Mass spectral study of polymorphism of the apolipoproteins of very low density lipoprotein

Pavel V. Bondarenko,[1] Steven L. Cockrill, Layle K. Watkins, Ingrid D. Cruzado, and Ronald D. Macfarlane[2]

Department of Chemistry, Texas A & M University, P.O. Box 300012, College Station, TX 77842-3012

Abstract **New isoforms of apolipoprotein (apo)C-I and apoC-III have been detected in delipidated fractions from very low density lipoprotein (VLDL) using matrix-assisted laser desorption (MALDI) and electrospray ionization (ESI) mass spectrometry (MS). The cleavage sites of truncated apoC-III isoforms have also been identified. The VLDL fractions were isolated by fixed-angle single-spin ultracentrifugation using a self-generating sucrose density gradient and delipidated using a newly developed C18 solid phase extraction protocol. Fifteen apoC isoforms and apoE were identified in the MALDI spectra and the existence of the more abundant species was verified by ESI-MS. The relative intensities of the apoCs are closely correlated in three normolipidemic subjects. A fourth subject with type V hyperlipidemia exhibited an elevated apoC-III level and a suppressed level of the newly discovered truncated apoC-I isoform. ApoC-II was found to be particularly sensitive to in vitro oxidation. The dynamic range and specificity of the MALDI assay shows that the complete apoC isoform profile and apoE phenotype can be obtained in a single measurement from the delipidated VLDL fraction.—**Bondarenko, P. V., S. L. Cockrill, L. K. Watkins, I. D. Cruzado, and R. D. Macfarlane. **Mass spectral study of polymorphism of the apolipoproteins of very low density lipoprotein.** ***J. Lipid Res.*** **1999.** 40: **543–555.**

Supplementary key words C apolipoproteins • truncated apolipoproteins • apoE • C18 solid phase extraction • molecular weight • proteolytic events • quantitative polymorphism • oxidized apolipoproteins

The C apolipoproteins constitute 60% of the protein fraction of human very low density lipoproteins (VLDL). The C apolipoproteins play a key role in lipoprotein metabolism. ApoC-II and apoC-III regulate triglyceride metabolism through activation and inhibition of several triacylglycerol lipases, respectively (1–3). ApoC-I modulates the interaction of apoE with VLDL (4) and inhibits phospholipase activity (5). ApoE is a minor component of VLDL and high density lipoprotein (HDL) apo fractions and acts as a ligand for the low density lipoprotein (LDL) receptor and for the special apoE chylomicron receptor. ApoE binds to the receptors and mediates catabolism of lipoprotein particles in the liver (6, 7). The quantitative and structural polymorphism of the apolipoproteins provide insight into the link between structural modifications and influence on function. Polymorphism can originate from mutations in the apolipoprotein genes (6, 8–10), proteolysis at the co- and post-translational levels and after maturation (11), glycosylation and deglycosylation (12). The amino acid sequences of the mature apolipoproteins, calculated molecular mass (MM) values, and post-translational glycosylation of apoC-III are included in this paper for reference and are summarized in **Fig. 1** (13–18).

ApoC polymorphism related to genetic variations and disorders at the translational level of gene expression is a relatively rare phenomenon. **Table 1** represents a list of the reported genetic mutations of the major serum apolipoproteins from the VLDL fraction, relative charge of genetic variants with respect to the normal isoform, MM value shift associated with each mutation, and influence of the mutation on lipoprotein metabolism.

The human gene for apoE is polymorphic, producing three major isoforms: apoE2, apoE3, and apoE4 (19–21). These mature isoforms have different amino acid residues at positions 112 and 158 and different MM values: apoE2 (Cys_{112}, Cys_{158}, MM = 34183.6 Da), apoE3 (Cys_{112}, Arg_{158}, MM = 34236.7 Da), and apoE4 (Arg_{112}, Arg_{158}, MM = 34289.8 Da). In addition to the most common isoforms, de Knijff et al.(22) have recently reviewed 30 rare apoE variants, 21 of which were found to be associated with different forms of dyslipidemia.

Several isoforms of C apolipoproteins and apoE are generally present in human blood as a result of glycosylation and deglycosylation (12, 23) and proteolytic activity

Abbreviations: ACN, acetonitrile; apo, apolipoprotein; BCA, bicinchoninic acid; CE, capillary electrophoresis; EDTA, ethylenediaminetetraacetic acid; ESI MS, electrospray ionization mass spectrometry; GE, gel electrophoresis; MALDI, matrix-assisted laser desorption ionization mass spectrometry; MM, molecular mass; SBB, Sudan Black B; SPE, solid phase extraction; TOF, time-of-flight; UC, ultracentrifugation; VLDL, very low density lipoprotein.

[1] Present address: Thermo BioAnalysis Corporation, 504 Airport Rd., Santa Fe, NM 87504-2108.

[2] To whom correspondence should be addressed.

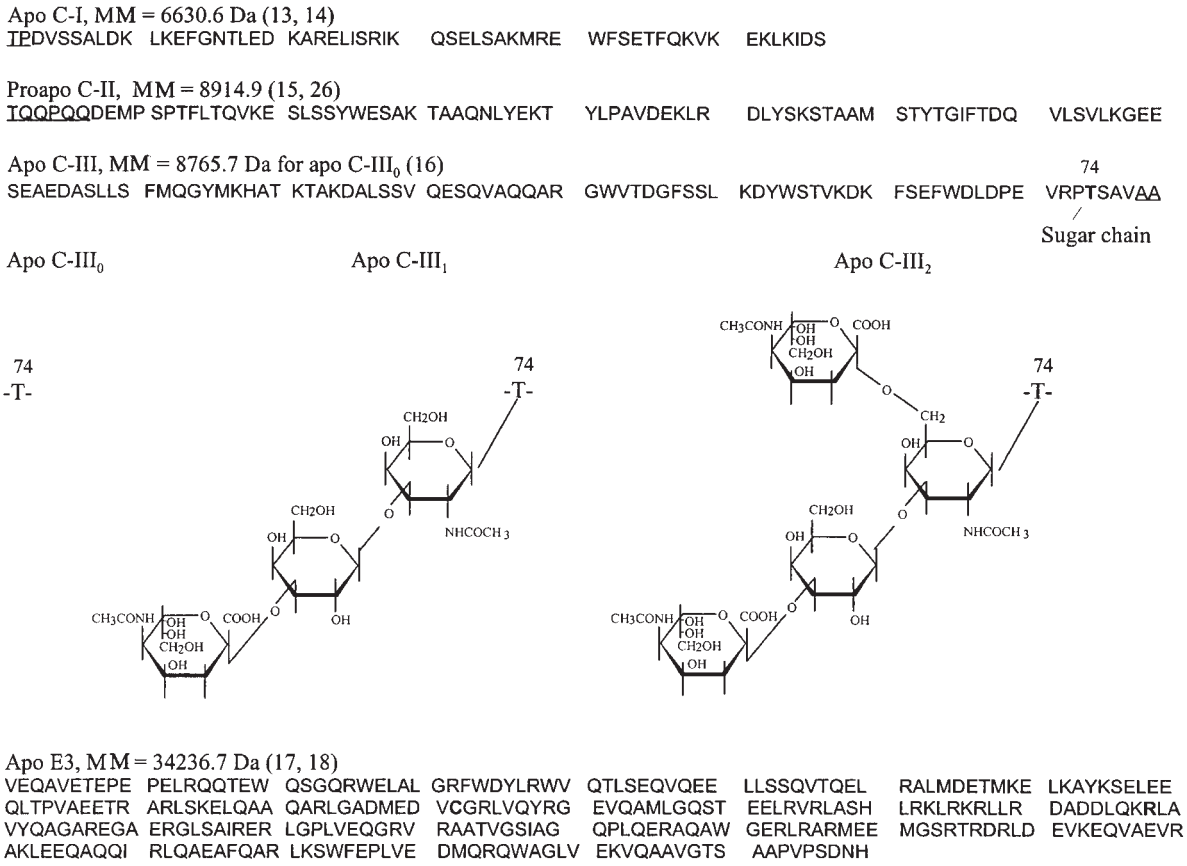

Fig. 1. Amino acid sequences and sugar chains of the major human serum apolipoproteins from VLDL fraction. One letter amino acid code: A, Ala; R, Arg; N, Asn; D, Asp; C, Cys; E, Glu; Q, Gln; G, Gly; H, His; I, Ile; L, Leu; K, Lys; M, Met; F, Phe; P, Pro; S, Ser; T, Thr; W, Trp; Y, Tyr; V, Val.

at the post-translational levels (11) leading to the final mature products (**Table 2**). Proteolysis of the mature C apolipoproteins can be initiated by the action of circulating enzymes in the blood leading to truncated forms which can serve as markers for a pathology associated with elevated enzyme levels. This idea has been proposed to explain the appearance of a truncated form of apoC-III in individuals with elevated carboxypeptidase A due to pancreatic diseases (24).

Originally, only a single form of apoC-II containing 79 amino acids was identified in human plasma (15). Later, it was suggested (11, 25) and subsequently established experimentally (26), that this species is actually proapoC-II. ProapoC-II undergoes proteolytic cleavage of its amino-terminal hexa-peptide (underlined in Figure 1) to generate the mature form of apoC-II which represents less than 5% of the plasma isoforms of apoC-II (26). It was suggested (11, 26) that the membrane-bound proteolytic enzyme responsible for this cleavage is the same one that cleaves proapoA-I to mature apoA-I, because residues 5, 6, and 7 are identical in both proapoC-II and proapoA-I.

ApoC-III occurs in human plasma in three isoforms depending on the number of post-translational sialyl groups added (27–29). The sugar moiety of apoC-III consists of 1 molecule of galactose, 1 molecule of N-acetyl-galactosamine, and 0, 1, or 2 molecules of sialic acid for apoC-III_0, apoC-III_1, and apoC-III_2, respectively. It was later determined (30, 31), that apoC-III_0 is missing not just sialic acid but the entire sugar moiety. An elevated apoC-III_2/apoC-III_1 ratio has been found in plasma of the uremic patients (32) and hypertriglyceridemic patients with secondary anemia (33). An apoC-III_3 isoform (over-sialylation) characterized by the addition of 3 molecules of sialic acid is also associated with hypertriglyceridemia (30). Several additional apoC-III isoforms have been identified using high resolution isoelectric focusing in immobilized pH gradients and have been positively correlated with an elevated serum triglyceride concentration (24). They appear to be in vivo hydrolysis products of carboxypeptidase A activity, which splits off the last four neutral amino acids from the C-terminus of the apoC-III.

The final example of polymorphism relevant to the current study is an important experimental concern in polymorphism studies in general discerning between in vivo polymorphism and events that occur after blood collection (34, 35). Several proteolytic enzymes, particularly carboxypeptidase A (36), increase their activity in pooled serum over time promoting extensive degradation. The apoC-II and apoC-III 'redox-forms' identified by HPLC appear to be due to the oxidation of methionine (37, 38). Storing plasma at 4°C for 12 days increases the percentage of oxidized forms of apoC-II and induces significant alter-

TABLE 1. Genetic isoforms of major human apolipoproteins from VLDL fraction

Isoform Code	Amino Acid Substitution	Relative Charge	ΔMMcalc, Da	Reference/Comments
ProapoC-II, MM = 8,914.9 Da	NO	0	0	(15, 26) normal proapoC-II (Table 1)
C-II Nijmegen	Val_{18}→stop	+2	−6939.7	(71) apoC-II deficiency, hyperlipidemia
C-II Padova	Tyr_{37}→stop	+2	−4845.4	(72, 73) apoC-II deficiency, hyperlipidemia
C-II Paris2	Lys_{19}→stop	+2	−6840.6	(74) apoC-II deficiency, hyperlipidemia
C-II Bari	Tyr_{37}→stop	+2	−4845.4	(75) apoC-II deficiency, hyperlipidemia
C-II Hamburg				(76) apoC-II deficiency, hyperlipidemia
C-II Paris	Met_{22}→Val	0	−32.0	(77) apoC-II deficiency, hyperlipidemia
C-II Toronto	T_{69}KFFLC_{74}	+3	−459.4	(78, 79) disfunctional, hyperlipidemia
C-II St. Michael				
P_{70} SSFCAEGRGV TARPPISGQG ESPLLP_{96}		+1	1608.8	(80) disfunctional, hyperlipidemia
C-II	Glu_{38}→Lys	+2	−1.0	(66) hyperlipidemia
C-II	Lys_{19}→Thr	−1	−27.0	(81)
C-II	Lys_{55}→Gln	−1	0.0	(63–65) a lower cholesterol level in 8% of Nigerian population
ApoC-III	NO	0	0	(16, 25) normal apoC-III (Table 1)
C-III	Asp_{45}→Asn	+1	−1.0	(82)
C-III	Thr_{74}→Ala	0	−30.0	(83) mutant apoC-III is not glycosylated
C-III	Lys_{58}→Glu	−2	1.0	(84) deficiency, hyperlipidemia
C-III Yucatan				(85) deficiency, low levels of cholesterol and triglycerides
ApoE				
E2	Cys_{112}, Cys_{158}	−1	−53.1	(19, 20, 86) frequency 10%, increased risk of type III hyperlipidemia
E3	Cys_{112}, Arg_{158}	0	0	(19, 86, 87) Fig. 1, frequency 75%
E4	Arg_{112}, Arg_{158}	+1	53.1	(19, 86, 87) frequency 15%, increased risk of Alzheimer's disease
Thirty rare apoE genetic variants				(22)

Isoform code name (if any), amino acid sequence modification, relative charge with respect to normal isoform, calculated molecular mass difference between genetic variant and normal apo (ΔMMcalc), references cited, and clinical significance of genetic mutation (if reported) are given.

ation in the apolipoprotein composition (38). Whether oxidation also occurs in vivo and is of physiological importance has not been established (37, 38). Because of the ease of oxidation of methionine, it has the potential for serving as an in vivo probe for monitoring the oxidation state of the lipid domains of lipoproteins, a factor linked to atherogenesis (39, 40).

Until recently, 2-D gel electrophoresis (GE) has been the most effective screening technique for the human apolipoprotein polymorphism (41–43). It has been used in the identification of almost all genetic and post-translational apo isoforms presented in Tables 1 and 2. Apolipoprotein phenotypes and genotypes have been assayed by this technique. Because of the high resolving power of the method in the IEF dimension, even minor structural differences in apolipoproteins can be detected if they are 'electrically non-neutral.' However, when one or even several neutral amino acid residues were cleaved from apoC-III (containing only 79 residues), the normal and truncated isoforms were difficult to resolve by the 2-D GE technique (24). This is because the second dimension, SDS gel electrophoresis, lacks the mass measurement accuracy and resolution needed to identify the truncated isoforms. As a result of this limitation of the 2-D GE technique, many cases of electrically neutral amino acid substitutions, oxidation, attachments of electrically neutral sugars and some other post-translational modifications remain "invisible" to the screening method. The introduction of ^{252}Cf-plasma desorption mass spectrometry in 1974 (44), its application to apolipoprotein characterization (31) and subsequent second generation methodologies (45, 46) has given us an opportunity to study apolipoprotein polymorphism based on accurate MM determination (47). One of the first mass spectrometric applications was the study of polymorphism of the apoC family after chromatographic or electrophoretic separation or the tryptic digests of the proteins (30, 31, 37, 48–50).

In this study, both MALDI and ESI-MS were investigated for their potential for screening for polymorphism in the apolipoproteins associated with VLDL. A method was developed for collecting and processing serum and isolation

TABLE 2. Post-translational polymorphism of major human apolipoproteins from VLDL fraction

Isoform Code[a]	Post-translational Isoform	Relative Charge	pI	ΔMMcalc, Da
$ApoC\text{-}II_0$	proapoC-II	0	4.82	0
$ApoC\text{-}II_{-1/2}$	mature apoC-II	$-1/_2$		−710.8
$ApoC\text{-}II_{-1}$	sialo-proapoC-II	−1		291.3
$ApoC\text{-}II_{-2}$	disialo-proapoC-II	−2	4.50	582.6
$ApoC\text{-}III_0$[b]	asialo-apoC-III	+2	4.95	−947.9
$ApoC\text{-}III_1$	sialo-apoC-III	+1	4.80	−291.3
$ApoC\text{-}III_2$	disialo-apoC-III	0	4.65	0
ApoE	mature apoE3	0	6.02	0
$ApoE_s$[c]	sialo-apoE3	−1	5.89	+291.3

Isoform code, relative charge with respect to the mature isoform in circulation, pI value, calculated molecular weight difference between minor isoform and mature apolipoprotein (ΔMMcalc) are given. Results from the following publications were used to compile this table: (12, 26, 43) for apoC-II, (12, 27–29, 43) for apoC-III, and (23) for apoE.

[a] The post-translational isoforms were assigned according to Anderson et al. terminology (88).

[b] The common subscript coding for apoC-III was introduced by Schmitz et al. (43).

[c] The subscript coding was introduced by Zannis et al. (23).

of VLDL apolipoproteins in a form suitable for mass spectrometric analysis. Polymorphism profiles determined by mass spectrometry were then correlated with previous findings and some initial results were obtained on subject variability.

MATERIALS AND METHODS

Materials

Chemicals used were: acetic acid (Fisher Scientific, Fair Lawn, NJ), acetonitrile (ACN; HPLC grade, EM Science, Gibbstown, NJ), benzamidine (Sigma Chemical Co., St. Louis, MO), α-cyano-4-hydroxycinnamic acid (Aldrich Chemical Co. Milwaukee, WI), dimethyl sulfoxide (Burdick and Jackson Laboratories Inc., Muskegon, MI), ferulic acid (Aldrich), formic acid (88% solution, EM Science), ethylenediaminetetraacetic acid (EDTA; J. T. Baker Inc., Phillipsburg, NJ), methanol (Mallinckrodt, Paris, KY), sinapinic acid (Aldrich), sodium azide (Aldrich Chemical Co., Milwaukee, WI), sodium bromide (NaBr; Fisher Scientific), Sudan Black B (SBB; Sigma), sucrose (Fisher Scientific), trifluoroacetic acid (TFA, Pierce Chemical Co., Rockford, IL), phenylmethylsulfonyl fluoride (PMSF, Sigma), and deionized water (HPLC grade, Burdick and Jackson). Protein standards were obtained from Sigma Chemical, St. Louis, MO. The proteins, their origin, and catalog number are, respectively: carbonic anhydrase I, human, C-4396; insulin, bovine serum, I-5500; and myoglobin, horse heart, M-1882.

Serum/plasma collection

The serum or plasma (12 h fast) from a normolipidemic, healthy 25-year-old white male (subject 1) was primarily used in the study. For measuring subject variability, a 26-year-old white female (subject 2), a 31-year-old Hispanic female (subject 3) and a 60-year-old white female with type V hyperlipidemia (subject 4) were included. At the time of blood collection, subject 4 presented a triglyceride level of 1600 mg/dL and a total serum cholesterol level of 360 mg/dL.

Serum samples were collected by a blood draw into an untreated 7-mL Vacutainer™ tube (Becton Dickinson Systems, Franklin Lakes, NJ). Blood was allowed to clot for 45 min at room temperature followed by 50 *g* centrifugation for 20 min at 4°C. The serum was recovered as the supernatant. For the analysis of plasma samples, blood was collected in tubes containing 12 mg K_2EDTA. A preservative mixture was added to plasma samples according to a procedure described previously (34). Each mL volume of sample was treated with 4 μL of 2.5% (w/v) sodium azide, 1 μL of 1.0 m benzamidine, and 5 μL of 0.2 m phenylmethylsulfonyl fluoride.

Isolation of VLDL by single-spin preparative ultracentrifugation (UC)

Details of the method used for separation of VLDL by ultracentrifugation are included in a recently published paper (51). A 200-μL volume of serum or plasma sample was stained with 7 μL of SBB in dimethyl sulfoxide (1% w/v) then diluted with deionized water to a volume of 400 μL. A 20% (w/v) sucrose solution containing sodium azide was used as a density gradient medium. An 800-μL aliquot of this solution was pipetted into a 11 × 34 mm polycarbonate open-top centrifuge tube (Beckman Instruments, Fullerton, CA) followed by placement of the sample layer on top of the sucrose solution. The tube was then centrifuged in a Beckman TL-100 ultracentrifuge equipped with a 30° fixed angle TLA100.2 rotor at 436,000 *g* and 20°C for 6 h. After centrifugation, a 200-μL volume of the VLDL fraction was collected from the top of the tube by aspiration ($d < 1.006$ g/ml).

The centrifugation was also performed using a NaBr density gradient. The preparation of the ultracentrifuge medium involved a series of solutions layered in the polycarbonate open-top tube in the following order: 250 μL of 1.26 g/mL NaBr solution, 200 μL of 1.10 g/mL NaBr solution, 207 μL of the stained plasma, and 600 μL of water. All of the layering solutions contained 0.01% EDTA and 0.01% sodium azide (34). The UC separation was carried out as described in the sucrose density gradient UC procedure with the exception that the samples were centrifuged for 3.5 h at 10°C.

Purity of VLDL fraction

For both ultracentrifuge procedures used to separate the VLDL fractions, contamination by serum proteins was investigated. Human serum albumin (HSA) was used as a marker for serum protein contamination because of its high serum level and affinity for associating with lipoprotein particles. Fractions were delipidated and then analyzed for HSA by capillary electrophoresis (51) and MALDI mass spectrometry. An HSA standard gives a peak with a characteristic effective electrophoretic mobility in the CE analysis and a protonated molecular ion in the MALDI mass spectrum. Neither analysis gave evidence for HSA in the VLDL fraction. We conclude from these findings that all of the proteins observed in the delipidated VLDL fraction are an integral part of the VLDL particle structure.

Delipidation of VLDL by solid phase extraction (SPE)

An SPE method was developed for fast delipidation of VLDL and recovery of the apolipoproteins for mass spectrometric analysis. A 500-μL aliquot of the VLDL fraction pooled from the VLDL fractions of three centrifuge tubes was acidified with 5% (v/v) aqueous acetic acid to pH 2.6 and transferred to a tC18 light cartridge (SEP-PAK, #51910, Waters Corp., Milford, MA), previously conditioned with 5% (v/v) acetic acid in ACN and 5% (v/v) aqueous acetic acid. The cartridge was washed with 5 mL of 5% aqueous acetic acid solution to remove the density gradient solute (sucrose or NaBr) and other water-soluble components. A series of isocratic elutions was carried out with 50-μL volumes of 5% acetic acid in ACN. Each eluant fraction was tested for protein using the bicinchoninic acid (BCA) method (52). The first and second fraction contained no protein but the third fraction tested positive. The protein concentration was found equal to ~0.2 mg/mL. All subsequent fractions tested negative.

Efficiency of the SPE delipidation method

In the development of this method, numerous studies were carried out to determine the efficiency of the method for the recovery of the apolipoproteins free from the lipid component. Using commercial insulin, apoA-I, apoA-II, apoC-I, II, III, apoE and apoB-100 standards, it was determined that all of these proteins except for apoB-100 elute in the 3rd 50-μL fraction and that the recovery of these proteins was between 70 and 90%. LDL fractions were also applied to the SPE cartridge and eluted. The eluant was then analyzed by CE. If apoB-100 is present in the sample, it gives a peak with a characteristic mobility in the electropherogram (51). No peak was detected. The conclusion is that apoB-100 is not eluted under these conditions. Analysis for the presence of cholesterol/cholesteryl esters and triglycerides in the protein fraction was carried out using commercial kits. None of these lipids was detected in the protein fraction. Continued elution of the cartridge by twenty 50-μL fractions of acidified ACN failed to elute the lipid fraction. Some of the VLDL lipids were eluted from the cartridge when the solvent was changed to chloroform. The conclusion of these findings is that the apolipo-

TABLE 3. Calculated and experimental molecular mass values of human serum apolipoproteins from delipidated VLDL fraction

Code	Identification	MMcalc, Da	MM ± SD, Da MALDI	MM ± SD, Da ESI	Reference
C-I′	apoC-I without N-terminus Thr-Pro-	6432.4	6432.4 ± 0.3	6432.3 ± 0.5	this study
C-I	apoC-I	6630.6	6630.6 calibr.	6630.6 calibr.	(13)
C-II	apoC-II	8204.1	8204.3 ± 0.4	8204.3 ± 1.0	(26)
$C\text{-}II_{Ox}$	oxidized apoC-II, apoC-II + 16 Da	8220.1	8220.2 ± 0.4	8219.6 ± 1.0	(37)
$C\text{-}III_0'$	$apoC\text{-}III_0$ without C-terminus -Ala	8693.6	8693.2 ± 1.0		(30, 31)
$C\text{-}III_0$	$apoC\text{-}III_0$	8764.7	8764.8 ± 0.7	8763.6 ± 2.0	(30, 31)
proC-II	proapoC-II	8914.9	8914.8 ± 0.4	8914.8 ± 0.5	(15, 25)
$proC\text{-}II_{Ox}$	oxidized proapoC-II, proapoC-II + 16 Da	8930.9	8930.8 ± 0.4	8931.0 ± 0.6	(37)
$C\text{-}III_1''$	$apoC\text{-}III_1$ without C-terminus -Ala-Ala	9279.1	9279.1 ± 1.2		this study
$C\text{-}III_1'$	$apoC\text{-}III_1$ without C-terminus -Ala	9350.2	9350.7 ± 0.9	9351.7 ± 2.0	this study
$C\text{-}III_1$	$apoC\text{-}III_1$	9421.3	9421.3 calibr.	9421.1 ± 0.3	(16, 25)
$C\text{-}III_2''$	$apoC\text{-}III_2$ without C-terminus -Ala-Ala	9570.4	9569.8 ± 0.8		(24)
$C\text{-}III_2'$	$apoC\text{-}III_2$ without C-terminus -Ala	9641.5	9642.4 ± 1.0		(24)
$C\text{-}III_2$	$apoC\text{-}III_2$	9712.6	9712.5 ± 0.7	9712.6 ± 0.6	(16, 25)
$C\text{-}III_x$	?		9933.8 ± 0.2	9933.0 ± 2.0	this study
E^{2+}	apoE3, two charges ($z = 2$)	34236.7	34238 ± 5		(17, 18)
	apoE2	34183.6	—		
E	apoE3	34236.7	34242 ± 10		(17, 18)
	apoE4	34289.8	—		

Amino acid sequences and reported post-translational modifications were used to obtain the calculated MM values (MMcalc). The experimental MM values were measured using the MALDI-TOF and ESI-TOF techniques.

protein fractions are essentially free from lipids. The standard methanol–ether delipidation protocol (53, 54) was also used for comparison. The recovery of the method was found to be not as high as for the SPE method. The mass spectra of apolipoproteins recovered by the methanol–ether method indicated that the level of water-soluble impurities was also higher. The first water rinsing step in the SPE procedure is particularly efficient in removing these impurities.

Mass spectrometry

The mass spectrometric measurements were performed using an ESI time-of-flight (TOF) instrument constructed in our laboratory (55) and a commercial MALDI-TOF (Voyager Elite XL, PerSeptive Biosystems, Framingham, MA). The apolipoprotein mixture eluted from the cartridge was injected directly into the ESI instrument. The MALDI analysis was performed following a published protocol (56). The matrices, including sinapinic acid, ferulic acid, and α-cyano-4-hydroxycinnamic acid were dissolved in methanol to a concentration of 20 mg/mL for each acid. Sinapinic acid was the most frequently used matrix because it produced fewer adduct and multiply charged molecular ions. Peak broadening due to the desorption/ionization process was lower than for the other two matrixes used to ascertain influences of the sample preparation method on the mass spectra. First, a matrix layer was prepared by applying 1 μL of the matrix solution to the surface of a smooth stainless steel sample plate and allowing it to dry. A finely divided crystalline layer was formed within a few seconds. A 1-μL aliquot of the apo fraction eluted from the tC18 cartridge was diluted with 7 μL of H_2O and 2 μL of matrix solution giving a solution with a composition of 20 mm matrix and an estimated total apolipoprotein concentration (C apolipoproteins and apoE) of 2 μm. An aliquot (~0.5 μL) of the matrix–sample solution was placed on the surface of the crystalline matrix deposition and dried in air at room temperature. A 2-μL droplet of deionized water was placed on top of the sample spot for 5 seconds to dissolve water-soluble impurities on the surface. The droplet was then shaken off and the sample spot was allowed to dry again at room temperature. The stainless steel sample plate was then inserted into the MALDI instrument. The plate was designed to accommodate several samples at one time. Serum bovine insulin (MM = 5,733.6) and horse heart myoglobin (MM = 16,951.5) were used as external MM standards for ESI and MALDI. The MM values of apoC-I (6630.6) and $apoC\text{-}III_1$ (9421.3) measured for all subjects were found to be accurate to within ± 0.1 Da of the literature value. Subsequently, these two apolipoproteins were used as an internal mass calibration for the remainder of the study. The standard deviations of the mass measurements for the proteins studied are given in **Table 3**.

RESULTS

Choice of protocol in sample purification for mass analysis

The first measurements carried out followed a widely used and established methodology: NaBr density gradient UC of plasma from subject 1 with added preservative followed by delipidation by solvent extraction, precipitation of the VLDL apolipoproteins, and re-dissolution in 5% aqueous acetic acid. The protein fraction was then analyzed by MALDI and ESI MS. The MALDI mass spectrum contained peaks identified as the protonated molecules of the C apolipoproteins as well as ions containing a variable number of Na^+ ions because of the high residual NaBr in the sample. The ESI spectrum contained no ions that could be attributed to the apolipoproteins presumably due to interferences from the high salt content in the sample.

An aliquot of the same sample was also purified using the tC18-SPE scheme and analyzed by MALDI and ESI. The MALDI mass spectrum showed only protonated species as the major species with no evidence for Na^+ adduct ion formation. The ESI spectrum, in the absence of salt contamination, contained a family of ions in the region m/z 700–1600 which were identified as multiply protonated molecules of the C apolipoproteins.

Next, results were obtained using the second protocol for recovering the apolipoproteins from VLDL where serum instead of plasma was used with no added inhibitors; NaBr was replaced by sucrose in forming the density gradient for UC separation, and the removal of water soluble impurities and delipidation were carried out in a single operation using the tC18- SPE cartridge. The MALDI and ESI spectra of the apolipoprotein fractions were found to be nearly identical to what was obtained using the (plasma + inhibitors)/NaBr-UC/ tC18-SPE procedure. There was no evidence for enzymatically induced degradation and the use of sucrose as the gradient solute did not alter the isoform distribution of the C apolipoproteins. The serum/ sucrose-UC/tC18- SPE method was adopted for the remainder of the study for recovering the apolipoproteins from the VLDL fraction.

MALDI analysis

Figure 2 shows a MALDI mass spectrum between m/z 6000 and 11,000 of the apolipoproteins derived from VLDL using sinapinic acid as the MALDI matrix. The spectrum is complex with 15 components identified in the region m/z 6400–11,000 that are linked to the family of C apolipoproteins based on their MM values. Peaks of weaker intensity were observed at higher MM with masses corresponding to apoE. A more definitive mass spectrum was obtained by processing a larger volume of the VLDL fraction (~1 mL) and removing the lower MM apolipoproteins by ultrafiltration because the presence of these higher abundance species appeared to be adversely influencing the ionization probability for the higher MM apolipoproteins. The ultrafiltration was carried out using 30,000 MM cut-off micro-concentrators equipped with hydrophilic membranes (volume 0.5 mL, model Microcon-30, Amicon, Inc., Beverly, MA). The low MM components from the delipidated VLDL fraction penetrated the membrane under a centrifugal force created by a Microcent compact centrifuge (Fisher Scientific Company, Pittsburgh, PA) spinning at 14,000 rpm for 10 min. Then, apoE and other high-MM species were recovered by placing the microconcentrator upside down in a new vial and spinning for 5 min. The mass spectrometric detection efficiency for the apoE protonated molecules was also increased by using an ion transmission gate and detecting only the ions with $m/z >$ 15,000. This mass spectrum is shown in **Fig. 3**. Two peaks were observed at m/z 34,243 ± 10 and m/z 17,120 ± 5 which are in agreement with the expected values for protonated (E) and doubly protonated (E^{2+}) apoE3 (MM = 34,236.7 Da). The doubly charged protonated molecules have a better accuracy and precision because they produce a higher intensity signal and were interpolated between the calibrants, horse heart myoglobin (MM + = 16,951.5 Da) and human carbonic anhydrase I (MM 28,781.1 Da), used for external calibration. ApoB-100 is not eluted from the tC18-SPE cartridge under the conditions for eluting the other VLDL apolipoproteins because of its high affinity for the stationary phase. Consequently, it was not present in the samples studied by MALDI and ESI. CE analysis of these fractions also confirmed that no apoB-100 was present based on the known effective electrophoretic mobility of this species (57).

The three major C apolipoprotein classes, apoC-I, apoC-II, and apoC-III including previously characterized proapo, glycosylated, and truncated forms were identified in the MALDI mass spectrum by correlation with their calculated molecular mass values (see Table 3). The truncated isoforms are labeled as apoC-X′ or apoC-X′′ in Table 3 and Fig. 2 and the previously identified glycosylated apoC isoforms are labeled as apoC-$III_{0,1,2}$. In addition, a new isoform was observed: a truncated form of apoC-I labeled apoC-I′. An additional peak with an intensity comparable to the apoC-III_2 peak was observed with MM 9933 Da which, based on additional evidence, has been assigned to a new isoform of apoC-III. This peak is labeled C-III_x. Table 3 lists the measured MM values for the truncated isoforms with the missing components of the sequences identified in the second column. In addition to the major apolipoproteins and their truncated isoforms, the two isoforms of apoC-II have satellite peaks 16 Da higher than the MM of the parent species. These peaks

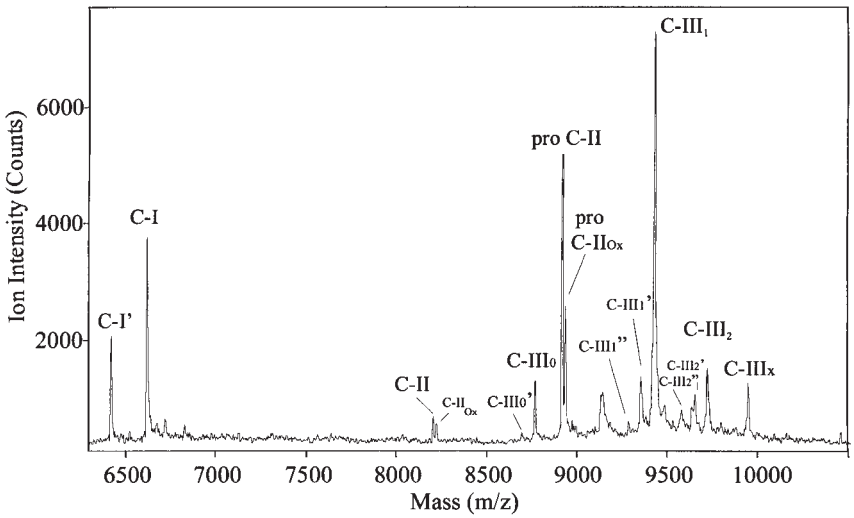

Fig. 2. Positive ion MALDI mass spectrum of human serum apolipoproteins from the delipidated VLDL fraction of subject 1. The molecular mass values of apolipoproteins from this spectrum, averaged for three independent measurements, are given in Table 3.

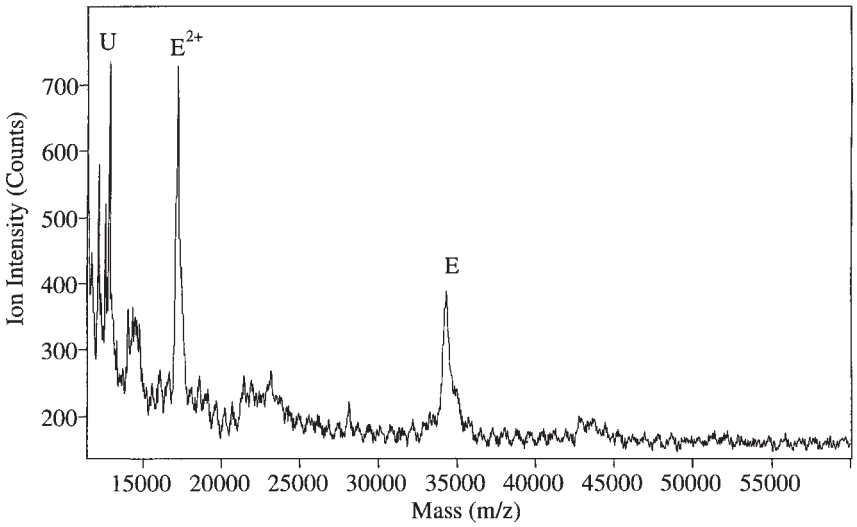

Fig. 3. Positive ion MALDI mass spectrum of human serum apolipoproteins from the delipidated VLDL fraction of subject 1: high-mass region.

are due to oxidation of one of the two methionine residues in apoC-II (37) and are included in the table as possible in vivo isoforms labeled proapoC-II_{ox} and apoC-II_{ox}. The peak labelled C-III_x was found in the serum of all subjects in both the VLDL and HDL delipidated fractions with essentially the same intensity relative to apoC-III_1 and apoC-III_2.

Several other low intensity peaks are present in the mass spectrum shown in Fig. 2 that are not consistently observed in the spectra of delipidated VLDL samples from other donors. The origin of these components is unknown. Some appear to be correlated to sample age while others appear to be donor dependent, possibly serum proteins sequestered by VLDL.

ESI analysis

Figure 4 shows the positive ion ESI mass spectrum of an aliquot of the same sample used to obtain the MALDI spectrum shown in Figs. 2 and 3. Multiply protonated molecules of the apolipoproteins with overlapping bell-shaped distributions of intensities dominate the ESI mass spectrum. The ESI molecular masses presented in Table 3 were determined using the averaging algorithm described earlier (58, 59). Briefly, after the charge states were assigned to the peaks in the mass spectrum (Fig. 4), the MM value of each compound was calculated using the following procedure. The mass-to-charge ratio (m/z) of a peak was multiplied by its charge state (z) to give the mass of multiply protonated molecules (m). The mass of all at-

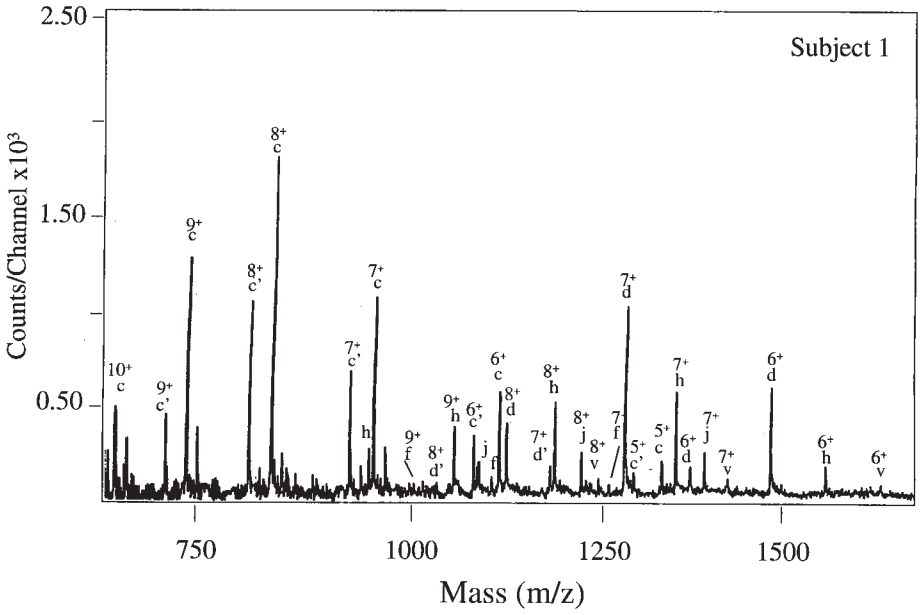

Fig. 4. Positive ion ESI mass spectrum of multiply protonated molecules of the C apolipoproteins from the VLDL fraction of subject 1. The m/z values of the multiply protonated molecules from this spectrum were used to calculate the molecular weight values of individual compounds. One letter code for C apolipoproteins: c′, apoC-I′; c, apoC-I; d′, apoC-II; f, apoC-III_0; d, proapoC-II; h, apoC-III_1; j, apoC-III_2; v, apoC-III_x.

TABLE 4. Numbers of the individual basic amino acid residues (see Fig. 1), total number of the basic amino acid residues along with an N-terminal amino group, MM value, pI values of the apos, and the experimentally observed maximum charge of the species in the ESI mass spectrum from Fig. 4

Apo	Arg (R)	Lys (K)	His (H)	Total number (+1)	pI	MM, Da	Maximum Charge
ApoC-I	2	9	0	12	6.5	6,630.6	11
ApoC-II	1	6	0	8	5.0	8,914.9	8
ApoC-III	2	6	0	9	4.8–5.1	~9,500	9

The $(M + 11H)^{11+}$ molecular ion of apoC-I is not shown in Fig. 4.

tached protons ($1.0079 \times z$) was subtracted from m to give the MM value of the compound, $m - 1.0079 \times z = MM$. These MM values calculated from each peak of the compound were averaged to produce the average MM value of the compound.

A feature of the ESI method was found to be particularly useful in this study. Earlier studies by others have shown that for a large number of proteins prepared in acidic solutions, an approximately linear correlation is observed between the maximum number of attached protons in the ESI spectrum and the number of basic amino acid residues (arginine, lysine, histidine) (60, 61). This correlation was tested for the C apolipoproteins. **Table 4** lists the following information related to each apoC species: numbers of the individual basic amino acid residues in the C apolipoproteins, total number of the basic amino acid residues including the N-terminal amino group, pI value, MM value, and the maximum number of protons added to apolipoprotein molecules. The total number of the basic amino acid residues is consistent with the maximum charge state. Hence, in addition to molecular mass, an electrospray mass spectrum provides additional information on amino acid composition of the unknown compounds. For example, the charge distribution of molecular ions of the truncated isoform of apoC-I which is identified as apoC-I′ in Table 3, is similar to the distribution of apoC-I which means that they have the same number of basic residues.

Correlation and summary of the MALDI and ESI mass-spectral interpretation

All of the isoforms observed by ESI were also observed by MALDI. Several isoforms observed by MALDI, all low intensity peaks, were not detected in the ESI analysis because of interferences from the high density of multiply charged ions of the more prominent species in the molecular ion manifold (Fig. 4). The same 17 apoC isoforms (within ±1 Da), were observed in the serum of the four subjects participating in the study but there were differences in relative intensity. The normal concentrations of apoC-I, apoC-II, and apoC-III in VLDL have been reported as 0.1, 0.9, and 2.4 mg/dL, respectively (62). The molar concentrations, more relevant to mass spectral peak intensities, were calculated as 0.2, 1.0, and 2.5 μm, respectively. The relative intensities of the peaks in the ESI and MALDI mass spectra (Fig. 2 and **Fig. 5**) for these apolipoproteins reflect these concentrations except for apoC-I. In both spectra, the relative intensity of apoC-I protonated species is higher than its known relative molar concentrations in VLDL. The precision of the relative molecular ion intensity measurements was found to be better from the ESI results. Consequently, the study of subject-to-subject variation in relative molecular ion intensities was obtained from ESI data.

Presentation of the ESI data as a deconvoluted mass spectrum

The complexity of the ESI spectrum due to the overlapping charge distributions of the C apolipoproteins can be reduced to a simpler histogram format where the peak intensities (heights) of the family of multiply charged ions for each component are summed. Figure 5 shows the resulting deconvoluted (58) ESI mass spectrum. The relative intensity of apoC-I in the mass spectrum is the highest of the three C apolipoproteins even though its molar con-

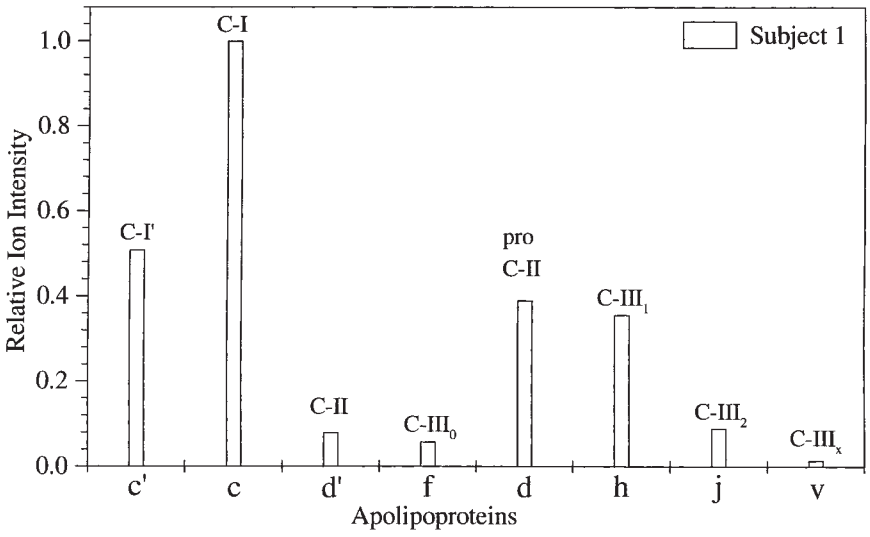

Fig. 5. The deconvoluted ESI mass spectrum of the C apolipoproteins from the VLDL fraction of subject 1. The height of each line in the histogram represents the relative intensity of a particular apoC isoform in the ESI mass spectrum.

centration in serum is the lowest, by an order of magnitude. The enhanced ion intensity can be attributed to two features of the ESI-TOF technique. First, the electrospray ionization efficiency of apoC-I is higher than other apolipoproteins. ApoC-I more readily accommodates protons, because it has a larger number of basic amino acid residues per mass unit than other apolipoproteins (see Table 4). This effect leads to the higher yield of molecular ions with lower m/z values in the mass spectrum (Fig. 4), and to the broader range of the charge states (from 5+ to 11+). Second, detection efficiency in the TOF mass spectrometry increases with decreasing m/z values. The same considerations can be used to explain an elevated intensity of the protonated apoC-I molecules in the MALDI mass spectrum. The histogram format of the presentation of the ESI data was adopted for comparing apoC profiles of different donors. Because of variability in the overall intensities of the mass spectra from run to run, an internal intensity standard was selected using the intensity of the apoC-I line in the histogram as a normalization intensity with an assigned value of 1 as shown in Fig. 5. ApoC-I was selected because it has the highest and least variable ion intensity and the fewest number of isoforms. The identification of these isoforms is included in the Discussion section.

ESI study of the C apolipoprotein intensity profile; subject variability

A compiled histogram representation of the data for all donors is shown in **Fig. 6** with the apoC-I intensity serving as an internal standard normalized to unity for each donor. Relative intensities of apoC-I', apoC-I, apoC-III$_0$, apoC-III$_1$, and apoC-III$_2$ showed a spread of ~20% between the three normolipidemic donors. The hyperlipidemic donor (subject 4) exhibited an approximately 3-fold enhancement in apoC-III$_1$ and apoC-III$_2$ levels and a 3-fold decrease in apoC-I′ level relative to the normolipidemic subjects.

Truncated isoforms

In the overview of the results obtained from the mass spectrometry studies, as described above, evidence for the presence of truncated isoforms for each of the C apolipoproteins was clearly indicated in the MALDI mass spectra. The minor truncated isoforms of apoC-III were not clearly detected in the ESI mass spectra because of low peak intensities (and concentrations) of these isoforms. The identification of the missing residues was made by comparing measured MM values with the amino acid sequence. On the basis of the calculated and experimental mass differences (Δ, Da), the truncated isoforms were identified as follows:

apoC-I′ apoC-I, missing Thr-Pro at the N-terminus,
$\Delta_{calc} = -198.2$; $\Delta_{MALDI} = -198.3 \pm 0.3$;
$\Delta_{ESI} = -198.3 \pm 0.5$;

apoC-II: proapoC-II, missing Thr-Gln-Gln-Pro-Gln-Gln at the N-terminus,
$\Delta_{calc} = -710.8$; $\Delta_{MALDI} = -710.5 \pm 0.4$;
$\Delta_{ESI} = -711.4 \pm 1.0$;

apoC-III$_0$′: apoC-III$_0$, missing Ala at the C-terminus,
$\Delta_{calc} = -71.1$; $\Delta_{MALDI} = -71.6 \pm 1.0$;

apoC-III$_1$′′: apoC-III$_1$, missing Ala-Ala at the C-terminus,
$\Delta_{calc} = -142.2$; $\Delta_{MALDI} = -142.4 \pm 1.2$;

apoC-III$_1$′: apoC-III$_1$, missing Ala at the C-terminus,
$\Delta_{calc} = -71.1$; $\Delta_{MALDI} = -70.6 \pm 0.9$;

apoC-III$_2$′′: apoC-III$_2$, missing Ala-Ala at the C-terminus,
$\Delta_{calc} = -142.2$; $\Delta_{MALDI} = -142.7 \pm 0.8$;

apoC-III$_2$′: apoC-III$_2$, missing Ala at the C-terminus,
$\Delta_{calc} = -71.1$; $\Delta_{MALDI} = -70.1 \pm 1.0$;

Most of these truncated isoforms have been previously identified. The truncated apoC-I isoform, apoC-I′, lacking a Thr-Pro at the N-terminus has not previously been reported.

DISCUSSION

Genetic polymorphism

The high mass accuracy and resolving power of mass spectrometric methods used in this study translates to a

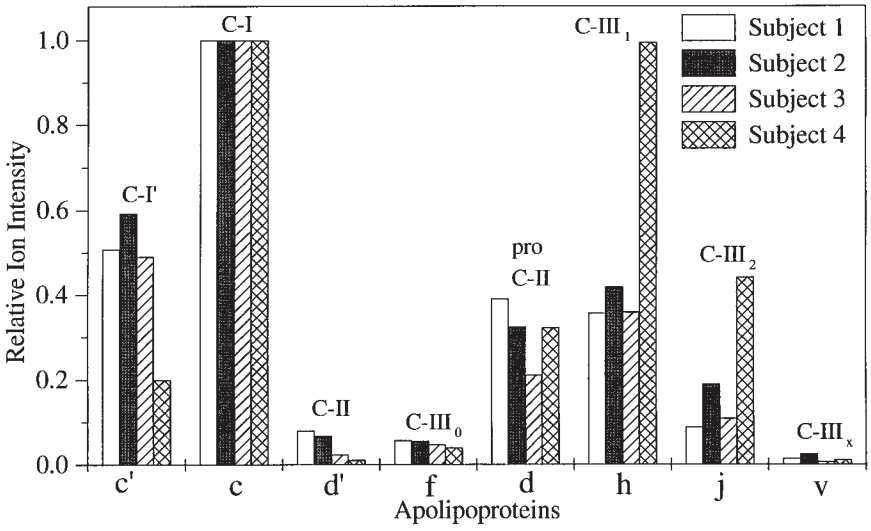

Fig. 6. The deconvoluted ESI mass spectrum of the C apolipoproteins from the VLDL fraction of the four subjects studied.

sensitivity of a single amino acid substitution if the mass shift is greater than ±1 Da. Among the known genetic variants, only the apoC-II variant, apoC-II (128.17 Da, Lys_{55} →128.13 Da, Gln_{55}) (63–65) falls within this uncertainty window. This substitution has been identified by isoelectric focusing gel electrophoresis (64) based on a detectable pI difference. The apoC-II variant, apoC-II (129.12 Da, Glu_{38}→128.17 Da, Lys_{38}) (66), if present, can be detected at the 60% confidence level (CL) based on the known accuracy and precision of the MM measurement. All other known variants of the C apolipoproteins can be identified at the greater than 99.9% CL on the basis of measured MM shifts.

The C apolipoproteins MM profiles for the four subjects participating in the study were measured and compared with the literature MM values. Within the mass accuracy window of the measurement, all of the subjects presented with the same and most frequently encountered genetic forms.

Post-translational modifications

An analysis of the mass spectra shows that apoC-II occurs as two isoforms: proapoC-II and mature apoC-II. The mature apoC-II constitutes ~10% of the relative intensity of the VLDL proapoC-II in both the MALDI (Fig. 2) and ESI (Fig. 5) mass spectra. This relative abundance is consistent with the relative plasma concentration of the mature apoC-II (5%) previously reported in relation to the total apoC-II population as determined by two-dimensional gel electrophoresis/immunoblot analysis (26). Two minor isoforms were also observed for both apoC-III_1 and apoC-III_2. Only one of these two isoforms types was detected for apoC-III_0 which has the lowest intensity of the apoC-III variants. Based on the MM values, these minor isoforms lack one (Ala) and two (Ala–Ala) C-terminal residues. Previous studies also reported additional isoforms of apoC-III based on high resolution 2-D GE measurements and it was postulated that they may be the result of carboxypeptidase cleavage within a quartet of neutral residues at the C-terminus (24). Our findings support this postulate and specifically identify the cleavage sites as C-terminal amino acids Ala_{78} and Ala_{79}. Both untreated serum and plasma containing proteolytic inhibitors showed equivalent relative intensities of these truncated isoforms suggesting that these deletions probably occur in vivo.

To our knowledge, this study is the first to observe the truncated apoC-I isoform, apoC-I′, lacking two electrically neutral amino acid residues Thr-Pro from the N-terminus. This truncated isoform was observed for all four subjects and the subject-to-subject variation in intensity of the apoC-I′ peak relative to the apoC-I peak was essentially the same for the normolipidemic subjects (1, 2, and 3). ApoC-I′ is a major component, constituting 30% of the apoC-I population based on both the MALDI and ESI data while for the hyperlipidemic subject (4) it was significantly lower (20%). The mechanism for processing apoC-I to apoC-I′ may involve the action of dipeptidyl peptidase IV (EC 3.4.14.5) in sera, a widely distributed proline-cleaving protease specific for NH_2-X-Pro- which is the N-terminal sequence for apoC-I or to post-proline cleaving enzyme (PPCE) (67, 68).

Isoforms due to glycosylation and the new isoform, apoC-III_x

Mass spectrometry has been particularly powerful in the study of glycoprotein structures through fragmentation studies and analysis of enzyme degradation products. In this study, mass spectrometry was used to identify apoC isoforms due to glycosylation by measurement of the added MM. All of the known apoC-III glycoproteins and their minor truncated isoforms were identified in the MALDI and ESI mass spectra.

One low intensity peak in the MALDI spectrum (Fig. 2) was identified and postulated to be a glycosylation isoform of apoC-III not previously observed. It is labeled apoC-III_x (MM = 9,933.8 Da). The charge distribution of species apoC-III_x ('v' in the ESI mass spectrum of Fig. 4) was found to be close to that for apoC-III_1, an indication that the two species have the same number of charged residues per mass unit and most likely the same protein backbone. It is possible that this isoform was not previously observed by IEF analysis because it has the same pI as apoC-III_1 or apoC-III_2.

Isoforms due to oxidation

Mass spectrometry is particularly sensitive to small differences or changes in the composition of the apolipoproteins. In order for mass spectrometry to be useful as part of an apolipoprotein analysis protocol, the relationship between the species identified in the mass spectrum and the in vivo species is an important and difficult issue to address. To minimize the perturbation, the time for analysis after blood draw was reduced to a minimum and samples requiring storage were kept at low temperature. The species most sensitive to in vitro oxidation were found to be proapoC-II, the most intense ion in the MALDI spectrum, and its truncated form, apoC-II, resulting in oxidation of one of the two methionine residues to an extent where they become the dominant forms in some samples. The other C apolipoproteins, under these circumstances, show some evidence for methionine oxidation but not to the same extent as proapoC-II and apoC-II. The spectrum shown in Fig. 2 shows the oxidized forms of proapoC-II and apoC-II as satellite peaks with no evidence for oxidation of the other apolipoprotein C species. The level of oxidized proapoC-II in the mass spectrum has been adopted as an internal marker for sample oxidation. The susceptibility of proapoC-II and apoC-II to methionine oxidation suggests that the conformation of this protein in the denatured state exposes these residues to oxidizing species while the other C apolipoproteins evidently adopt conformations that protect their methionines.

When samples analyzed by both MALDI and ESI-MS showed high levels of oxidized proapoC-II and apoC-II, another peak also appeared in the mass spectrum with MM of 9131 ± 2 Da in the MALDI spectrum and 9130 ± 1 Da in the ESI spectrum (not shown in Fig. 4). This MM value is consistent with an isoform of apoC-III_1 with the terminal sialic group missing from the sugar side chain, apoC-$III_{0,glyc}$. This species does not appear to be an in vivo form because it is present in the mass spectra only when the ox-

idized proapoC-II and apoC-II forms are present in high abundance. Most likely, it is formed by an oxidative process resulting in vitro desialylation of apoC-III_1.

Quantitative polymorphism

Quantitative polymorphism is defined as a subject-to-subject variation in apolipoprotein concentrations. In this study, the quantitative polymorphism measurement was restricted to the relative ion intensity measurement of the C apolipoproteins in VLDL. The objective of this component of the analysis is to develop a base line relative intensity pattern for normolipidemic individuals which is then compared with a dyslipidemic individual to identify species in the pattern that exhibit relative intensities that appear to be different from the normal distribution.

As shown in Fig. 6, the relative ion intensities of the C apolipoproteins, after normalizing to the intensity of apoC-I, are within a narrow range of ±20% for the three normolipidemic subjects. Subject 4, who had a lipoprotein pattern consistent with type V hyperlipidemia, had an apoC-III level that is 3 times that of the normolipidemic subjects. ApoC-III enhancement for hyperlipidemic individuals has been documented in the literature using immunoassay (69). In addition to apoC-III enhancement, subject 4 also exhibited a decrease by a factor of 2 of the level of apoC-I′, the new truncated isoform identified in this study. This finding for one subject warrants a more in-depth clinical assessment to establish this link, but it suggests a possible diminishing of dipeptidyl peptidase IV activity, the enzyme suggested to be active in the truncation. A reduction of this enzyme activity has been linked to aging (67).

The limited data set obtained in this study suggests that efforts to improve quantitation in mass spectrometry are warranted and could provide additional markers for dyslipidemias. Based on the results obtained in this study, MALDI appears to offer higher sensitivity for the minor isoforms as well as generating mass spectra that do not require deconvolution of multiple charge states. A MALDI protocol incorporating high mass accuracy and good precision in relative ion intensity measurements has the potential of being an important addition to apolipoprotein analysis.

Biochemical and clinical applications of mass spectrometry of the C apolipoproteins and apoE

The most important feature that mass spectrometry brings to the apolipoprotein analysis is that it is a multicomponent analysis with high specificity over the entire isoform distribution and in a single measurement requiring a few minutes of data acquisition time. The analysis is not dependent on antibody reactivity and is sensitive to isoforms that have similar MM values and the same pI ('electrically neutral' polymorphism (70)). The new truncated isoform of apoC-I detected in this study illustrates how isoforms with the same pI and similar MM values can be resolved and identified. Mass spectrometry makes more accessible the 'electrically neutral' polymorphism of the apolipoproteins. Many of these isoforms may constitute a part of the normal population and are invariant to any pathology. From a different perspective, the better accessibility of these isoforms to analysis provided by mass spectrometry may give more insights in the role of these species in human metabolism.

Phenotyping apoE by accurate MM measurement has the potential for a more specific assay of this important apolipoprotein. There is a 53 Da mass difference between apoE2, E3, and E4. This mass difference is larger than the resolution and accuracy of the MM mass measurement. As an example, the subject who provided the serum for the apoE mass spectrum shown in Fig. 3 was not genotyped for apoE. However, it is possible to phenotype the individual based on mass spectral evidence. The spectrum consists of a single apoE component with MM 34242 ± 10 Da. Based on these observations, we identify this individual as an E3 homozygote.

This work was sponsored by the National Institutes of Health (GM 26096 and HL 54566) the Robert A. Welch Foundation (A-258), and the Texas A & M College of Science Research Enhancement Program. We are particularly grateful for the medical expertise and perspective provided by Catherine J. McNeal, MD, Ph.D. A. M. Spiekerman, Ph.D. and his staff at the Scott & White Hospital, Pathology Department, have been important for numerous discussions in lipoprotein analysis and assisting in the transport of serum samples. The valuable support of Professor David Russell, Director of the Laboratory for Biological Mass Spectrometry, Department of Chemistry at Texas A&M, and Dr. Ricky Edmundson and their capable staff in carrying out the MALDI analyses is gratefully acknowledged.

Manuscript received 21 April 1998 and in revised form 29 October 1998.

REFERENCES

1. Kinnunen, P. K., R. L. Lackson, L. C. Smith, A. M. Gotto, Jr., and J. T. Sparrow. 1977. Activation of lipoprotein lipase by native and synthetic fragments of human plasma apolipoprotein C-II. *Proc. Natl. Acad. Sci. USA.* **74:** 4848–4851.
2. Wang, C. S., W. J. McConathy, H. U. Kloer, and P. Alaupovic. 1985. Moditaion of lipoprotein lipase activity by apolipoproteins. Effects of apolipoprotein C-III. *J. Clin. Invest.* **75:** 384–390.
3. Mahley, R. W., T. L. Innerarity, S. C. Rall, Jr., and K. H. Weisgraber. 1984. Plasma lipoproteins: apolipoprotein structure and function. *J. Lipid Res.* **25:** 1277–1292.
4. Swaney, J. B., and K. H. Weisgraber. 1994. Effect of apolipoprotein C-I on the apolipoprotein E content and receptor-binding properties of beta-migrating very low density lipoproteins. *J. Lipid Res.* **35:** 134–142.
5. Poensgen, J. 1990. Apolipoprotein C-I inhibits the hydrolysis by phospholipase A2 of phospholipids in liposomes and cell membranes. *Biochim. Biophys. Acta.* **1042:** 188–192.
6. Mahley, R. W. 1988. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science.* **240:** 622–630.
7. Dominiczak, M. H. 1997. Apolipoproteins and lipoproteins in human plasma. *In* Handbook of Lipoprotein Testing. N. Rifai, G. R. Warnick, and M. H. Dominiczak, editors. AACC Press, Washington, DC. 1–24.
8. Sirtori, C. R., G. Franceschini, H. B. Brewer, Jr., and G. Assmann. 1988. Human Apolipoprotein Mutants 2: From Genes Structure to Phenotypic Expressions. Plenum Press, New York and London.
9. Fojo, S. S., and H. B. Brewer, Jr. 1992. Hypertriglyceridaemia due to genetic defects in lipoprotein lipase and apolipoprotein C-II. *J. Intern. Med.* **231:** 669–677.
10. Reina, M., J. D. Brunzell, and S. S. Deeb. 1992. Molecular basis of familial chylomicronemia: mutation in the lipoprotein lipase and apolipoprotein C-II gene. *J. Lipid Res.* **33:** 1823–1832.

11. Scanu, A. M., R. E. Byrne, and C. Edelstein. 1984. Proteolytic events affecting plasma apolipoptroteins at the co- and post-translational levels and after maturation. *J. Lipid Res.* **25:** 1593–1601.
12. Hussain, M. M., and V. I. Zannis. 1990. Intracellular modification of human apolipoprotein AII (apo AII) and sites of apoAII mRNA synthesis: comparison of apoAII with apoCII and apoCIII isoproteins. *Biochemistry.* **29:** 209–217.
13. Jackson, R. L., J. T. Sparrow, H. N. Baker, J. D. Morrisett, O. D. Taunton, and A. M. Gotto, Jr. 1974. The primary structure of apolipoprotein-serine. *J. Biol. Chem.* **249:** 5308–5313.
14. Shulman, R. S., P. N. Herbert, K. Wehrly, and D. S. Fredrickson. 1975. The complete amino acid sequence of C-I (ApoLp-Ser), an apolipoprotein from human very low density lipoproteins. *J. Biol. Chem.* **250:** 182–190.
15. Hospattankar, A. V., T. Fairwell, R. Ronan, and H. B. Brewer, Jr. 1984. Amino acid sequence of human plasma apolipoprotein C-II from normal and hyperlipoproteinemic subjects. *J. Biol. Chem.* **259:** 318–322.
16. Hospattankar, A. V., H. B. Brewer, Jr., R. Ronan, and T. Fairwell. 1986. Amino acid sequence of human plasma polipoprotein C-III from normolipidemic subjects. *FEBS Lett.* **197:** 67–73.
17. McLean, J. W., N. A. Elshourbady, D. J. Chang, R. W. Mahley, and J. M. Taylor. 1984. Human apolipoprotein E mRNA. cDNA cloning and nucleotide sequencing of a new variant. *J. Biol. Chem.* **259:** 6498–6504.
18. Rall, S. C., Jr., K. H. Weisgraber, and R. W. Mahley. 1982. Human apolipoprotein E. The complete amino acid sequence. *J. Biol. Chem.* **257:** 4171–4178.
19. Utermann, G., Langenbeck, U. Beisiegel, and W. Weber. 1980. Genetics of the apolipoprotein E system in man. *Am. J. Hum. Genet.* **32:** 339–347.
20. Utermann, G., I. Kindermann, H. Kaffarnik, and A. Steinmetz. 1982. Apolipoprotein E phenotypes and hyperlipidemia. *Hum. Genet.* **60:** 232–236.
21. Zannis, V. I., and J. L. Breslow. 1981. Human very low density lipoprotein apolipoprotein E isoprotein polymorphism is explained by genetic variation and posttranslational modification. *Biochemistry.* **20:** 1033–1041.
22. de Knijff, P., A. M. J. M. van den Maagdenberg, R. R. Frants, and L. M. Havekes. 1994. Genetic heterogeneity of apolipoprotein E and its influence on plasma lipid and lipoprotein levels. *Hum. Mutat.* **4:** 178–194.
23. Zannis, V. I., J. McPherson, G. Goldberger, S. K. Karathanasis, and J. L. Breslow. 1984. Synthesis, intracellular processing, and signal peptide of human apolipoprotein E. *J. Biol. Chem.* **259:** 5495–5499.
24. Haase, R., I. Menke-Mollers, and K. Oette. 1988. Analysis of human apolipoproteins C by isoelectric focusing in immobilized pH gradients. *Electrophoresis.* **9:** 569–575.
25. Sharpe, C. R., A. Sidoli, C. S. Shelley, M. A. Lucero, C. C. Shoulder, and F. E. Baralle. 1984. Human apolipoproteins AI, AII, CII and CIII. cDNA sequence and mRNA abundance. *Nucleic Acids Res.* **12:** 3917–3932.
26. Fojo, S. S., L. Taam, T. Fairwell, R. Ronan, C. Bishop, M. S. Meng, J. M. Hoeg, D. L. Sprecher, and H. B. Brewer, Jr. 1986. Human preproapolipoprotein C-II. Analysis of major plasma isoforms. *J. Biol. Chem.* **261:** 9591–9594.
27. Brown, W. V., R. I. Levy, and D. S. Fredrickson. 1970. Further characterization of apolipoproteins from human plasma very low density lipoproteins. *J. Biol. Chem.* **245:** 6588–6594.
28. Albers, J. J., and A. M. Scanu. 1971. Isoelectric fractionation and characterization of polypeptides from human serum very low density lipoproteins. *Biochim. Biophys. Acta.* **236:** 29–37.
29. Brewer, H. B., Jr., R. S. Shulman, P. N. Herbert, R. Ronan, and K. Wehrly. 1974. The complete amino acid sequence of alanine apolipoprotein (apo C-III), an apolipoprotein from human plasma very low density lipoproteins. *J. Biol. Chem.* **249:** 4975–4984.
30. Jabs, H-U. and G. Assmann. 1987. Characterization of an apolipoprotein C-III mutant by high-performance liquid chromatography and time-of-flight secondary ion mass spectrometry. *J. Chromatogr.* **414:** 323–333.
31. Ito, Y., J. L. Breslow, and B. T. Chait. 1989. Apolipoprotein C-III_0 lacks carbohydrate residues: use of mass spectrometry to study apolipoprotein structure. *J. Lipid Res.* **30:** 1781–1787.
32. Atger, V., F. Duval, K. Frommherz, T. Drueke, and B. Lacour. 1988. Anomalies in composition of uremic lipoproteins isolated by gradient ultracentrifugation: relative enrichment of HDL in apolipoprotein C-III at the expense of apolipoprotein A-I. *Atherosclerosis.* **74:** 75–83.
33. Stocks, J., G. Holdsworth, and D. Galton. 1979. Hypertriglyceridaemia associated with an abnormal triglyceride-rich lipoprotein carrying excess apolipoprotein C-III_2. *Lancet.* **8144:** 667–671.
34. Edelstein, C., and A. M. Scanu. 1986. Precautionary measures for collecting blood destined for lipoprotein isolation. *Methods Enzymol.* **128:** 151–155.
35. Schumaker, V., and D. L. Puppione. 1986. Sequential flotation ultracentrifugation. *Methods Enzymol.* **128:** 155–170.
36. Saruta, H., Y. Ashihara, M. Sugiyama, M. Roth, E. Miyagawa, Y. Kido, and Y. Kasahara. 1986. Colorimetric determination of carboxypeptidase A activity in serum. *Clin. Chem.* **32:** 748–751.
37. von Eckardstein, A., M. Walter, H. Holz, A. Benninghoven, G. Assmann, A. Roetrige, and R. Kock. 1991. Site-specific methionine sulfoxide formation is the structural basis of chromatographic heterogeneity of apolipoproteins A-I, C-II, and C-III. *J. Lipid Res.* **32:** 1465–1476.
38. Hughes, T. A., M. A. Moore, P. Neame, M. F. Medley, and B. H. Chung. 1988. Rapid quantitative apolipoprotein analysis by gradient ultracentrifugation and reversed-phase high performance liquid chromatography. *J. Lipid Res.* **29:** 363–376.
39. Esterbauer, H., J. Gebicki, H. Puhl, and G. Jurgens. 1998. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radical Biol. Med.* **13:** 341–390.
40. Nagano, Y., H. Arai, and T. Kita. 1991. High density lipoprotein loses its effect to stimulate efflux of cholesterol from foam cells after oxidative modification. *Proc. Natl. Acad. Sci. USA.* **88:** 6457–6461.
41. O'Farrell, P. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250:** 4007–4021.
42. Sprecher, D. L., L. Taam, and H. B. Brewer, Jr. 1984. Two-dimensional electrophoresis of human plasma apolipoproteins. *Clin. Chem.* **30:** 2084–2092.
43. Schmitz, G., K. Ilsemann, B. Melnik, and G. Assmann. 1983. Isoproteins of human apolipoprotein A-II: isolation and characterization. *J. Lipid Res.* **24:** 1021–1029.
44. Torgerson, D. F., R. P. Skowronski, and R. D. Macfarlane. 1974. New approach to the mass spectrometry of non-volatile compounds. *Biochem. Biophys. Res. Commun.* **60:** 616–621.
45. Hillenkamp, F., M. Karas, R. C. Beavis, and B. T. Chait. 1991. Matrix-assisted lazer desorption/ionization mass spectrometry of biopolymers. *Anal. Chem.* **63:** 1193A–1202A.
46. Fenn, J. B., M. Mann, C. K. Meng, S. F. Wong, and C. M. Whitehouse. 1989. Electrospray ionization for mass spectrometry of large biomolecules. *Science.* **246:** 64–71.
47. Beavis, R. C., and B. T. Chait. 1990. Rapid, sensitive analysis of protein mixtures by mass spectrometry. *Proc. Natl. Acad. Sci. USA.* **87:** 6873–6877.
48. Jabs, H-U., G. Assmann, D. Greifendorf, and A. Benninghoven. 1986. High performance liquid chromatography and time-of-flight secondary ion mass spectrometry: a new dimension in structural analysis of apolipoproteins. *J. Lipid Res.* **27:** 613–621.
49. Weinmann, W., C. E. Parker, L. J. Deterding, D. I. Papac, J. Hoyes, M. Przybylski, and K. B. Tomer. 1994. Capillary electrophoresis-matrix-assisted laser-desorption ionization mass spectrometry of proteins. *J. Chromatogr. A.* **680:** 353–361.
50. Wang, C. S., D. Downs, A. Dashti, and K. W. Jackson. 1996. Isolation and characterization of recombinant human apolipoprotein C-II expressed in *Escherichia coli*. *Biochim. Biophys. Acta.* **1302:** 224–230.
51. Smith, R. D., C. J. Barinaga, and H. R. Udseth. 1989. Tandem mass spectrometry of highly charged cytohrome *c* molecular ions produced by electrospray ionization. *J. Phys. Chem.* **93:** 5019–5022.
52. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150:** 76–85.
53. Osborne, J. C. 1986. Delipidation of plasma lipoproteins. *Methods Enzymol.* **128:** 213–222.
54. Cruzado, I. D., S. Song, S. F. Crouse, B. C. O'Brien, and R. D. Macfarlane. 1996. Characterization and quantitation of the apoproteins of high-density lipoprotein by capillary electrophoresis. *Anal. Biochem.* **243:** 100–109.
55. Bondarenko, P. V., and R. D. Macfarlane. 1997. A new electrospray ionization time-of-flight mass spectrometer with electrostatic wire ion guide. *Int. J. Mass Spectrom. Ion Processes.* **160:** 241–258.
56. Edmondson, R. D. and D. H. Russell. 1996. Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass measure-

ment accuracy by using delayed extraction. *J. Am. Soc. Mass Spectrom.* **7:** 995–1001.
57. Cruzado, I. D., S. L. Cockrill, C. J. McNeal, and R. D. Macfarlane. 1998. Characterization and quantitation of apolipoprotein B-100 by capillary electrophoresis. *J. Lipid Res.* **39:** 205–217.
58. Mann, M., C. K. Meng, and J. B. Fenn. 1989. Interpreting mass spectra of multiply charged ions. *Anal. Chem.* **61:** 1702–1708.
59. Smith, R. D., J. A. Loo, C. G. Edmonds, C. J. Barinaga, and H. R. Udseth. 1990. New development in biochemical mass spectrometry: electrospray ionization. *Anal. Chem.* **62:** 882–899.
60. Loo, J. A., H. R. Udseth, and R. D. Smith. 1989. Peptide and protein analysis by electrospray ionization-mass spectrometry and capillary electrophoresis-mass spectrometry. *Anal. Biochem.* **179:** 404–412.
61. Smith, R. D., J. A. Loo, R. R. Ogorzalek Loo, M. Busman, and H. R. Udseth. 1991. Principles and practice of electrospray ionization-mass spectrometry for large polypeptide and proteins. *Mass Spectrom. Rev.* **10:** 359–451.
62. Gotto, A. M., Jr., H. J. Pownall, and R. J. Havel. 1986. Introduction to the plasma lipoproteins. *Methods Enzymol.* **128:** 3–41.
63. Menzel, H. J., J. P. Kane, M. J. Malloy, and R. J. Havel. 1986. A variant primary structure of apolipoprotein C-II in individuals of African descent. *J. Clin. Invest.* **77:** 595–601.
64. Sepehrnia, B., M. I. Kamboh, and R. E. Ferrell. 1988. Genetic studies of human apolipoproteins. III. Polymorphism of apolipoprotein C-II. *Hum. Hered.* **38:** 136–143.
65. Sepehrnia, B., M. I. Kamboh, L. L. Adams-Campbell, C. H. Bunker, M. Nwankwo, P. P. Majumder, and R. E. Ferrell. 1989. Genetic studies of human apolipoproteins. XI. The effect of the apolipoprotein C-II polymorphism on lipoprotein level in Nigerian blacks. *J. Lipid Res.* **30:** 1349–1355.
66. Pullinger, C. R., B. R. Zysow, L. K. Hennessy, P. H. Frost, M. J. Malloy, and J. P. Kane. 1993. Molecular cloning and characteristics of a new apolipoprotein C-II mutant identified in three unrelated individuals with hypercholesterolemia and hypertriglyceridemia. *Hum. Mol. Genet.* **2:** 69–74.
67. Gotoh, H., M. Hagihara, T. Nagatsu, H. Iwata, and T. Miura. 1988. Activity of dipeptidyl peptidase IV and post-proline cleaving enzyme in sera from osteoporotic patients. *Clin. Chem.* **34:** 2499–2501.
68. Vanhoof, G., F. Goossens, I. De Meester, D. Hendriks, and S. Scharpe. 1995. Proline motifs in peptides and their biological processing. *FASEB J.* **9:** 736–744.
69. Bury, J., W. DeKeersgieter, M. Rosseneu, F. Belpaire, and J. Christophe. 1985. Immunonephelometric quantitation of the apolipoprotein C-III in human plasma. *Clin. Chim. Acta.* **145:** 249–258.
70. von Eckardstein, A., H. Funke, M. Walter, K. Altland, A. Benninghoven, and G. Assmann. 1990. Structural analysis of human apolipoprotein A-I variants. Amino acid substitutions are nonrandomly distributed throughout the apolipoprotein A-I primary structure. *J. Biol. Chem.* **265:** 8610–8617.
71. Fojo, S. S., A. F. Stalenhoef, K. Marr, R. E. Gregg, R. S. Ross, and H. B. Brewer, Jr. 1988. A deletion mutation in the apoC-II gene (ApoC-II Nijmegen) of a patient with a deficiency of apolipoprotein C-II. *J. Biol. Chem.* **263:** 17913–17916.
72. Gabelli, C., C. Bilato, S. S. Fojo, S. Martini, H. B. Brewer, Jr., G. Crapaldi, and G. Baggio. 1993. Heterozygous apolipoprotein C-II deficiency: lipoprotein and apolipoprotein phenotype and RsaI restriction enzyme polymorphysm in the apo C-II_{Padova} kindred. *Eur. J. Clin. Invest.* **23:** 522–528.
73. Fojo, S. S., P. Lohse, C. Parrott, C. Gabelli, F. Thomas, J. Hoffman, and H. B. Brewer, Jr. 1989. A nonsense mutation in the apolipoprotein C-II_{Padova} gene in a patient with apolipoprotein C-II deficiency. *J. Clin. Invest.* **84:** 1215–1219.
74. Parrott, C. L., N. Alsayed, R. Rebourcet, and S. S. Fojo. 1992. ApoC-II_{Paris2}: a premature termination mutation in the signal peptide of apoC-II resulting in the familial chylomicronemia syndrome. *J. Lipid Res.* **33:** 361–367.
75. Crecchio, C., A. Capurso, and G. Pepe. 1990. Identification of the mutation responsible for a case of plasmatic apolipoprotein C-II deficiency (apoC-II_{Bari}). *Biochem. Biophys. Res. Commun.* **168:** 1118–1127.
76. Beil, F. U., S. S. Fojo, H. B. Brewer, Jr., H. Greten, and U. Beisiegel. 1992. Apolipoprotein C-II deficiency syndrome due to apo C-$II_{Hamburg}$: clinical and biochemical features and *Hph*I restriction enzyme polymorphism. *Eur. J. Clin. Invest.* **22:** 88–95.
77. Fojo, S. S., J. L. deGennes, J. Chapman, C. Parrott, P. Lohse, S. S. Kwan, J. Truffert, and H. B. Brewer, Jr. 1989. An initiation codon mutation in the apoC-II gene (ApoC-II_{Paris}) of a patient with a deficiency of apolipoprotein C-II. *J. Biol. Chem.* **264:** 20839–20842.
78. Connelly, P. W., G. F. Maguire, T. Hofmann, and J. A. Little. 1987. Structure of apolipoprotein C-$II_{Toronto}$, a nonfunctional human apolipoprotein. *Proc. Natl. Acad. Sci. USA.* **84:** 270–273.
79. Connelly, P. W., G. F. Maguire, C. Vezina, R. A. Hegele, and J. A. Little. 1993. Identification of disulfide-linked apolipoprotein species in human lipoproteins. *J. Lipid Res.* **34:** 1717–1727.
80. Connelly, P. W., G. F. Maguire, and J. A. Little. 1987. Apolipoprotein C-$II_{St. Michael}$: familial apolipoprotein C-II deficiency associated with premature vascular disease. *J. Clin. Invest.* **80:** 1597–1605.
81. Zysow, B. R., C. R. Pullinger, L. K. Hennessy, R. V. J. Farese, M. Ghassemzadeh, and J. P. Kane. 1994. The apolipoprotein C-II variant apo C-II(Lys19→Thr) is not associated with dyslipidemia in an affected kindred. *Clin. Genet.* **45:** 292–297.
82. Luttmann, S., A. von Eckardstein, W. Wei, H. Funke, E. Kohler, R. W. Mahley, and G. Assmann. 1994. Electrophoretic screening for genetic variation in apolipoprotein C-III: identification of a novel apoC-III variant, apoC-III(Asp_{45}→Asn_{45}), in a Turkish patient. *J. Lipid Res.* **35:** 1431–1440.
83. Maeda, H., R. K. Hashimoto, T. Ogura, S. Hiraga, and H. Uzawa. 1987. Molecular cloning of a human apoC-III variant: Thr74→Ala74 mutation prevents O-glycosylation. *J. Lipid Res.* **28:** 1405–1409.
84. von Eckardstein, A., H. Holz, M. Sandkamp, W. Weng, H. Funke, and G. Assmann. 1991. Apolipoprotein C-III(Lys58→Glu). Identification of an apolipoprotein C-III variant in a family with hyperalphalipoproteinemia. *J. Clin. Invest.* **87:** 1724–1731.
85. Ferrell, R. E., M. I. Kamboh, P. P. Majumder, R. Valdez, and K. M. Weiss. 1990. Genetic studies of human apolipoproteins. XIII. Quantitative polymorphism of apolipoprotein C-III in the Mayans of the Yucatan peninsula. *Hum. Hered.* **40:** 127–135.
86. Menzel, H. J., R. G. Kladetzky, and G. Assmann. 1983. Apolipoprotein E polymorphism and coronary artery disease. *Arteriosclerosis.* **3:** 310–315.
87. Lundqvist, M., P. Baltzer, D. Edvardsson, L. Karlsson, and B. Wannberg. 1995. Novel time-of-flight instrument for doppler free kinetic energy release spectroscopy. *Phys. Rev. Lett.* **75:** 1058–1061.
88. Anderson, N. L., R. P. Tracy, and N. G. Anderson. 1984. High-resolution two-dimensional electrophoretic mapping of plasma proteins. *In* The Plasma Proteins. Structure, Function, and Genetic Control. F.W. Putman, editor. Academic Press, Inc. London. 222–270.