Enzyme Linked Immunosorbent Assay for Human Apolipoprotein C-III

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Summary: A sandwich enzyme linked immunosorbent assay (ELISA) for apolipoprotein C-III was developed. Anti apolipoprotein C-III immunoglobulins were used both for coating of microtitre plates and for the preparation of an anti apolipoprotein C-III horse-radish peroxidase conjugate.

Under optimized assay conditions, the sensitivity lies around 0.3 ng apolipoprotein C-III with a working range of 1 to 6 ng. Standard curves are parallel for purified apolipoprotein C-III, for untreated plasma and for lipoprotein fractions. Delipidation did not affect the content of apolipoprotein C-III in plasma. The assay was evaluated by comparison with an immunonephelometric assay for apolipoprotein C-III, yielding a correlation coefficient of 0.982 (n = 79). The mean intra- and interassay CV for the whole working range of the assay were 3.6% and 4.2% respectively.

Enzyme linked immunosorbent assay (ELISA) für menschliches Apolipoprotein C-III

Zusammenfassung: Ein sandwich enzyme linked immunosorbent assay (ELISA) für Apolipoprotein C-III wurde entwickelt. Für die Beschichtung von Mikrotiterplatten sowie die Herstellung eines anti-Apoliprotein C-III-Meerrettichperoxidase-Konjugats wurden anti-Apolipoprotein C-III-Immunglobuline benutzt.

Unter optimierten Bedingungen beträgt die Empfindlichkeit des Bestimmungsverfahrens etwa 0,3 ng Apolipoprotein C-III bei einem Einsatzbereich von 1—6 ng. Die Standardkurven für gereinigtes Apolipoprotein C-III, unbehandeltes Plasma und Lipoproteinfraktionen verlaufen parallel. Die Lipidabtrennung beeinflußte den Gehalt von Apolipoprotein C-III im Plasma nicht. Das Bestimmungsverfahren wurde durch Vergleich mit einer immunnephelometrischen Methode zur Bestimmung von Apolipoprotein C-III geprüft; der Korrelationskoeffizient betrug 0,982 (n = 79). Die mittleren Variationskoeffizienten betrugen innerhalb der Serie 3,6% und von Tag zu Tag 4,2%.

Introduction

Until recently, the investigation of various types of dyslipoproteinaemia was mainly confined to the measurement of the plasma lipid concentrations. A better understanding of the major physiological functions of the apolipoproteins has stressed the importance of their quantification in plasma (1, 2). Most studies have focused on the major apolipoproteins of HDL (apolipoproteins A-I and A-II) and LDL (apolipoprotein B).

The C- and E apolipoproteins play an important role in lipoprotein metabolism. Apolipoprotein C-III, being a major constituent of triglyceride-rich lipoproteins and a minor constituent of HDL, is known as an inhibitor of lipoprotein lipase in vitro (3, 4) and might also prevent the hepatic uptake of triglyceride-rich lipoproteins (5, 6).

Immunological assays have been developed mainly for the apolipoproteins A-I, A-II and B proteins by a variety of techniques (7, 8). Quantitation of

apolipoprotein C-III by radioimmunoassay (RIA) (9, 10), electroimmunodiffusion (EID) (11), a competitive enzyme linked immunosorbent assay (ELISA) (12) and an immunonephelometric assay (INA) (13) have been previously reported.

This report describes the technical optimization and the validation of a sandwich enzyme linked immunosorbent assay (ELISA), which was developed for the measurement of human apolipoprotein C-III in plasma and in lipoprotein fractions. Results are evaluated by comparison with other techniques, described previously.

Material and Methods

Isolation of apoliproteins and production of antisera

The three apolipoprotein C-III isoforms (i.e. apolipoproteins C-III$_0$, C-III$_1$ and C-III$_2$) were isolated from human apolipoproteins of Very Low Density Lipoproteins by a combination of gel filtration on a Sephacryl S 200 column and ion-exchange chromatography on a DEAE-cellulose column, as described previously (13). The homogeneity of the apolipoprotein C-III preparation, used as primary standard and immunogen, was checked by electrophoresis on polyacrylamide gels, containing either 8 mol/l urea (14) or 1 g/l sodium dodecyl sulphate (15), by immunodiffusion with antisera against apolipoprotein A-I, A-II, B, C-III, E and albumin, and by amino acid analysis, based on the composition of purified apolipoprotein C-III (16).

Apolipoprotein A-I and apolipoprotein A-II were purified from apo High Density Lipoproteins by ion exchange chromatography (17, 18). Water soluble apolipoprotein B was prepared from Low Density Lipoproteins (d = 1.030 − 1.050 kg/l) as described by Cardin et al. (19) and apolipoprotein E were isolated from apolipoproteins of Very Low Density Lipoproteins by high performance liquid chromatography using an Ultro-Pak TSK-G 3000 SW column (LKB, Broma, Sweden) (20, 21).

The antisera to these purified apolipoproteins were raised in rabbits as previously described (13, 18, 22, 23).

Preparation of the coater and the enzyme-antibody conjugate

An apolipoprotein C-III affinity column was prepared by covalent linkage of 8 mg apolipoprotein C-III to 1 g CNBr-activated Sepharose 4 B (Pharmacia, Uppsala, Sweden) (24). Anti human apolipoprotein C-III immunoglobulins were isolated from a rabbit antiserum by affinity chromatography, according to the standard procedure, and were used both for coating and for the preparation of an enzyme-antibody conjugate. For this purpose 2 mg of horse-radish peroxidase was covalently linked to 4 mg purified anti-apolipoprotein C-III immunoglobulin by the periodate coupling procedure described by Nakane (25).

The immunoglobulins, used for coating, were stored at 4°C in 0.05 mol/l sodium borate, 0.15 mol/l NaCl, containing 1 g/l NaN$_3$, while the antibody-enzyme conjugate was stored in aliquots at −20°C, mixed with an equal volume of glycerol (26). This preparation was stable for at least six months, without any loss of activity.

Buffer system

A sodium phosphate buffer 10 mmol/l pH 7.4, containing 0.15 mol/l NaCl was used throughout the whole procedure for coating, incubations and washings. In addition the coating buffer contained 1 g/l NaN$_3$, whereas the assay buffer contained 10 g/l bovine serum albumin (27). To reduce non-specific binding, the wash buffer contained 0.5 ml/l Tween 20 (26).

Coating of the solid phase

Purified anti-apolipoprotein C-III immunoglobulins (110 μl) at a concentration of 25 mg/l were pipetted into each well of a polystyrene microtitre plate (Flat Bottom micro ELISA plates, 129 B, Dynatech Lab Inc., Alexandria, Virginia, 22314), excluding the outer rows which tend to produce edge effects (28). The plates were sealed (Sealing tape, Dynatech), and incubated for 3 h at 37°C before an overnight incubation at 4°C. The plates were used either immediately or stored with the coater at 4°C for a maximum of two weeks. Before use the plates were washed three times with wash buffer and incubated one hour at room temperature with the assay buffer, in order to block any residual coupling sites with albumin (29). The plates were subsequently washed three times and shaken dry.

Apolipoprotein C-III quantitation by sandwich ELISA

Standard or plasma samples (100 μl), representing 5000-fold dilutions (Microlab 1000 dilutor, Hamilton, Bonaduz, Switzerland), were pipetted into the wells of a precoated microtitre plate. The plates were covered and incubated at 37°C for 2 h. The content of each well was aspirated and the plates were washed five times. After 10000 dilution with assay buffer, the affinity purified anti-apolipoprotein C-III peroxidase conjugate (100 μl) was added to each well and plates were incubated for 2 h at 37°C. The wells were subsequently emptied and washed five times. The o-phenylenediamine hydrochloride substrate (Sigma chemical Co, St. Louis, MO 63178), at a concentration of 3 g/l, was freshly prepared in a 0.01 mol/l sodium-phosphate-citrate buffer pH 5.6, containing 0.2 g/l H$_2$O$_2$. An aliquot of this solution (100 μl) was pipetted into each well at timed intervals and left at room temperature in the dark. After 30 min the reaction was blocked by adding 100 μl of 2.5 mol/l sulphuric acid (Analytical Grade, Merck, Darmstadt, FRG). The developed colour was read within two hours on a Chromo Scan EIA Reader (Bio-Tech Instrument, Burlington, VT 05401) at 490 nm.

Calibration curves were obtained from the appropriate dilutions of purified apolipoprotein C-III$_2$, whose concentration was assessed by amino acid analysis. This primary standard was solubilized in the sodium phosphate buffer, containing the nonionic detergent Apovax (Ortho Diagnostics, Beerse, Belgium) at a concentration of 0.1 g/l, then further diluted with the ELISA assay-buffer. Sera from normolipidaemic donors, aliquoted and stored at −20°C, were used as a suitable secondary standard and control (7, 8). Standard curves were obtained by plotting the absorbance at 490 nm as function of the logarithm of the apolipoprotein C-III concentration.

Apolipoprotein C-III distribution in lipoprotein fractions

Lipoproteins were separated from 1 ml fresh disodium EDTA-plasma (1 g/l) by gel filtration on a Sepharose 6BCL column (1.6 × 90 cm), equilibrated with 2 mmol/l Na$_3$PO$_4$, 0.2 mol/l NaCl, 1 mmol/l disodium EDTA and 1 g/l NaN$_3$, pH 7.4 and eluted at 9.2 ml/h. Apolipoprotein C-III was assayed in all fractions by the ELISA procedure described above and cholesterol was assayed enzymatically (30).

Results

Optimization of the assay conditions

Optimal coating concentration and conjugate dilution were determined by using the chequerboard titration,

described by *Voller* et al. (26). A good sensitivity, together with a low zero-dose response were achieved, using a coating concentration of 25 μg purified anti-apolipoprotein C-III immunoglobulins per ml coating buffer and a final antibody-peroxidase dilution of 10000 fold.

A second coating with albumin, to block any residual binding sites on the polystyrene plates (29) decreased the intra-assay CV by a factor two. This procedure, in combination with the use of 0.5 ml Tween 20 per l wash buffer, yielded a four-fold increase of the intra-assay precision from 13.5% to 3.8%.

Standardization

As shown in figure 1, the calibration curve constructed with purified apolipoprotein C-III$_2$ and with the plasma obtained from a normolipidaemic subject and from a subject with a severe *Fredrickson* type V hyperlipoproteinaemia (triglycerides 47.25 g/l and cholesterol 8.85 g/l) were identical. Delipidation of the plasma samples (31) did not increase the apolipoprotein C-III immunoreactivity, indicating that all apolipoprotein C-III epitopes are accessible to titration in native plasma.

The calibration curves, obtained with lipoprotein fractions freshly isolated from plasma by sequential ultracentrifugation, paralleled the standard curve constructed with purified apolipoprotein C-III$_2$ (fig. 2). Identical curves were obtained after delipidation of chylomicra, Very Low Density Lipoproteins and High Density Lipoproteins.
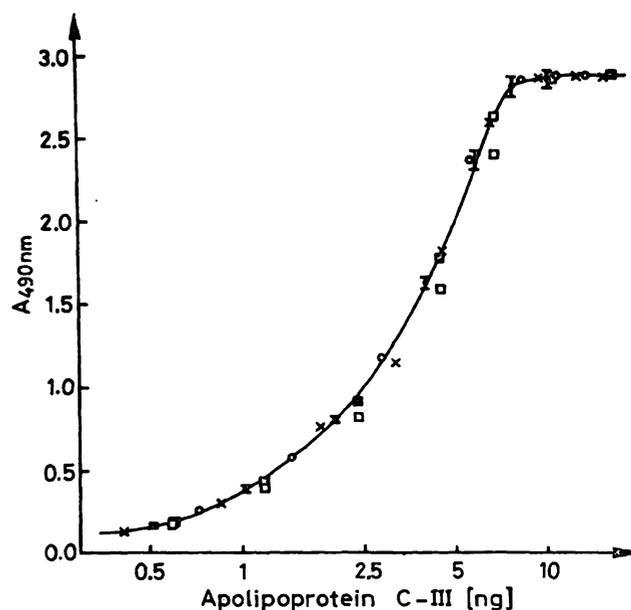

Fig. 1. Calibration curves for apolipoprotein C-III quantitation by ELISA: purified apolipoprotein (×), normolipidaemic plasma (⊕) (mean ± 1 SD), normolipaemic delipidated plasma (O), plasma from a *Fredrickson* type V patient native (■) and delipidated (□).

Sensitivity, precision and accuracy

The enzyme linked immunosorbent assay is sensitive down to 0.3 ng apolipoprotein C-III with a working range of 1 to 6 ng apolipoprotein C-III. Using a 5000 fold dilution, this range corresponds to apolipoprotein C-III plasma concentrations between 0.05 and 0.30 g/l.
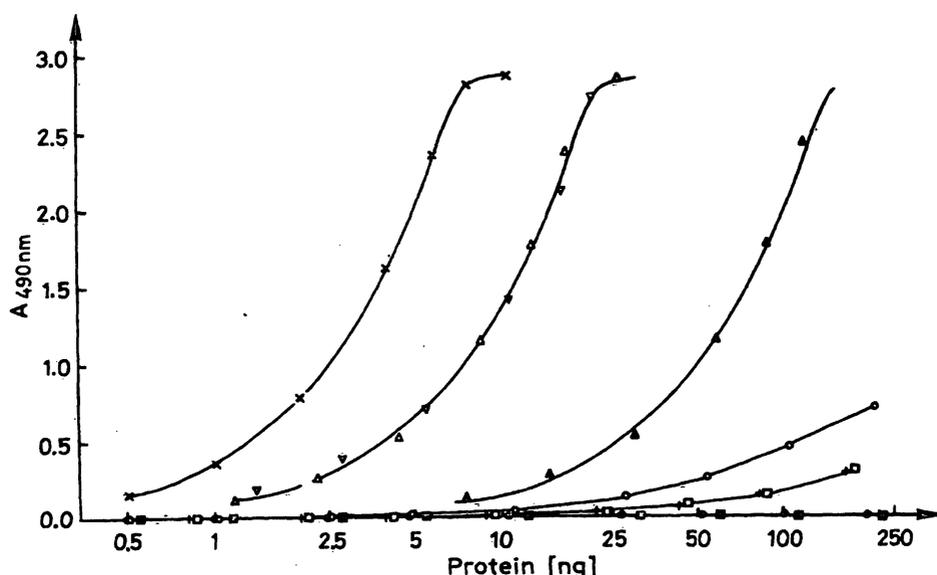

Fig. 2. Standard curves, constructed with purified apolipoprotein C-III (×), chylomicra (△), VLDL (▽) and HDL$_2$ (▲). apolipoproteins A-I (O), A-II (⬤), B (□), E (+) and human albumin (▣).

The selectivity of the ELISA for apolipoprotein C-III was investigated by constructing calibration curves with purified apolipoproteins A-I, A-II, B, C-III$_2$, E and human albumin. As shown in figure 2, the cross-reactivity of these antigens with anti-apolipoprotein C-III immunoglobulins, amounted to 1.02, 0.00, 0.51, 100.00, 0.56 and 0.00% respectively.

Five plasma samples, with low, intermediate and high concentrations of apolipoprotein C-III, were analysed twenty times in the same assay and twice a week during five weeks to calculate the assay precision. As shown in table 1, the average intra- and inter-assay CV amounted to 3.6% and 4.2% respectively.

Tab. 1. Precision of the apolipoprotein C-III ELISA assay.

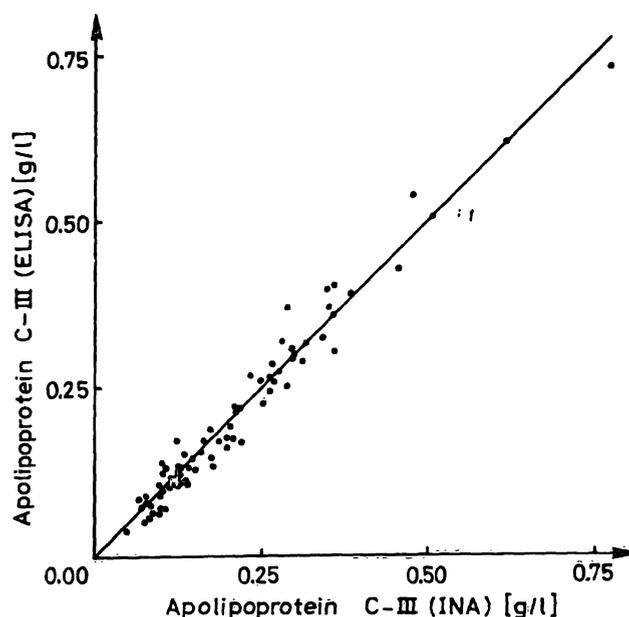| Apolipoprotein C-III concentration (g/l)	Intra-assay CV (n = 20) (%)	Inter-assay CV (n = 10) (%)
0.090	4.33	2.84
0.126	4.14	2.39
0.144	3.83	4.87
0.220	3.81	5.67
0.303	1.86	5.36

Fig. 3. Correlation between the enzyme linked immunosorbent assay (ELISA) and the immunonephelometric assay (INA) for the quantitation of plasma apolipoprotein C-III.
y = 0.988x − 0.002.   r = 0.982.   N = 79.

In order to investigate the accuracy of the assay, apolipoprotein C-III was assayed in 79 plasma samples both by immunonephelometry (13) and by the ELISA procedure described above. The regression line between the two techniques (fig. 3) has a correlation coefficient of 0.982 and is represented by the equation y = 0.988x − 0.002. A paired t-test failed to detect any significant difference (p < 0.05) between the two immunoassays.

Apolipoprotein C-III concentrations in human plasma

Table 2 summarizes the plasma concentrations of apolipoprotein C-III in normal and hyper-lipoproteinaemic subjects as determined by the ELISA procedure. The mean apolipoprotein C-III concentration in 34 normolipaemic subjects was 0.101 ± 0.03 g/l. *Fredrickson* type IIa patients had only slightly

elevated apolipoprotein C-III levels, whereas all other hyperlipoproteinaemic subjects had significantly higher (p<0.001) apolipoprotein C-III values than normals and type IIa patients. The highest apolipoprotein C-III concentrations were observed in *Fredrickson* type V hyperlipoproteinaemic patients.

Investigations on 76 patients with a wide range of total plasma triglycerides (0.27−47.25 g/l), and total plasma cholesterol (1.02−8.85 g/l) showed that apolipoprotein C-III plasma concentrations were positively correlated with plasma triglycerides (r = 0.93) and total plasma cholesterol (r = 0.83).

Distribution of apolipoprotein C-III among the lipoprotein fractions

The apolipoprotein C-III profile was investigated in 3 normal and 3 severe *Fredrickson* type V subjects

Tab. 2. Concentrations of apolipoprotein C-III (mean ± SD) in plasma from normal and hyperlipoproteinaemic subjects.

Subjects Phenotype	number	Triglycerides g/l	Cholesterol g/l	Apolipoprotein C-III g/l
N	34	0.73 ± 0.29	1.97 ± 0.40	0.101 ± 0.031
I	2	22.90	2.58	0.311
IIa	17	1.05 ± 0.31	3.33 ± 0.55	0.168 ± 0.31
IIb	14	2.72 ± 1.06	3.56 ± 0.60	0.307 ± 0.047
IV	6	3.88 ± 1.63	2.49 ± 0.35	0.338 ± 0.081
V	6	21.08 ± 8.85	6.12 ± 1.47	0.716 ± 0.223

as described under methods. In the normolipaemic subjects apolipoprotein C-III was distributed as follows: 15.4 ± 0.8% (mean ± SD) in the Very Low Density Lipoproteins, 11.1 ± 0.6% in Low Density Lipoproteins and 73.5 ± 2.9% in High Density Lipoproteins. In the three *Fredrickson* type V patients 86.8 ± 4.9% of the apolipoprotein C-III was found in the triglyceride-rich lipoproteins (i. e. chylomicra and Very Low Density Lipoproteins) while High Density Lipoproteins cholesterol contained only 5.6 ± 1.7% apolipoprotein C-III (cf. fig. 4).
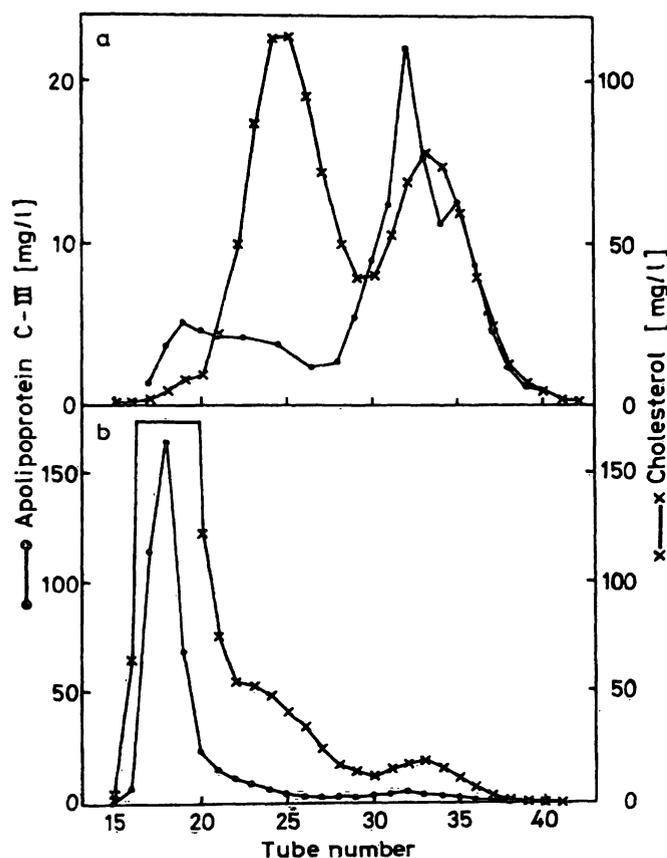

Fig. 4. Apolipoprotein C-III profile in a normolipaemic subject (a) and a patient with a *Fredrickson* type V hyperlipoproteinaemia (b). Fresh plasma (1 ml) was separated on a Sepharose 6B column and apolipoprotein C-III (⊙) and cholesterol (×) were measured in all fractions.

Discussion

Several immunological assays have been described for the measurement of human apolipoprotein C-III (9—13) each of which has its own characteristics and limitations. For the electro-immunodiffusion assay (EID) the selection of a primary standard with the same migration properties as the unknown sample is

critical. The major limitation of laser nephelometric assays is the sample turbidity. This is caused by the presence of triglyceride-rich lipoproteins, and necessitates sample delipidation prior to the assay (13). Radioimmunoassay is the most sensitive technique: down to 0.8 ng apolipoprotein C-III (10). However, it suffers from various major drawbacks, including expensive counting equipment, unstable reagents and biohazards, associated with the handling of radioisotopes. These disadvantages can easily be circumvented by replacing the isotope label by an enzyme.

For this purpose a non-competitive enzyme linked immunosorbent assay was developed for the measurement of apolipoprotein C-III. The sandwich type assay was selected, as both coater and conjugate can be prepared from immunoglobulins with a high stability i. e.: the anti-apolipoprotein C-III-peroxidase conjugate was stable for at least 6 months, without loss of activity.

Standard curves, constructed either with plasma as a secondary standard or with lipoprotein fractions, paralleled the calibration curves, constructed with purified apolipoprotein C-III, which served as the primary standard. In contrast with the electro-immunoassay described by *Curry* et al. (11), the apolipoprotein C-III immunoreactivity was not enhanced by delipidation. Therefore, the ELISA assay does not detect any difference in the exposure of the apolipoprotein C-III epitopes, on the free or on the lipid-bound protein.

Compared with previously described techniques (CV: 6—13%), the use of affinity purified antibodies yielded a high assay sensitivity (0.3 ng apolipoprotein C-III) and standard curves with a steep slope, together with a high assay precision (CV: 4%).

The ELISA assay was evaluated by comparison with an immunonephelometric assay for apolipoprotein C-III, yielding a correlation coefficient of 0.982 (n = 79). Plasma apolipoprotein C-III and triglyceride concentrations were well correlated (r = 0.93, n = 76) as shown by a 3-fold increase of apolipoprotein C-III levels in patients with hypertriglyceridaemia (*Fredrickson* types I, IIb and IV), and by a 5 to 10-fold increase in patients with a *Fredrickson* type V hyperlipoproteinaemia as compared to normolipidaemic subjects.

Apolipoprotein C-III, which plays an important role in triglyceride catabolism, is distributed among triglyceride-rich lipoproteins and HDL. This distribution can easily be influenced by both

endogeneous and exogeneous factors (32). Due to its sensitivity and precision this ELISA assay is especially suited to the study of apolipoprotein C-III profiles after plasma lipoprotein separation by either column chromatography or gradient ultracentrifugation.

This was demonstrated on normolipaemic and hypertriglyceridaemic plasma samples separated on a Sepharose 6B column. In agreement with previous reports (9, 11 – 13), the hypertriglyceridaemic state is attented by a higher amount of apolipoprotein C-III in the triglyceride-rich lipoprotein fractions, suggesting

that the apolipoprotein C-III profile could become a useful marker of triglyceride metabolism.

The characterization of the apolipoprotein C-III content and distribution in plasma should provide a better insight in to the function of this apolipoprotein in lipoprotein metabolism.   · ı

Acknowledgement

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