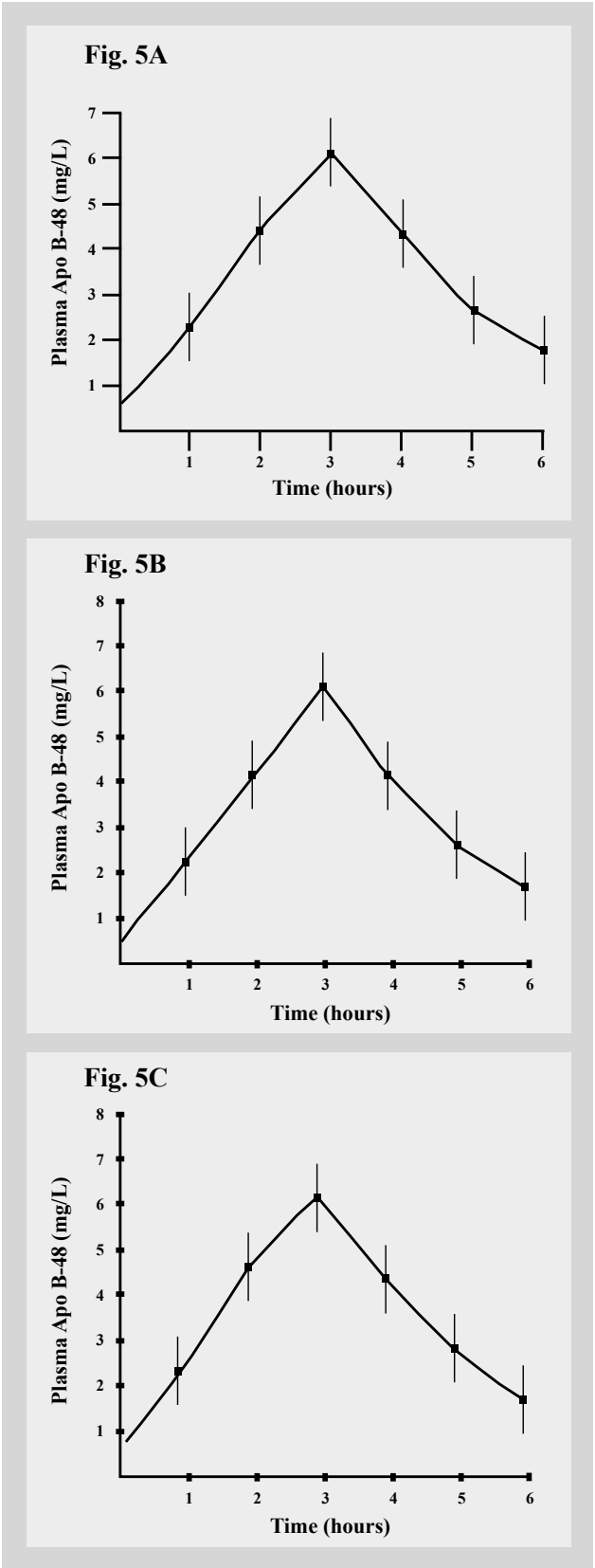

Figure 5. Postprandial Apo B-48 levels in the whole sample and discriminated by sex. Values are in mean ± DS.

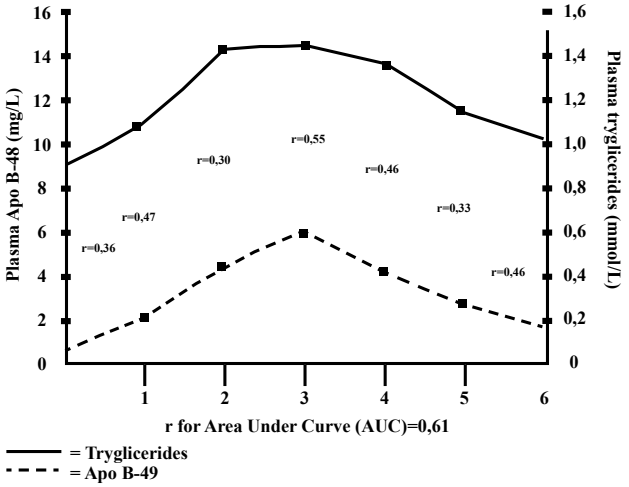

Figure 6. Correlation between plasma tryglicerides and Apo B-48 during postprandial lipemia; r for area under curve = 0,61.

fractions obtained by ultracentrifugation and classified according to their flotation coefficient, using different methodologies;[6, 20-21] however, all differ in the reported concentrations, perhaps as consequence of the different methodologies used in their extraction. This hinders the task of corroborating the obtained results.

The kinetics shown by Apo B-48 in this study are very similar to those of triglycerides, Illustrating well that the chance in the measured of Apo B-48 correspond to the expected transient accumulation of chylomicron particles in the circulation after lipid meal. However, as noted above, the correlation was not absolute, and hence there is an important range of variability in the lipid content of chylomicrons and its remnants among individuals.

The finding of a very strong association between third hour Apo B-48 and BMI indicates that body mass is related to chylomicron synthesis and clearance, suggesting another link between overweight and atherosclerosis.

The development of this quick and direct method for the quantification of the Apo B-48 in serum it is constituted on the dot of departure for future studies about the lipids kinetic and the importance of Apo B-48 quantification ace an indirect but easier marker of triglyceride-rich lipoproteins lipid content in the postprandial state.

Other potential applications of the method are several circumstances where Apo B-48 measurement is highly relevant, these include:

- Evaluation of the effect of lipid-lowering medications on postprandial lipemia.[22]
- Several mutations Effect and genetic polymorphisms in postprandial lipemia.[23]
- Impact of dietary interventions in characteristics of postprandial lipemia.[24]

- Relation between insulin resistance, hyperinsulinism and characteristics of postprandial lipemia.[25]
- Ability of fasting plasma Apo B-48 to predict postprandial lipemia.[26]
- Relationships between clinical features (BMI, WHR, body weight, fat distribution pattern) and characteristics of postprandial lipemia.[27,28]

Prospective studies are guaranteed to determine the role of fasting and postprandial Apo B-48 as a cardiovascular disease predictor, and the way it could interact with other risk factors.

Anyway are need next studies to determine the way the above described method is modificated by hemolysis, high plasma lipid concentrations, sample preservation procedures and other variables, but we believe that it is an important step ahead in the postprandial lipid metabolism study.

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