Chapter 5

Mapping O-glycosylation of apolipoprotein C-III in MALDI-FT-ICR protein profiles

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ABSTRACT

Ultrahigh resolution MALDI-FT-ICR profiles were obtained from human serum samples that were processed using a fully automated RPC18-based magnetic bead method. Proteins were profiled from *m/z* value 6630 with a resolving power of 73000 up to *m/z* value 12600 with a resolving power of 37000. In this study, a detailed evaluation was performed of the isoforms of apolipoprotein C-III, *i.e.* the different mucin-type core 1 O-glycans with the addition of one or two sialic acid residues. The MALDI-FT-ICR profiles are discussed with regard to reproducibility of the signal intensities as well as the accurate mass measurements. ESI-FTICR-MS/MS analyses of the same serum samples were performed to confirm the identity of apolipoprotein C-III glycoforms.

INTRODUCTION

Human serum peptide and protein profiling by MALDI coupled with TOF-MS is a well-established discovery strategy for disease-related biomarkers [1]. Studies reported in the previous decade have resulted in a large number of candidate peptides and proteins that were found to correlate with the presence and/or a stage of a certain disease. However, most of these discoveries have not been translated into a diagnostic clinical assay [2–5]. The obvious explanation for this setback is the inherent lack of "depth" in profiling studies, *i.e.* of the existing ten orders of magnitude of concentration range in protein abundance, only the first three or four layers are mapped [5]. Another important reason for the hitherto disappointing outcome of MS-based biomarker studies relates to validation, as robustness of the analytical platform at high-throughput use is essential. It has been shown that the reproducibility of MALDI profiling methods improves using highly standardized sample collection and work-up protocols [6–9]. The use of a fully automated liquid-handling robotic platform results in reproducible SPE peptide purification steps as well as in robust MALDI spotting procedures; thus, standardizing the entire serum profiling workflow. Sample purification and/or separation procedures are needed prior to analysis by MS, because body fluids such as serum are highly complex mixtures. With each additional procedure that is included in the workflow, more effort is required to achieve good robustness of the analytical protocols. While MALDI profiling protocols include off-line peptide or protein purification methods [10], the application of LC together with ESI allows separation of peptide and proteins with on-line MS measurement. The separation efficiency of LC has led to both a larger number of profiled peptides and proteins and to a more quantitative analysis when triple quadrupole (Q) MS was used [11,12]. However, relatively low throughput is inherent to most LCMS methods, which makes the analysis of large sample cohorts difficult and thus impedes the process of biomarker validation. High-throughput approaches pose a large demand on LC equipment. Obviously, chromatographic runs can be shortened (to a certain extent) to alleviate the problem of running into weeks or even months of analysis time. Another way to tackle sample complexity involves the use of ultrahigh resolution instruments such as FT-ICR or Orbitrap R MS. These provide the highest mass measurement precision and accuracy and allow separation of peptides and proteins that would overlap in lower resolution mass spectrometers [13–15]. We recently reported serum peptide profiles (*m/z* range from 1000

to 6500 Da) with low parts per million (ppm) mass measurement errors (MMEs) obtained from an MALDI-FT-ICR system equipped with a 15 tesla magnet [16]. It was shown that the sub-ppm mass measurement precision improved the alignment of multiple spectra that are needed for comparative analysis and thus allowed a robust and standardized high-throughput screening of hundreds to thousands of patient samples. In almost any biomarker discovery study larger peptides and proteins are profiled by low resolution MALDI-TOF-MS. Unfortunately, because of the lower resolving power at higher masses as well as the intrinsic more complex isotopic distribution, such larger peptides and proteins are detected as broad peaks. In these cases, the identification and quantification of single species is very challenging with respect to robustness of the method. Moreover, when protein signals in profiles are isotopically resolved the mass differences between corresponding variants can be determined with great accuracy, thus allowing the determination of a point mutation or a PTM [17]. In the current study, we have used a new MALDI-FT-ICR method for profiling of large serum peptides and proteins in the mass range from 6000 to 15 300 Da with high sensitivity and high resolution. Based on the previously reported identifications in MALDI profiles, we focused on apolipoproteins that were observed in the current profiles, namely apolipoprotein C-I (Apo-CI), C-II (Apo-CII), and C-III (Apo-CIII), including its glycosylated isoforms [1, 18, 19]. Apolipoproteins C (Apo-Cs) are present in blood associated, at different degrees, with various lipoproteins particles [20–22]. Several studies have been carried out in order to elucidate the role of Apo-Cs in the metabolism of lipoproteins and results have been comprehensively reviewed [23–25]. Briefly, Apo-Cs are involved in the metabolism of lipoproteins through the activation or inhibition of several triacylglycerol lipases and interaction with different lipoprotein receptors [26]. It is known that Apo-Cs levels are abnormal with certain diseases. For example, individuals affected by diabetes as well as those subjects with hypertriglyceridemia have increased concentration of Apo- CIIIs [27,28]. The risk of coronary heart disease has been also correlated with Apo-CIIIs levels in blood [29, 30]. Cohen et al. recently reported on an MALDI-TOF profiling study showing that Apo-CI and Apo-CIII extracted on C8-coated beads were significantly lower in serum samples of stomach cancer patients as compared to healthy individuals [31]. In addition, the glycosylation pattern of Apo-CIII is being used as a marker for certain congenital disorders of glycosylation (CDGs). In fact, Wopereis et al. found that Apo-CIII

IEF can be used to detect abnormalities in the biosynthesis of core 1 mucin-type O-glycans and reported that patients with CDG type IIe and IIf as well as part of patients with an unspecific CDG type II had hypoglycosylated Apo-CIII isoform profiles [32, 33]. Density gradient ultracentrifugation procedures have been applied for the separation of the lipoprotein particles containing Apo-Cs [34]. Other techniques such as delipidation, 2DE [35–37], IEF [38], and LC [39, 40] have been used to further purify and separate Apo-Cs. In the first approach, long centrifugation times are often required, thus decreasing the throughput of the analysis. Apo-Cs have been successfully extracted and profiled using a combination of SPE and MALDI-TOF MS [18, 19, 41–43]. In the present study, we perform magnetic bead (MB) based SPE of serum samples followed by the acquisition of ultrahigh resolution MALDI-FT-ICR profiles including Apo-CI, Apo-CII, and glycosylated Apo-CIII isoforms.

MATERIALS AND METHODS

Sample collection. Human blood samples were collected as previously described [44]. Briefly, blood samples were collected in 10-mL BD Vacutainer tubes (containing a clot activator and a gel for serum separation) from healthy volunteers by antecubital venipuncture and centrifuged for 1 h. Serum was then transferred to a 1-mL cryovial and stored at –80 °C until further aliquoting. To this end, 96 cryovials were thawed and serum was distributed over eight racks using an eight-channel liquid-handling robot (Hamilton, Bonaduz, Switzerland). Each rack was stored again at –80 °C and thawed only for the automated peptide isolation procedure.

Serum protein isolation. Serum samples were anonymized before purification of proteins by SPE either based on magnetic beads (MBs) or on cartridges. Previously, it was shown that these two SPE protocols provided similar results for peptide isolation from serum [45]. In the cartridge-based protocol, serum samples were thawed at room temperature for 1 h, diluted four times with 0.1% acetic acid and loaded (100 µL) on preconditioned C18-cartridges (SPARK). Each cartridge was washed with 2 mL of 1% acetic acid/2% acetonitrile (ACN) solution and peptides/proteins were eluted with 100 µL of a 50% ACN/0.1% acetic acid solution. This procedure was implemented on a customized SPARK Symbiosis SPE system, specifically adapted to increase sample throughput. The

MB-based protocol was performed for the purification of 96 serum samples from 96 different individuals using a 96-channel liquid handling robot as described previously [16]. After both types of SPE methods, MALDI spotting was performed on the same liquid handling robot by mixing 2 µL of the sample eluates with 10 µL of an α-cyano-4-hydroxycinnamic acid solution (0.3 g/L in ethanol/acetone 2:1). Each sample was spotted either in duplicate (MBseluates) or quadruplicate (cartridge eluates) onto an MALDI AnchorChip (600 µm; Bruker Daltonics, Bremen, Germany). Cartridge-based SPE was used for method optimization of the MALDI-FT-ICR experiments (Fig. 1), whereas MBs were used to evaluate the biological variation of the Apo-Cs isoforms that were stored at −80 °C for 6 months and thawed at room temperature prior to MALDI spotting (Fig. 3). For identification purposes, proteins were purified from serum samples by RPC4 SPE. Briefly, 300 µL of serum was diluted with 600 µL of a 0.05% formic acid (FA) solution, vortexed and kept at room temperature for 15 min for standardization reasons. The diluted serum was then loaded on a 1 mL BioSelect SPE C4 cartridge (Grace, Deerfield, Illinois, USA) that was prewashed two times with 900 µL of a 95% ACN/4.95% water/ 0.05% FA solution and preconditioned with 900 µL of a 0.05% FA solution. The cartridge was then washed three times with 900 µL of a 0.05% FA solution and the adsorbed peptides and proteins were stepwise eluted with 900 µL of 5, 10, 15, and 20% ACN solution. These five fractions were analysed by MALDI-FT-ICR-MS to determine which fractions contained the relevant apolipoproteins (data not shown). To this end, 1 µL of each of the RPC4 cartridge eluates was mixed with 15 µL α-cyano-4-hydroxycinnamic acid solution (0.3 g/L in ethanol/acetone 2:1) and 1 µL of the mixture was spotted on an MALDI AnchorChip. Direct infusion ESI-FT-ICR analysis was performed only on the RPC4 cartridge fractions that contained the Apo-CIII isoforms. Prior to ESI-FT-ICR MS, 50 µL of each suitable eluate was diluted with 50 µL of a 95% ACN/4.95% water/0.05% FA solution and analyzed by direct infusion.

MALDI- and ESI-FT-ICR MS. Both MALDI- and ESI-FT-ICR experiments were performed on a Bruker 15 tesla solariX^{TM} FT-ICR mass spectrometer. The MALDI-FT-ICR system was controlled by Compass solariX control software and equipped with a Bruker Smartbeam-II^{TM} Laser System that operated at a frequency of 1000 Hz. The “medium” predefined shot pattern was used for spectrum acquisition. Each mass

spectrum was acquired from *m/z* values 6000 to 15000 and obtained from the average of ten scans of 150 laser shots each using 256 K data points. The random walk option was allowed on a diameter of 300 µm. Typically, the target plate offset was 100 V with the deflector plate set at 180 V. The ions were trapped and measured in the ICR cell using the Gated Trapping option. Both the front and the back trapping potentials were set to 1 V while the front and the back detection voltages were set to 0.4 and 0.45 V, respectively. The ramp time was 0.01 s. The required excitation power was 45% with a pulse time of 15.0 µs. DataAnalysis Software 4.0 SP 3 (Bruker Daltonics) was used for the visualization and the calibration of the spectra while the IsotopePattern software (Bruker Daltonics) was used for the simulation of the isotopic distribution of the Apo-CIIIs isoforms. Direct infusion ESI-FT-ICR experiments were performed using the same instrumental settings described previously [46]. Briefly, a Q was used for precursor ion selection and a hexapole collision cell for CID. Direct infusion ESI experiments were carried out at an infusion rate of 2 µL/min. The ion funnels operated at 100 and 6.0 V, respectively, with the skimmers at 15 and 5 V. The trapping potentials were set at 0.60 and 0.55 V, the analyzer entrance was maintained at –7 V, and side kick technology was used to further optimize peak shape and signal intensity. The required excitation power was 28% with a pulse time of 20 µs. MS/MS experiments were performed with the Q at an isolation window of 10 mass units followed by CID and fragment ion mass analysis in the ICR cell. For CID experiments, both the collision energy and the accumulation time in the hexapole collision cell were optimized for each precursor ion. Collision energies varied from 5 to 33 V while the accumulation times varied from 1 to 5 s.

RESULTS AND DISCUSSION

MALDI-FT-ICR serum profiles. A stringent protocol was used for the collection of human blood samples to allow for a valid evaluation of peptide and protein signals in MALDI profiles, and for later comparison of different patient cohorts in a high-throughput manner. All serum samples were processed through a fully automated and standardized workup procedure applying SPE with RPC18 functionalized MBs as described previously [45]. Then, for screening purposes, 1 µL of each serum eluate (*i.e.* obtained after SPE) was measured on an MALDI-TOF system and the remainder of the eluates was stored at –80 °C (data not shown). In this study, these serum eluates were

further used to obtain profiles of apolipoproteins. To this end, the eluates were thawed and anonymized, and then spotted onto an MALDI target plate after premixing with matrix solution using the same 96-channel liquid handling platform. MALDI-FT-ICR spectra (profiles) were obtained through an automated acquisition procedure on the solariX^{TM} system (see Section 2). A typical example of an ultrahigh resolution MALDI-FT-ICR serum protein profile recorded from *m/z* values 6000 to 15000 is shown in Fig. 1. Note that all species (*i.e.* proteins) in the profile in Fig. 1 are isotopically resolved, with a resolving power of 73000, 51000, and 37000 at *m/z* values 6630, 9420, and 12607, respectively. Furthermore, a resolving power of 29000 for the signal at *m/z* 15193 was determined in the MALDI-FT-ICR protein profile obtained from an RPC4 cartridge eluate, as shown in the inset in Fig. 1 (note that this signal was not observed in the RPC18 MALDI-FTICR protein profile). Recently, we have reported on ultrahigh resolution MALDI-FT-ICR serum peptide profiles up to *m/z* value 6500 from similar samples with identical workup. It was shown that MMEs of multiple MS acquisitions were at the sub-ppm level both for individual samples (repeatability) and for hundreds of different serum samples after alignment. Hence, these FT-ICR spectra were established as precision peptide profiles. In the current profile, the *m/z* range has been extended up to *m/z* value 15000. From Fig. 1, it is evident that the use of an FT-ICR-system allows accurate mass analysis of small proteins in MALDI protein profiles because all species are fully resolved up to an *m/z* value of 12615 (or even at *m/z* value 15193, Fig. 1 inset). This was not possible on previous MALDI equipment (often TOF mass analyzers).

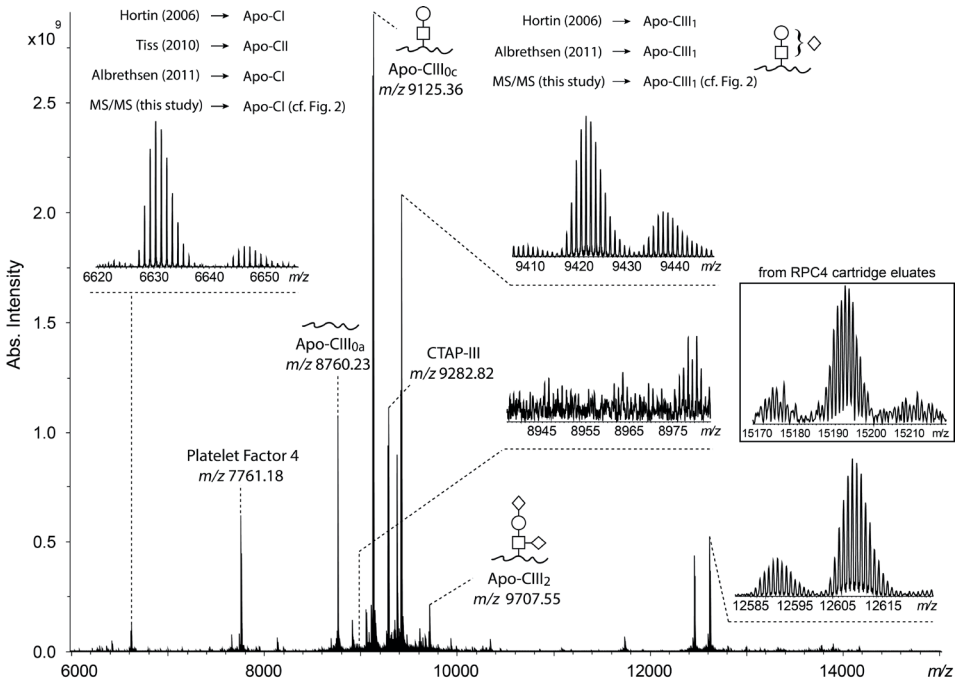

Figure 1. Ultrahigh resolution 15T MALDI-FT-ICR protein profile obtained from human serum purified by RPC18 cartridge-based SPE. Proteins were profiled from *m/z* value 6630 with a resolving power of 73000 up to *m/z* value 12600 with a resolving power of 37000. The inset shows an enlargement of an MALDI-FT-ICR protein profile obtained from an RPC4 cartridge eluate. The resolving power for the protein at 15193 *m/z* was 29000. This signal was not observed in the RPC18 MALDI-FT-ICR protein profile. The identification was performed by comparing the observed *m/z* values to previously reported lists of (proteolytically degraded) proteins (or polypeptides) (Table 1). The most abundant peaks were identified as O-glycosylated isoforms of apolipoprotein-CIII. ESI-FT-ICR MS/MS experiments confirmed the identity of both Apo-CI and Apo-CIII$_1$ (Fig. 2).

Moreover, possible peak overlap can be determined in ultrahigh resolution profiles, and peak intensities at high *m/z* values can be measured with greater precision [16]. Obviously, the more accurate mass differences between various species within one profile increase confidence levels of their identification (as will be further discussed at the end of Section 3.2, *i.e.* Fig. 3).

Apolipoprotein identifications. Initially, all *m/z* values of species observed in the ultrahigh resolution high-mass MALDI-FT-ICR profile (Fig. 1) were compared to previously reported lists of (proteolytically degraded) proteins (or polypeptides) [1, 18, 19]. From these lists it became clear that several apolipoproteins were present in the profile, such as the monoisotopic peak of apo-CI (calculated *m/z* value 6627.5131), the monoisotopic peak of apo-CIII (calculated *m/z* value 8689.1879), and the monoisotopic peak of apo-CII (calculated *m/z* value 8910.3856). A summary of all species in Fig. 1 that could be assigned based on known proteins is given in Table 1. The monoisotopic peak values in our data were in low ppm agreement with the previously reported O-glycosylated isoforms of Apo-CIII, with the remark that glycan linkages cannot be determined from the current experiments. These previously reported Apo-CIII isoforms consist of a single mucin-type core-1 disaccharide galactose linked to N-acetylgalactosamine (Galβ1–3GalNAc), as such, or include a terminal modification with one (NeuAcα2–3Galβ1–3GalNAc or Galβ1–3[NeuAcα2–6]GalNAc) or two (NeuAcα2–3Galβ1–3[NeuAcα2–6]GalNAc) sialic acid residues (or N-acetylneuraminic acid (NeuAc)). These three different isoforms of Apo-CIII are commonly referred to as Apo-$CIII_0$, Apo-$CIII_1$, and Apo-$CIII_2$, respectively, the index pointing at the number of sialic acid residues bound to the protein as determined by IEF [32]. Note that in such an IEF profile the Apo-$CIII_0$ band can include three different isoforms, namely nonglycosylated Apo-CIII, Apo-CIII with one GalNAc monosaccharide, and Apo-CIII with the GalNAc-Gal disaccharide. Since all these three species lack any sialic acids (and are thus referred to as Apo-$CIII_0$), it has been proposed to add an index a, b, or c [43]. In this study we did not find any evidence for the presence of Apo-$CIII_{0b}$ (calculated *m/z* value 8963.3044) in any of the acquired MALDI-FT-ICR profiles of all measured serum samples (Fig. 1). Interestingly, a conflict in assignment of the *m/z* value 6627.5 in Fig. 1 was noted, which was typed as Apo-CII by Tiss et al., whereas Hortin and Albrethsen reported Apo-CI. From our ESI-FT-ICRMS/MS data it was concluded that this species (in MALDI observed at *m/z* value 6627.5, in ESI selected as precursor ion $[M+10H]^{10+}$) matched the Apo-CI amino acid sequence (part of the CID-spectrum is depicted in Fig. 2A).

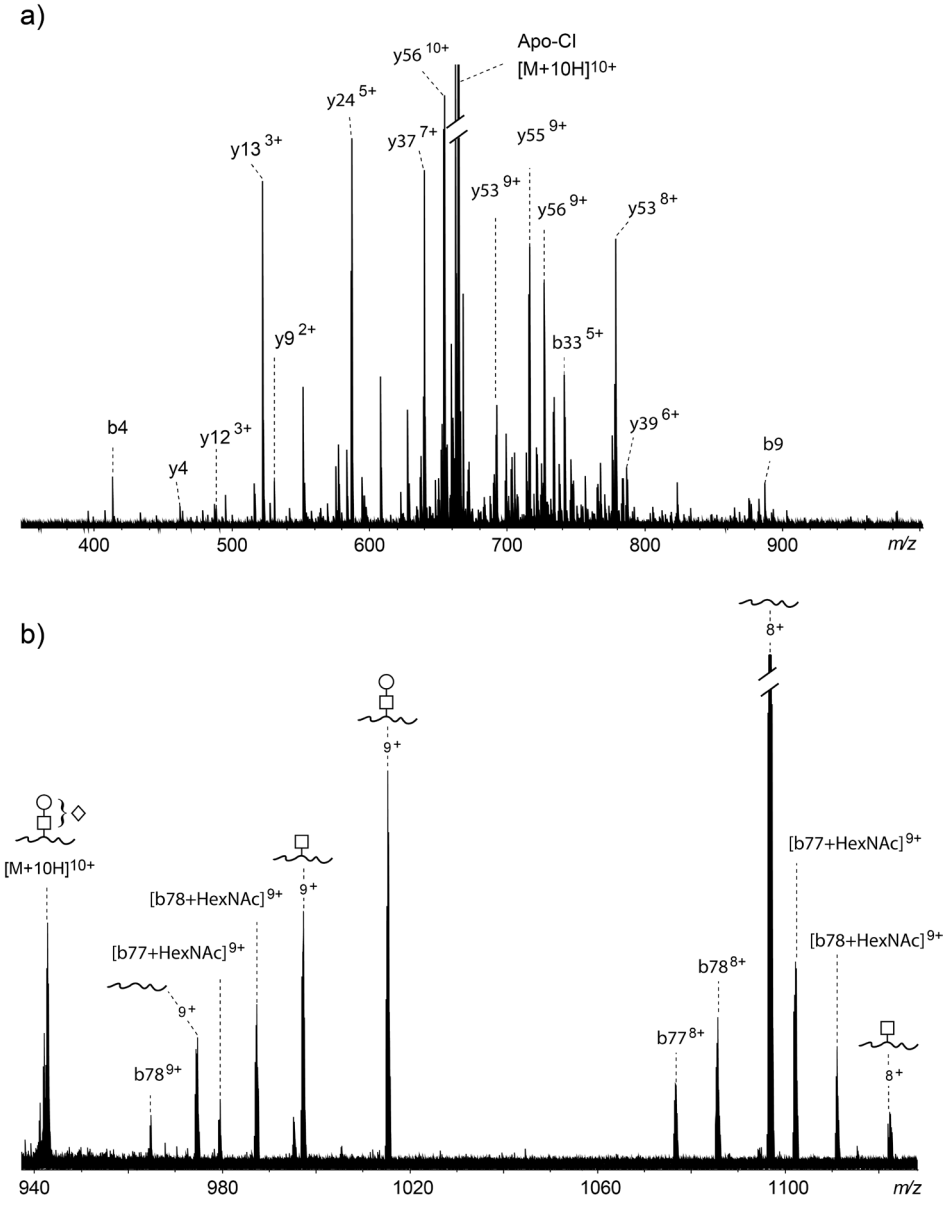

Figure 2. Enlarged parts of the CID spectra of Apo-CI (A) and Apo-CIII$_1$ (B) obtained with direct infusion ESI-FT-ICR-MS/MS of serum samples that were purified by RPC4 cartridge-based SPE. In both cases, the [M+10H]$^{10+}$ precursor ion was selected in the quadrupole (Q) to perform MS/MS experiments. In the MS/MS spectrum of Apo- CIII$_1$ (B) all fragment ions lack the O-glycan, unless indicated explicitly.

Table 1. List of identified proteins observed in RPC18 magnetic bead and RPC18 cartridge MALDI-FTICR serum profiles in the *m/z*-range of 6,000-15,000. Twelve out of 15 identified proteins belong to the apolipoprotein family including O-glycosylated isoforms of apolipoprotein-CIII. The identity of the two proteins written in bold, namely ApoCI (27-83) and ApoCIII$_1$ (21-99) was confirmed by ESI-FTICR MS/MS experiments (Fig. 2).

Calculated monoisotop. $[M+H]^+$	Unipro t Entry	Name (fragment)	Peptide sequences
6429.4127	P02654	ApoCI (29-83)	DVSSALDKLKEFGNTLEDKARELISRIKQSELSAKMREWFSETFQKVKEKL KIDS
6627.5131	P02654	**ApoCI (27-83)**	TPDVSSALDKLKEFGNTLEDKARELISRIKQSELSAKMREWFSETFQKVKE KLKIDS
7761.1843	P02776	Platelet Factor 4 (32-101)	EAEEDGDLQCLCVKTTSQVRPRHITSLEVIKAGPHCPTAQLIATLKNGRKIC LDLQAPLYKKIIKKLLES
8200.0509	P02655	ApoCII (29-101)	DEMPSPTFLTQVKESLSSYWESAKTAAQNLYEKTYLPAVDEKLRDLYSKST AAMSTYTGIFTDQVLSVLKGEE
8689.1879	P02656	ApoCIII$_{0a}$ (21-98)	SEAEDASLLSFMQGYMKHATKTAKDALSSVQESQVAQQARGWVTDGFSS LKDYWSTVKDKFSEFWDLDPEVRPTSAVA
8703.4457	P02652	ApoA2 (24-100)	QAKEPCVESLVSQYFQTVTDYGKDLMEKVKSPELQAEAKSYFEKSKEQLT PLIKKAGTELVNFLSYFVELGTQPATQ
8760.2250	P02656	ApoCIII$_{0a}$ (21-99)	SEAEDASLLSFMQGYMKHATKTAKDALSSVQESQVAQQARGWVTDGFSS LKDYWSTVKDKFSEFWDLDPEVRPTSAVAA
8910.3856	P02655	ApoCII (23-101)	TQQPQQDEMPSPTFLTQVKESLSSYWESAKTAAQNLYEKTYLPAVDEKLR DLYSKSTAAMSTYTGIFTDQVLSVLKGEE
9054.3201	P02656	ApoCIII$_{0c}$ (21-98)	SEAEDASLLSFMQGYMKHATKTAKDALSSVQESQVAQQARGWVTDGFSS LKDYWSTVKDKFSEFWDLDPEVRPT(GalNAcGal)SAVA
9125.3572	P02656	ApoCIII$_{0c}$ (21-99)	SEAEDASLLSFMQGYMKHATKTAKDALSSVQESQVAQQARGWVTDGFSS LKDYWSTVKDKFSEFWDLDPEVRPT(GalNAcGal)SAVAA
9282.8208	P02775	CTAP-III (44-128)	NLAKGKEESLDSDLYAELRCMCIKTTSGIHPKNIQSLEVIGKGTHCNQVEVI ATLKDGRKICLDPDAPRIKKIVQKKLAGDESAD
9345.4155	P02656	ApoCIII$_1$ (21-98)	SEAEDASLLSFMQGYMKHATKTAKDALSSVQESQVAQQARGWVTDGFSS LKDYWSTVKDKFSEFWDLDPEVRPT(GalNAcGalneu5Ac)SAVAA
9416.4526	P02656	**ApoCIII$_1$ (21-99)**	SEAEDASLLSFMQGYMKHATKTAKDALSSVQESQVAQQARGWVTDGFSS LKDYWSTVKDKFSEFWDLDPEVRPT(GalNAcGalneu5Ac)SAVAA
9707.5480	P02656	ApoCIII$_2$ (21-99)	SEAEDASLLSFMQGYMKHATKTAKDALSSVQESQVAQQARGWVTDGFSS LKDYWSTVKDKFSEFWDLDPEVRPT(GalNAcGalnNeu5AcNeu5Ac)SAVAA
11676.5003	P02735	SAA1 (19-122)	RSFFSFLGEAFDGARDMWRAYSDMREANYIGSDKYFHARGNYDAAKRGP GGVWAAEAISDARENIQRFFGHGAEDSLADQAANEWGRSGKDPNHFRPA GLPEKY

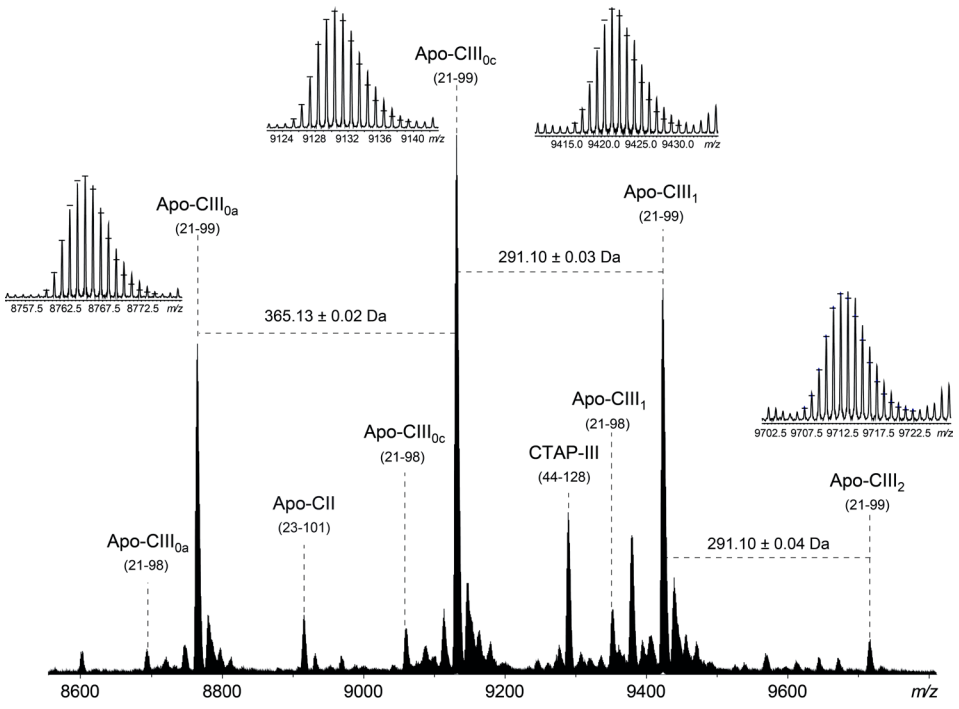

Figure 3. Enlarged part of the ultrahigh resolution MALDI-FT-ICR protein profile obtained from human serum purified by RPC18 cartridge-based SPE. In this *m/z* range, Apo-CIII isoforms were identified as the most abundant peaks and Apo-CIII isoforms truncations (minus the C-teminal alanine), Apo-CII and the connective tissue-activating peptide III (CTAP-III) were observed at lower intensities. The upper inlets show the comparison between the observed isotopic distribution and those calculated from the amino acid sequences of the Apo-CIII isoforms (as indicated). The mass differences (365.13 and 291.10) between the Apo-CIII isoforms are in excellent agreement with the theoretical values (for HexNAcHex and NeuAc are 365.1322 and 291.0954 Da). The SDs of the mass differences were calculated from 96 RPC18 MB-based MALDI-FT-ICR protein profiles.

Furthermore, it should be stressed that the small glycoprotein Apo-$CIII_1$ can actually be a mixture of two isomers, namely the sialic acid residue can be linked to either the terminal Gal or to the GalNAc (NeuAcα2–3Galβ1–3GalNAc or Galβ1–3[NeuAcα2– 6]GalNAc, respectively). The observed ESI-FT-ICR-MS/MS analysis is in full accordance with both structures (Fig. 2B). Therefore, no conclusions can be drawn from these data on the

relative contribution of these two isomers to the Apo-$CIII_1$ species. Possibly electron transfer dissociation MS/MS experiments can differentiate between these two isomers [46]. As a final remark it becomes clear from MALDI-FT-ICR profiles that the mass difference between species nonglycosylated Apo-CIII, Apo-$CIII_0$, Apo-$CIII_1$, and Apo-$CIII_2$ provides additional evidence for the peak identities. This is illustrated in Fig. 3, where the observed Δm are 365.13, 291.10, and 291.10 Da, respectively (theoretical differences for HexNAcHex and NeuAc are 365.1322 and 291.0954 Da). Note that the mass difference of 0.02 Da corresponds to a MME of 2 ppm based on the mass of Apo-$CIII_1$ (calculated *m/z* 9416.4526).

Quantitative analysis of four apo-CIII isoforms. In order to evaluate the biological variation in abundance of the apo-CIII isoforms in human sera, we analysed the MALDI-FT-ICR MS spectra obtained from 96 differentindividuals. A 96-well-plate containing RP18 MBs eluates and stored at –80C was thawed and each eluate was spotted in duplicate onto a MALDI target plate as described in Section 2. From this plate, 192 spectra were obtained and visualized using the DataAnalysis software (Bruker). First, the technical variation in the Apo-CIII isoform distributions was determined from each pair of spectra (replicate spots). Recently, we reported in more detail on the technical variation of similarly obtained MALDI-FT-ICR profiles [45, 47]. It was found that the reproducibility of peak areas of peptides and proteins between 1 and 9 kDa varied for each signal, with on average a coefficient of variation of approximately 15%. In the current profiles, similar technical variation was observed upon considering duplicate measurements of the same sample. The spectrum with highest intensities was used for further evaluation. In Fig. 3, a typical MALDI-FT-ICR serum protein profile in the mass range of 8550–9820 *m/z* value is depicted. In this *m/z* range, Apo-CIII isoforms were identified as the most abundant peaks while Apo-CIII isoforms truncations (minus the C-teminal alanine), Apo-CII and the connective tissue-activating peptide III were identified as minor components (Table 1). The high confidence in the identifications derived from literature search or obtained fromMS/MSanalysis follows from the observed isotopic distribution of the Apo-CIII isoforms when compared with those calculated from the amino acid sequence using the IsotopePattern tool (Bruker). As shown in the upper insets of Fig. 3, the two observed and calculated isotopic distributions of all the Apo-CIII

isoforms have a good match with each other. Further note the excellent agreement of the mass differences between the Apo-CIII isoforms with the theoretical values. To calculate those mass differences, the *m/z* values of the most abundant isotope peak in the observed isotopic distribution of each of the Apo-CIII isoforms were extracted using the MassList tool (Bruker), copied in an Excel sheet and compared with the most abundant isotope peak in the calculated isotopic distribution of the corresponding Apo-CIII isoform. Thus, the average and SD of the mass differences in 96 spectra were calculated.

In order to evaluate the biological variation quantitative data on the abundances of the four species non-glycosylated Apo-CIII (Apo-$CIII_{0a}$), Apo-$CIII_{0c}$, Apo-$CIII_1$ and Apo-$CIII_2$ were obtained as follows. The signal intensity of the extracted *m/z* values was calculated using the Xtractor tool [16] and exported in an Excel sheet. These intensities are summarized in the box-plot depicted in Fig. 4. It is clear from this box-plot that significant variation is present in isoform abundances. This was confirmed through an ANOVA, which resulted in an F value of 87with a p-value less than 2×10^{-16}. In view of the important role of apolipoproteins in various disease pathways and of the fact that the present analytical procedure allows high-throughput screening [45] while providing very detailed information, further studies are warranted. Therefore, future studies will be performed in which MALDI-FT-ICR profiling of the Apo-CIII isoforms will be applied in a clinical cohort. This requires a systematical evaluation of abundances and identities with respect to both biological and technical variations in a fully standardized setting.

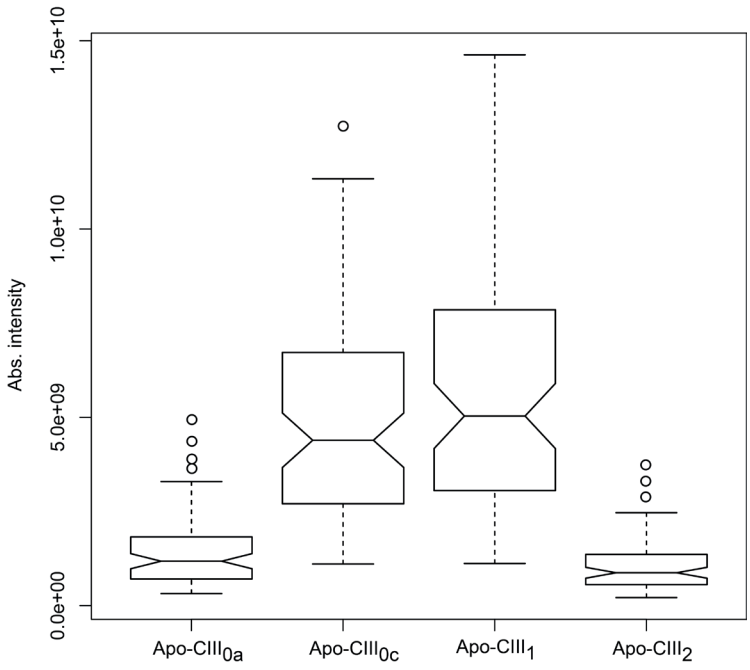

Figure 4. Box-plot of the signal intensities of Apo-$CIII_{0a}$, Apo-$CIII_{0c}$, Apo-$CIII_1$, Apo-$CIII_2$, as obtained from 96 ultrahigh resolution MALDI-FT-ICR protein profiles. Large biological variation in the abundance of the apolipoprotein-C isoforms in 96 different individuals is evident, ANOVA resulted in an F-value of 87 with a p-value less than 2×10^{-16}.

CONCLUSIONS

Peptide and protein profiles from human body fluids can be obtained from ultrahigh resolution MALDI instruments such as FT-ICR-MS with sub-ppm mass precision and four orders of magnitude in dynamic concentration range. Provided sample collection as well as SPE is performed according to highly standardized protocols these profiles hold great potential for screening (larger) cohorts of patient samples. Moreover, single species (*e.g.* proteins) in complex mixtures can be identified and quantified using ultrahigh resolution MS. In this study a fully automated RPC18-based magnetic bead method was applied for SPE of 96 different human serum samples. Proteins were profiled from *m/z* value 6000 to *m/z* value 15 000. The FT-ICR-system allowed accurate mass analysis

of middle-down proteins in MALDI protein profiles since all species were fully resolved in this mass range. It was shown that isoforms of apo-CIII could be mapped, namely the ones with the different mucin type core 1 O-glycans including one or two sialic acid residues. No evidence was found for the presence of Apo-CIII-GalNAc ("truncated" mucin-type core 1) in any of the acquired MALDI-FT-ICR profiles of all 96 different serum samples. Finally, ESI-FT-ICR-MS/MS identified the species observed in the MALDI-profile at *m/z* value 6627.5 as the monoisotopic peak of apo-CI.

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