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Identification of apolipoprotein C-III as a potential plasmatic biomarker associated with the resolution of hepatitis C virus infection

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Understanding the virus-host interactions that lead to approximately 20% of patients with acute Hepatitis C Virus (HCV) infection to viral clearance is probably a key towards the development of more effective treatment and prevention strategies. Acute hepatitis C infection is usually asymptomatic and therefore rarely diagnosed. Nevertheless, HCV nucleic acid testing carried out on all blood donations detects donors who have resolved their HCV infection after seroconversion. Here we have used SELDI-TOF-MS technology to compare, at a proteomic level, plasma samples respectively from donors with HCV clearance, from donors with chronic HCV infection and from unexposed healthy donors ($n = 15$ per group). A candidate marker of about 9.4 kDa was detected as differentially expressed in the three groups. After purification we identified by nanoLC-Q-TOF-MS/MS this candidate marker as Apolipoprotein C-III (ApoC-III). The identification was confirmed by western blot analysis. Levels of ApoC-III were then determined in the 45 plasma samples by immunoturbidimetric assay. ApoC-III was found to be higher in donors who had resolved their HCV infection than in donors with chronic infection, results which were consistent with SELDI-TOF-MS data. ApoC-III is the first reported candidate biomarker in plasma associated with the spontaneous resolution of HCV infection.

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Abbreviations: ApoC-III, apolipoprotein C-III; HCV, hepatitis C virus; HDL, high density lipoprotein; LDL-C, low density lipoprotein; NAT, nucleic acid testing; SRBI, scavenger receptor class B type I; TG, triglycerides; VLDL, very low density lipoprotein

1 Introduction

Acute hepatitis C virus (HCV) infection is usually a mild and asymptomatic disease, making early diagnosis difficult [1]. Spontaneous resolution of HCV infection occurs in 10–30% of acutely infected patients and is associated with a broad, specific and vigorous cellular immune response [1–4]. In 70–90 % of patients, however, HCV persists and causes chronic hepatitis with its life-threatening complications, including liver failure and hepatocellular carcinoma [1]. An estimated 170 million people worldwide are chronically infected with HCV including five million in Europe [5]. Furthermore, current anti-viral therapy is expensive, relatively toxic and effective in only 50–60% of treated patients [3]. There is as yet no vaccine against HCV but the development of at least a partly effective vaccine seems feasible [6]. Many aspects of HCV infection remain enigmatic. A better understanding of the early stages of the HCV infection is probably a key to the development of more effective treatment and prevention strategies [4].

The systematic screening of viral markers in blood donations has decreased the risk of transfusion-associated hepatitis to a negligible level in developed countries [5]. New screening methods such as the nucleic acid testing (NAT) of blood and plasma donors has yielded important insights into the epidemiology, pathogenesis and prognosis of HCV infection [7, 8]. Recently, in a transfusion study, viral and host factors in early HCV infection have been studied among 94 donor-recipient pairs in which there was transmission. It appears that host factors are more important determinants of acute HCV infection dynamics than virus-associated factors [9]. Since plasma potentially contacts every cell as it circulates through the body, it may carry clues both to diagnosis and treatment of disease [10].

It is commonly expected that recent techniques leading to the detection of a growing number of trace proteins within biological fluids will result in the discovery of new biomarkers. Recent advances in proteomics associating protein separation technologies and MS have provided opportunities for biomarker identification and characterization [11]. SELDI-TOF-MS is a variant of MALDI-TOF-MS, in which the samples are directly applied to a chip coated with a specific chemical or biochemical matrice. The bound proteins are then analyzed by MS to obtain the protein fingerprint of the sample [12, 13]. Consequently, SELDI-TOF-MS has an excellent potential for protein profiling. This method, which is suitable for the analysis of a large number of biological samples, has shown promise for biomarker discovery in HCV infection and hepatocellular carcinoma [14–18].

The aim of this project was to identify biomarkers associated with the resolution of acute HCV infection. The main difficulty encountered in comparing normal with pathological patterns is linked to the choice of the relevant samples. HCV NAT detects donors with viral clearance after sero-conversion, as demonstrated both by HCV RNA negativity and HCV seropositivity. In consequence, we have applied

SELDI-TOF-MS to analyze the plasma from blood donors with well-defined serological and virological profiles. We have compared the plasma proteome for three groups of donors (i) individuals who cleared their HCV infection, (ii) chronic HCV carriers, and (iii) unexposed healthy individuals to investigate whether the differential expression of certain proteins could be associated with the resolution of HCV infection. Several differentially expressed protein peaks have been detected. After purification, proteins of interest were analyzed by nanoLC-Q-TOF-MS/MS. A candidate biomarker was characterized and its identity was validated by western blot analysis and immunoturbidimetric assay in all plasma samples.

2 Materials and methods

2.1 Samples

Human plasma samples ($n = 45$) were obtained from the Etablissement Français du Sang Pyrénées-Méditerranée; 15 samples from blood donors who tested negative for all HCV markers, 15 samples from donors with resolved HCV infection, and 15 from donors with chronic hepatitis C. Plasma samples were aliquoted and then stored at -80°C until use. None of the individuals had received an antiviral treatment prior to donation. All plasma samples were screened for anti-HCV antibodies with the Ortho HCV v3.0 ELISA test system (Ortho clinical diagnostics, Raritan, NJ, USA). The RIBA HCV 3.0 immunoblot assay (Chiron, Emeryville, CA, USA) was used as the confirmatory test. Screening of HCV RNA was systematically performed by PCR using the Cobas Ampliscreen HCV v2.0 assay (Roche Molecular Systems, Branchburg, NJ, USA). We further quantified HCV RNA levels in plasma samples from donors with chronic hepatitis by the Cobas Amplicor HCV Monitor v2.0 test (Roche Molecular Systems). The clinical characteristics of donors are shown in Table 1.

2.2 Profiling of plasma using SELDI-TOF-MS

Optimization studies on a hydrophobic surface were carried out prior to profiling analysis. All plasma samples were albumin depleted with Cibacron Blue before analysis (Sigma, Saint Louis, Mo, USA). On H50 ProteinChip arrays, optimal conditions were found to be a 1:10 dilution of depleted plasma with the binding buffer ($1 \times \text{PBS}$, 0.1% TFA). H50 arrays were pre-washed twice with $5 \mu\text{L}$ of 50% ACN for 5 min. After air drying, the surface was loaded twice with $2 \mu\text{L}$ of binding buffer for 2 min. and then $5 \mu\text{L}$ of 1:10 diluted plasma was added to each spot and incubated for 1 h in a humidified chamber at room temperature. Plasma was removed and each spot was individually washed three times for 2 min with $5 \mu\text{L}$ washing buffer (10% ACN, 0.1% TFA) followed by one quick wash with deionized water. The surface was allowed to air dry, and $1 \mu\text{L}$ of SPA (Ciphergen Bio-

systems, Palo Alto, CA, USA) in 50% v/v ACN and 0.5% v/v TFA was added twice to each spot. H50 arrays were then read in a ProteinChip Reader system, PBSIIc serie (CIPHERGEN Biosystems). The spectra were generated by the accumulation of 80 laser shots through the spot at a laser intensity of 180 arbitrary units. Spectra were collected and analyzed using the CIPHERGEN ProteinChip software (v3.0). All mass spectra were then normalized using TIC normalization before proceeding to statistical analysis. The TIC method assumes that on average, the total number of proteins being expressed is constant across the samples being normalized. The process takes the TIC used for all the spots, averages the intensity, and adjusts the intensity scales for all the spots in order to display data on the same scale. The m/z ratio of each of the peaks to be quantified was determined according to externally calibrated standards (CIPHERGEN Biosystems). Peak clustering in the range from 1000 to 20 000 m/z ratio was performed using Biomarker Wizard Software (CIPHERGEN Biosystems) at settings that provide a 0.3% mass window and 5% S/N determination. Statistics were performed using the nonparametric Mann-Whitney U -test on the maximal intensity of each peak. Significant threshold was set at $p < 0.05$.

2.3 Enrichment of biomarkers candidates

We performed a fractionation of the plasma proteins with increased concentrations of ammonium sulfate (Sigma) [19, 20]. This phenomenon of protein precipitation in the presence of excess salt is known as salting-out. We used increasing salt concentrations to reach 20, 40, 60, 80 and 100% saturation of ammonium sulfate as follows. Ammonium sulfate was added to 50 mL of undiluted plasma to reach 20% saturation. After incubation at room temperature with gentle agitation for 1 h, the tube was then centrifuged at $11\ 300 \times g$ for 15 min at 20°C. The supernatant was transferred to another tube and ammonium sulfate was added to reach 40% saturation. The mixture was treated as above. The proteins in the supernatant were further precipitated with 60, 80 and 100 % ammonium sulfate saturation respectively. The precipitated proteins were collected and categorized according to the concentration of the salt used for saturation. Each of the five pellets was resuspended in 2 mL of biomolecular grade water and then aliquoted and stored at -80°C until use. The precipitated proteins were analyzed by SELDI-TOF-MS on normal phase NP20 arrays as recommended by the manufacturer in order to compare their protein profile with the original unfractionated plasma.

2.4 Recovery of enriched proteins using passive elution

A 1:10 diluted plasma sample from a donor with resolved HCV infection and the 1:5 diluted precipitates recovered from the precipitation steps were separated by SDS-PAGE under reducing conditions on a 12% acrylamide NuPAGE Bis-tris gel (Invitrogen, Carlsbad, CA, USA). The apparent

molecular masses were determined by running the SeeBlue pre-stained protein standard (Invitrogen). Bands of molecular weight approximating to candidate biomarkers were cut out from the CBB stained gel. Passive elution was effected as previously described by Currid *et al.* [21]. Briefly, gel pieces were washed with 50% ACN/ 50 mM ammonium bicarbonate and then dehydrated with 100% ACN. The gel pieces were heated to 50°C, before the addition of a 45% formic acid/30% ACN/10% isopropanol solution and incubation in a sonicating waterbath for 30 min at room temperature. The passively eluted material was then spotted on an H50 array, followed by addition of saturated sinapinic acid.

2.5 Identification of the proteins by nanoLC-Q-TOF-MS/MS

The 1:5 diluted precipitate recovered from the 80% ammonium sulfate saturation was analyzed by 1-DE under reducing conditions as described in Section 2.4. The band of interest and a blank area of the gel were excised from the CBB stained gel before in-gel tryptic digestion. The resulting peptides were analyzed by nanoflow-HPLC-Q-TOF-MS/MS using a CapLC coupled with a Q-TOF Ultima Global Instrument (Waters/Micromass UK, Manchester, UK). The MS/MS fragment data were integrated using MASCOT (Matrix Science, Boston, MA, USA) searching the National Center for Biotechnology Information and Swiss-Prot databases. The parameters for the query were: species of origin *Homo sapiens*, digestion by trypsin allowing for no more than one missed cleavage, peptide mass tolerance 50 ppm, fragment mass tolerance 0.6 Da, possible charge +1, +2, +3. The threshold of significance ($p < 0.05$) was given with MASCOT by a score of 55.

2.6 Western blot analysis

A 1:10 diluted sample from the plasma used in the enrichment step was albumin depleted before analysis by electrophoresis under reducing conditions as described in Section 2.4. A 1:5 diluted precipitate recovered from the 80% ammonium sulfate saturation and a sample of purified human apolipoprotein C-III (ApoC-III) protein from human plasma (Chemicon, Temecula CA, USA) were used as controls. After transfer to a NC Hybond ECL membrane (GE Healthcare, Buckinghamshire, UK) and immunodetection with a 1:20 000 dilution of a polyclonal rabbit anti-human ApoC-III antibody (Biogenesis, Poole, UK), the rabbit IgG was then probed with 1:10 000 dilution of HRP-labeled goat anti-rabbit antibodies (Sigma) and the proteins were visualized using an enhanced chemiluminescence detection method (GE Healthcare).

2.7 Plasma lipid analysis

ApoC-III levels were measured by immunoturbidimetric assay (Kamiya biomedical company, Seattle, USA) adapted

on the Konepro analyzer (Thermo Electron, Cergy-Pontoise, France) with a linearity ranging from 0.03 to 0.3 g/L. ApoA-I and apoB concentrations were determined by immunonephelometric assay (Image 800, Beckman Coulter, Villepinte, France). ApoA-II level was determined by immunonephelometry on a BN II nephelometer analyzer (Dade Behring, Marburg GmbH, Germany). ApoC-I level was determined by competitive Elisa on Vitros 950 as previously described by Dautin *et al.* [22]. ApoC-II and Apo-E levels were measured by immunoturbidimetric assay (Randox, Mauguio, France) adapted on the Olympus AU 640 analyser (Olympus, Rungis, France). Total cholesterol (TC), high density lipoprotein (HDL)-cholesterol, low density lipoprotein (LDL)-cholesterol, and triglycerides (TG) levels were measured in plasma samples by routine enzymatic methods on the Konepro analyzer. Very low density lipoprotein (VLDL)-cholesterol was estimated as TC-(HDL-cholesterol+LDL-cholesterol). Statistical differences between groups were determined using Wilcoxon's test (www.u707.jussieu.fr/biostatgv/index.html). Significant threshold was set at $p < 0.05$.

3 Results

3.1 Plasma screening on H50 proteinchip arrays

For the purposes of our study, we analyzed archived plasma samples, from blood donors, which had been screened for HCV infection markers. Acute hepatitis C infection is usually asymptomatic and therefore rarely diagnosed. Nevertheless, HCV NAT, implemented on all blood donations since July 2001 in France, detects asymptomatic donors. Based on screening tests, the samples were divided into three groups (Table 1). To minimize unrelated variables, samples used in this study have been restricted with respect to sex and age. The "Negative" group ($n = 15$) represents blood donors tested negative for all HCV markers. Individuals in the "Resolved" group ($n = 15$) show evidence of resolution of HCV infection (anti-HCV antibodies (+), HCV RNA (-)). The "Chronic" group ($n = 15$) comprises patients with chronic HCV infection (anti-HCV antibodies (+), HCV RNA (+)). HCV RNA levels were evaluated in the last group; their values ranged from 2×10^4 to $>50 \times 10^4$ IU/mL.

Plasma samples ($n = 45$) from the three groups (Negative, Resolved, Chronic) were albumin depleted and diluted 1:10 before analysis on six H50 arrays using SELDI-TOF-MS technology. All mass data were baseline subtracted, normalized using total ion current, and peak clusters were generated by Biomarker Wizard software. The representative profile of plasma samples is presented in Fig. 1. Four peaks were found to be differentially expressed between the three sample groups with significant p -values ($p < 0.05$) or very significant p -values ($p < 0.01$), and one peak with highly significant p -value ($p < 0.001$) ($1500\text{--}20\,000$ m/z ratio was our optimal range setting). The average m/z values associated with these

Table 1. Characteristics of donors involved in the study

Donor group ^{a)}	Negative	Resolved	Chronic
Sex (Male/Female)	(7/8)	(8/7)	(7/8)
Age (mean)	36.07 ± 14.00	38.47 ± 9.73	35.60 ± 9.77
Anti HCV antibody	–	+	+
Screening test	–	4.91 ± 1.57	7.81 ± 1.11
Confirmatory test	–	++	++++
HCV RNA	–	–	+
Qualitative PCR	–	–	+
Quantitative PCR	–	–	$2 \times 10^4\text{--}50 \times 10^4$ IU/mL

a) Negative (unexposed), Resolved and Chronic HCV infection respectively.

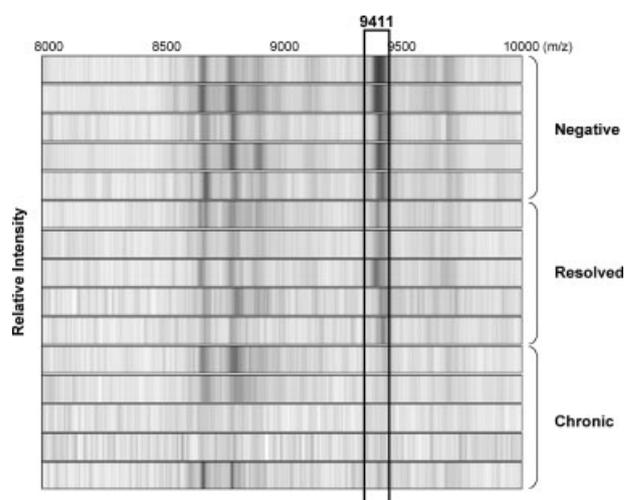


Figure 1. SELDI-TOF-MS protein spectra of human plasma samples from negative donors *versus* donors with resolved or chronic HCV infection (five representatives out of the 15 plasma samples *per* condition) in the 8000–10000 m/z range. The H50 chip-processed profiles show the relative abundance of chip-binding proteins. A boxed region identifies a cluster near 9411 m/z ratio differentially expressed in the three groups ($p < 0.05$, Mann-Whitney U -test).

protein peaks were 8674.51 ($p = 0.0284$), 8789.99 ($p = 0.0334$), 8909.35 ($p = 0.0059$), 9411.68 ($p = 0.0003$) and 9689.32 ($p = 0.0015$) respectively. We identified a peak of about 9411 Da ($z = 1$) that was highly differentially expressed in the plasma from donors who had resolved their HCV infection. A scatter plot of the normalized linear intensity of the candidate 9.4 kDa marker in all plasma is shown in Fig. 2 and the mean intensities of cases within each group are indicated by the bar. The mean intensities \pm SD are as follows: 4.78 ± 1.93 (Negative), 3.03 ± 0.77 (Resolved) and 2.29 ± 0.90 (Chronic) respectively. Table 2 shows the p -values for the mean intensities of the 9.4 kDa marker for pairwise comparisons between the different sample groups. The mean intensity of the 9.4 kDa marker was higher in negative donors than in donors who had resolved their HCV

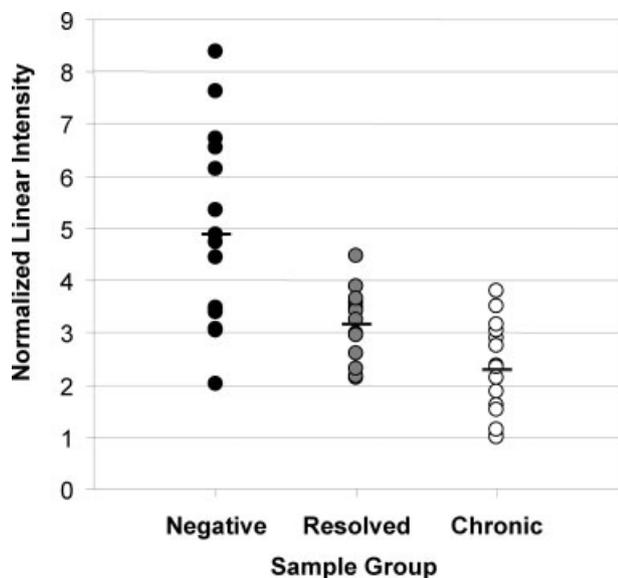


Figure 2. Distribution of the normalized linear intensity of the 9.4 kDa marker of each sample in all of the sample groups. Bio-marker Wizard generated normalized linear intensity. Software-Bar indicates the mean intensity of all samples within each group.

Table 2. Comparisons of means (Wilcoxon test) between the groups of donors for the 9.4 kDa marker

Group pairs	<i>p</i> -value
Negative vs. Resolved	0.01141
Negative vs. Chronic	0.00022
Resolved vs. Chronic	0.02910

infection ($p = 0.0114$) or in donors with chronic HCV infection ($p = 0.0002$). The mean intensity of this marker was higher in donors who had resolved their HCV infection than in donors with chronic HCV infection ($p = 0.0291$). This 9.4 kDa polypeptide may be considered as a candidate marker associated with the resolution of HCV infection.

3.2 Enrichment of the 9.4 kDa candidate marker

We developed a salting-out strategy for enrichment and purification of the candidate marker. Plasma fractionation was performed using increased concentrations of salt to reach 20, 40, 60, 80 and 100% saturation of ammonium sulfate. The precipitated proteins obtained from the plasma of a donor who had resolved his HCV infection were analyzed by 1-D SDS-PAGE. As shown in Fig. 3a, two bands (*a* and *b*) migrating with an apparent mass near 9.4 kDa were visualized on the CBB stained gel in the 80% saturation fraction but not in the original unfractionated plasma. A control by SELDI-TOF-MS was performed on a normal phase NP20 array and confirmed the enrichment of the 9.4 kDa marker in the 80% saturation

fraction (Fig. 3b). The two bands of interest were then excised for passive elution and were analyzed by SELDI-TOF-MS on a H50 array. Figure 3c depicts the SELDI-TOF-MS spectra for each eluted protein from the *b* band, demonstrating that one protein gave a singly charged 9.4 kDa peak similar to the candidate protein identified in the original profiling study.

3.3 Identification of the 9.4 kDa candidate marker

The *b* band migrating on SDS-PAGE with an apparent 9.4 kDa mass was excised, digested with trypsin and analyzed by nanoflow-HPLC-Q-TOF-MS/MS. Three peptides pointed to the identification of ApoC-III (Swiss-Prot accession No P02656) (Fig. 4 a, b and c). Ions with m/z of 572.97 MH^{3+} , 858.95 MH^{2+} and 449.74 MH^{2+} were identified as a fragments of ApoC-III with MASCOT probability score of 316 (Fig. 4 d) and a 33% sequence coverage of the secreted form. This form of ApoC-III corresponds to the mature secreted form with signal peptide cleaved. We purchased purified ApoC-III protein and analyzed the SELDI-TOF-MS profil on a NP20 array (Fig. 4e). Pure ApoC-III resulted in three peaks at 9153.5, 9443.1 and 9730.8 m/z ratios corresponding to the three isoforms termed as ApoC-III₀, ApoC-III₁ and ApoC-III₂ that have been shown to contribute, respectively, to approximately 10, 55 and 35% of the total ApoC-III levels in circulation [23].

3.4 Validation of identified ApoC-III

Identity of ApoC-III was validated by western blot analysis and by immunoturbidimetric assay. Western blot analysis using a specific rabbit anti-human ApoC-III antibody detected ApoC-III in unfractionated human plasma and in the 80% saturation fraction (Fig. 5). A heavier band at approximately 20 kDa was also observed for the purified ApoC-III as specified by the manufacturer.

To further confirm the western blot result, ApoC-III levels were measured by immunoturbidimetric assay on the 45 human plasma samples previously described. Differential expression of ApoC-III was validated by the significant decrease in ApoC-III levels observed when comparing the Negative group (0.088 ± 0.032 g/L) to the Resolved group (0.053 ± 0.019 g/L) or to the Chronic group (0.040 ± 0.001 g/L) (Fig. 6). The *p*-values for the mean concentrations of ApoC-III for pairwise comparisons between the different sample groups are indicated by a bar (Fig. 6). However, no obvious clinical correlation was found between the ApoC-III levels and the HCV viral loads (data not shown). The results from immunoassay are consistent with SELDI-TOF-MS data.

3.5 Plasma lipid parameters

The comparison of plasma lipid profiles between unexposed donors, donors with resolved HCV infection and with chronic HCV infection confirmed the key role for ApoC-III

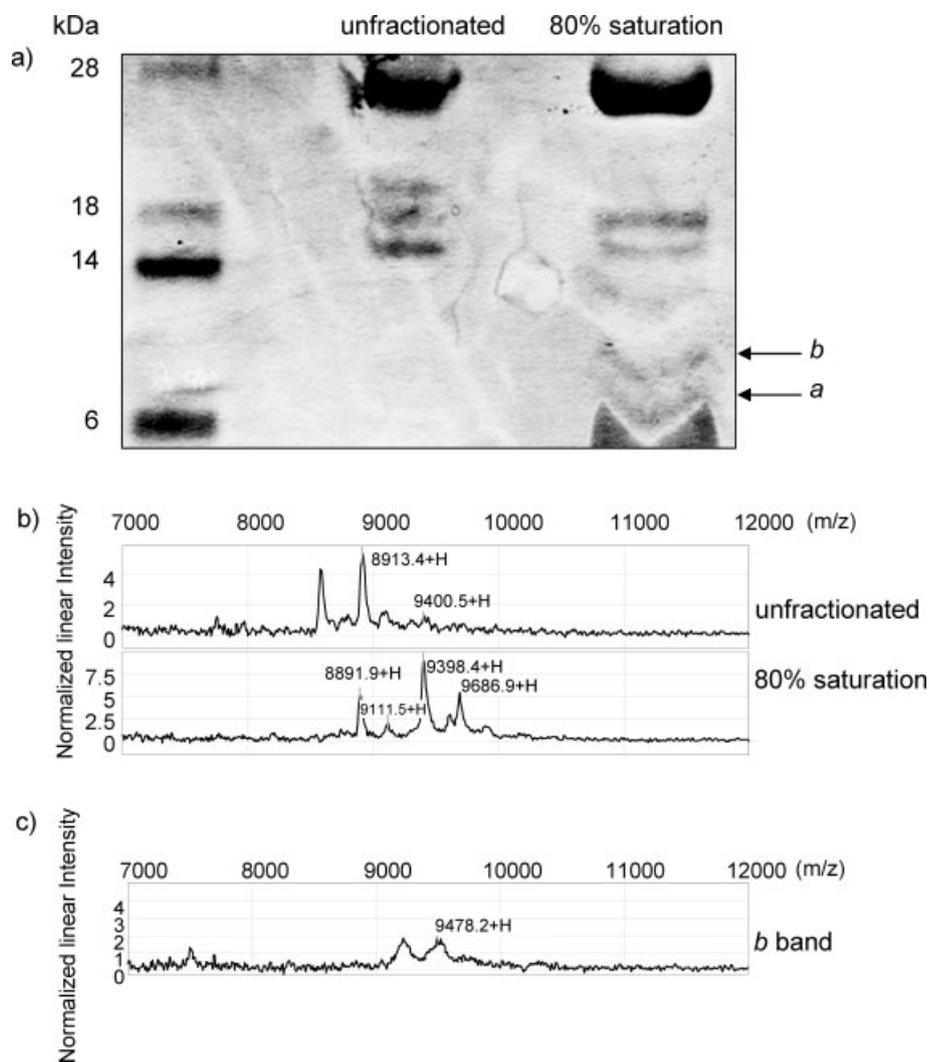


Figure 3. Results of the enrichment process. (a) SDS-PAGE analysis of both unfractionated plasma from a donor with resolved HCV infection and of one enriched fraction obtained by salting out with 80% saturation of ammonium sulfate. The separated proteins were stained with CBB. Molecular weight protein markers are indicated. (b) Confirmation of enrichment of the 9.4 kDa marker by SELDI-TOF-MS analysis on NP20 array in unfractionated plasma and enriched fraction respectively. Gel bands *a* and *b* from the enriched fraction were excised and subjected to passive elution. (c) The proteins eluted from the *b* band bound to H50 array and SELDI-TOF-MS analysis of the spectra indicate a 9.4 kDa peak.

as this protein is the only one which shows a statistically different expression between the three groups (Table 3). Indeed decreased ApoC-III levels in the Resolved group were not linked to variations in other measured apolipoproteins (ApoA-I, ApoA-II, Apo B, ApoC-I, ApoC-II, ApoE), nor in total cholesterol, HDL-cholesterol, LDL-cholesterol, VLDL-cholesterol and TG levels when compared to the Negative group. By contrast, the Chronic group exhibited a significant lipid metabolism impairment when compared to the Negative group. This was characterized by a decrease in total cholesterol, LDL-cholesterol and TG levels, associated with lower levels of ApoB, ApoC-II and ApoC-III (Table 3).

4 Discussion

The classical separation techniques, including 2-D PAGE, are time consuming and lead to a poor resolution of hydrophobic proteins as well as of low molecular mass polypep-

tides (<20 kDa). The tremendous complexity of the plasma proteome is paradoxically the source of both its extraordinary value in diagnosis and of great difficulties in analysis. The challenge is to detect the proteins present only in low abundances, since 99% of the protein in plasma is made up of about 20 highly abundant species. New biomarkers are expected to be found among trace proteins [10]. To circumvent these limitations, we have used SELDI-TOF-MS technology (www.bio-rad.com/proteinchip), a proteomic technique that rapidly performs the analysis of proteins at the femtomole level and provides a research platform to compare many different plasma samples.

Hepatitis C infection represents a serious health problem worldwide. The mechanisms by which HCV enters and infects host cells are incompletely understood. The LDL-receptor has been suspected to play a role in HCV infection on the basis of the well-documented interaction between HCV and lipoprotein [24–26]. LDL-receptor has been identified as a receptor candidate for HCV [27–30]. We have

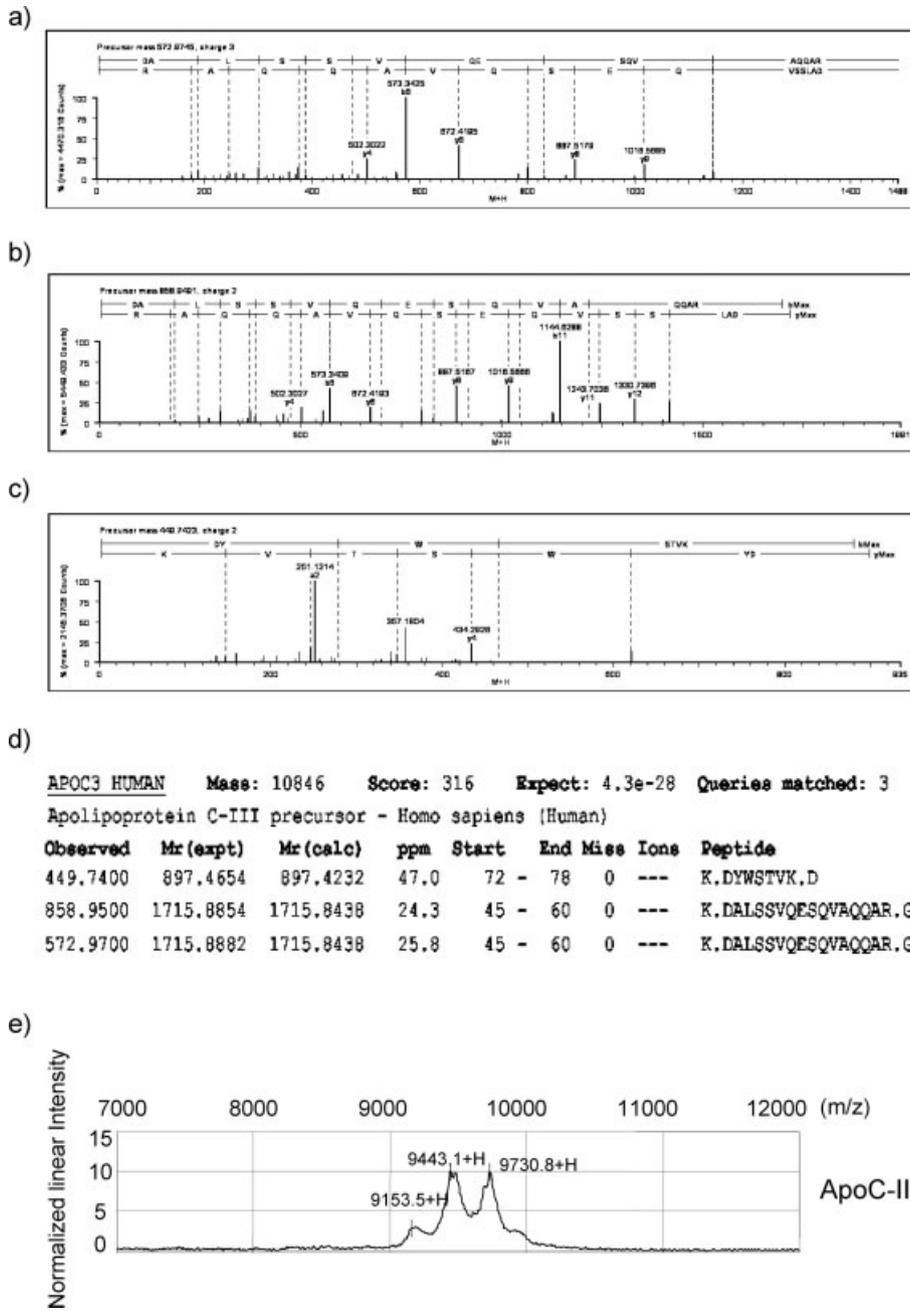


Figure 4. Identification of the 9.4 kDa marker by nanoLC-Q-TOF-MS/MS. MS/MS analysis of ion with m/z (a) 572.97 MH^{3+} , (b) 858.95 MH^{2+} and (c) 449.74 MH^{2+} . (d) Ions were identified as tryptic fragments of ApoC-III (P02656) with probability based Mowse Score of 316. (e) SELDI-TOF-MS analysis on NP20 array of purified ApoC-III.

recently shown that the LDL-receptor plays a role in the infection of primary human hepatocytes by HCV from human serum [31]. Other receptors including CD81 [32], the scavenger receptor class B type I (SRBI), a receptor of HDL, LDL and VLDL [33–37] and more recently Claudin-1 [38], have also been implicated in cell virus entry. In recent genomic analysis of liver biopsies from acutely infected chimpanzees and of subgenomic replicon models, the accumulation of free fatty acids associated with transcriptional changes in host genes involved in lipid metabolism was

reported to have a positive effect on the HCV replicon and may have a similar effect on HCV replication [39]. Furthermore, Jacobs *et al.* demonstrated that transfection of hepatoma permissive Huh-7.5 cells with a full length HCV replicon induces several changes in protein abundance indicative of disturbances in lipid metabolism [40]. It was proposed that accumulation of free fatty acids benefits the virus. Consistent with these data, Kapadia *et al.* demonstrated that elements of the cholesterol and fatty-acid-biosynthetic pathways are required for HCV RNA replication in Huh-7 cells [41].

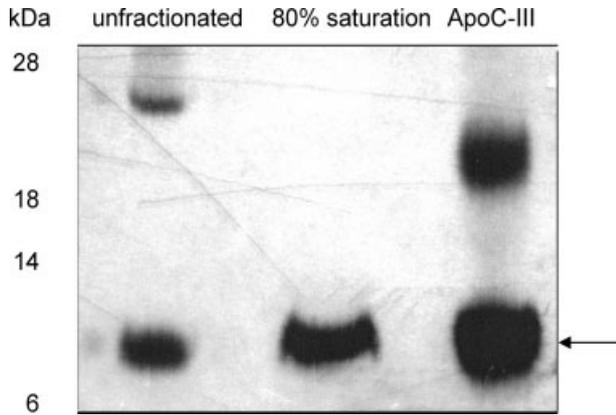


Figure 5. Western blot analysis of ApoC-III levels of both unfractionated plasma from a donor with resolved HCV infection and of one enriched fraction obtained by salting out with 80% saturated ammonium sulfate. A human purified ApoC-III (2 μ g) was used as control. ApoC-III antibody was used at a 1:20 000 dilution and secondary anti-rabbit HRP at 1:10 000. Molecular weight protein markers are indicated.

The accessibility of archived plasma samples in optimal storage conditions fully characterized in blood transfusion services makes these samples the ideal candidates for the identification of biomarkers for clinical studies [11, 42]. To identify candidate biomarkers associated with the resolution of HCV infection, protein expression profiles from plasma samples from blood donors who have cleared the virus, *versus* those from HCV negative donors and from donors with chronic hepatitis were compared by SELDI-TOF-MS. We found a 9.4 kDa protein that was highly differentially expressed in the three groups. This peak was identified as ApoC-III and the identity was validated by western blot analysis and by immunoassay. Consistent with SELDI-TOF-MS

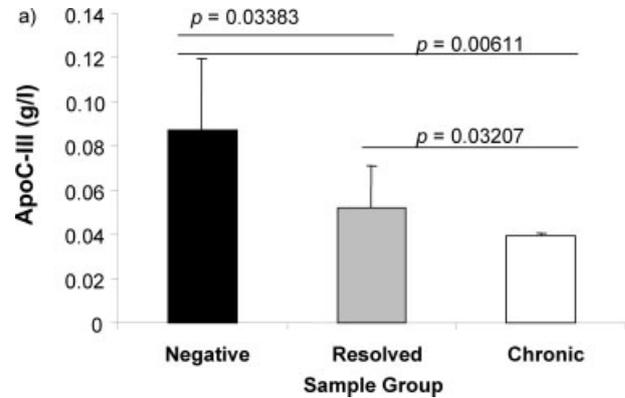


Figure 6. Plasmatc ApoC-III levels assayed by immunoturbidimetric experiments on 15 samples from negative donors, 15 samples from donors with resolved HCV infection and 15 samples from chronic carriers.

data, the mean concentration levels of ApoC-III were higher in unexposed donors than in those who had resolved their HCV infection and in donors with chronic HCV infection. The analysis of lipid profiles of all plasma samples showed that the ApoC-III level was the only statistically different parameter between the three groups. ApoC-III levels were significantly lower in patients with resolved infection than in unexposed donors, while no significant difference was observed in other apolipoproteins (ApoA-I, ApoA-II, ApoB, ApoC-I, ApoC-II, and ApoE), nor in cholesterol and TG concentrations, suggesting that the reduction is linked to HCV and not only to liver damage. Finally, no correlation was found between the ApoC-III values and that of the HCV viral load in chronically infected patients. This suggests that ApoC-III could play a role during the acute phase of HCV infection.

Table 3. Plasma lipid parameters

	Negative	Resolved	Chronic	Normal Range
ApoA-I (g/L)	1.63 \pm 0.40	1.64 \pm 0.29	1.56 \pm 0.26	1.10–1.80
ApoA-II (g/L)	0.38 \pm 0.08	0.37 \pm 0.10	0.37 \pm 0.07	0.32–0.54
ApoB (g/L) ^{a),b)}	0.93 \pm 0.20	0.95 \pm 0.20	0.75 \pm 0.19	0.50–1.82
ApoC-I (g/L)	0.073 \pm 0.018	0.079 \pm 0.014	0.076 \pm 0.014	0.057–0.089
ApoC-II (g/L) ^{a),b)}	0.032 \pm 0.017	0.029 \pm 0.013	0.013 \pm 0.010	0.016–0.042
ApoC-III (g/L) ^{a),b),c)}	0.088 \pm 0.032	0.053 \pm 0.019	0.040 \pm 0.001	0.05–0.12
ApoE (g/L)	0.038 \pm 0.015	0.034 \pm 0.009	0.036 \pm 0.011	0.027–0.045
Total cholesterol (g/L) ^{a),b)}	2.10 \pm 0.42	2.04 \pm 0.30	1.73 \pm 0.35	1.60–2.21
HDL-cholesterol (g/L)	0.66 \pm 0.29	0.56 \pm 0.12	0.63 \pm 0.13	>0.45
LDL-cholestérol (g/L) ^{a),b)}	0.97 \pm 0.22	0.97 \pm 0.24	0.75 \pm 0.21	<1.60
VLDL-cholesterol (g/L) ^{a)}	0.46 \pm 0.22	0.49 \pm 0.14	0.35 \pm 0.16	0.30
TG (g/L) ^{a),b)}	1.73 \pm 0.96	1.42 \pm 0.67	0.94 \pm 0.34	0.53–1.49

Values are mean \pm SD. Statistics by Wilcoxon test ($n = 15$ per group)

a) $p < 0.05$: Resolved vs. Chronic

b) $p < 0.05$: Negative vs. Chronic

c) $p < 0.05$: Negative vs. Resolved

Many proteins of the apolipoprotein groups were used as biomarkers in cancer [44–47]. ApoA-I could be a useful biomarker for HIV diagnosis [48]. ApoC-I and ApoC-III were reported as potential plasmatic markers to distinguish between ischemic and hemorrhagic stroke [49]. In our study, we discovered a significant variation in the average differential expression of ApoC-III between unexposed donors, donors with resolved HCV infection and chronic HCV carriers. These findings suggest that ApoC-III can potentially be used as a biomarker associated with the resolution of HCV infection. ApoC-III, a protein secreted mostly by the liver, is associated with both triglyceride-rich lipoproteins and HDL in peripheral circulation [23, 50, 51]. This is the most abundant apolipoprotein C in human plasma [50, 51]. ApoC-III is present in three isoforms that are termed ApoC-III₀, ApoC-III₁ and ApoC-III₂ depending on the number of sialic molecules (0 to 2) terminating the oligosaccharidic portions of the protein. Each isoform has been shown to contribute, respectively, to approximately 10, 55 and 35% of the total ApoC-III levels in circulation [23]. Rossi *et al.* previously described that ApoC-III isoforms should be associated to three dominant peaks in SELDI spectra with averaged *m/z* values at 9162.31, 9411.96 and 9707.00 using strong anionic exchange chips [43]. Thus, we have analyzed the peaks obtained from our profiling study with *m/z* value at position 9126.76, 9411.68 and 9689.32 that were suspected to be associated to ApoC-III₀, ApoC-III₁ and ApoC-III₂ respectively. The comparative analysis between the mean intensities of these three peaks in each group (*n* = 15 *per* group) and the normal distribution in circulation showed no significant difference in the distribution of the three potential ApoC-III isoforms within each group (data not shown).

Proposed mechanisms underlying the hypertriglyceridemic effect of ApoC-III comprise inhibition of lipoprotein lipase activity, disruption of interaction of triglyceride-rich lipoproteins with vessel wall heparan sulfate proteoglycans and lower clearance of ApoB-containing lipoproteins by LDL-receptor and LDL-related receptors [52]. Previous studies have shown that ApoC-III completely abolishes the ApoB-mediated binding of lipoproteins to the LDL-receptor and this inhibitory action is probably due to the masking of the receptor domain of ApoB by ApoC-III [50]. ApoB₁₀₀, exclusively secreted by the liver, is an obligatory constituent of VLDL, HDL and LDL [53]. Interestingly, evidence suggests that the LDL-receptor, which recognizes ApoB and ApoE apolipoproteins exposed on lipoproteins, mediates the binding and endocytosis of native HCV particles isolated from patient's blood, most likely via their association with LDL or VLDL [37]. Furthermore, another report has also shown that ApoB mediates interaction of natural HCV with SRBI [34]. In addition, Andréo *et al.* suggested recently that lipoprotein lipase mediates HCV cell entry by a mechanism similar to hepatic clearance of triglyceride-rich lipoproteins from the circulation, promoting a non-productive virus uptake [54]. Another ligand of SRBI, serum amyloid A, has an antiviral activity against HCV [56, 57] and HIV [58]. A recent report

described that ApoC-I, an exchangeable apolipoprotein that predominantly resides in HDL, increases the fusion rates between viral and target membranes via a direct interaction with HCV particles [55]. Finally, Kapadia *et al.* recently demonstrated that HCV infection is dependent on a cooperative interaction between CD81 and SRBI and that cellular cholesterol content has a significant impact on HCV entry in Huh-7 cells [59]. Taken together, these *in vitro* studies outlined the strong relationship between the lipid metabolism and the evolution of HCV infection also observed *in vivo* [24, 60–62].

Given the correlation between HCV infection and lipid metabolism, we can hypothesize that ApoC-III is involved in differential HCV infection evolution. Moreover, decreased ApoC-III level was previously reported in HCV infection in regard to HCV genotype [63], underlining a complex relationship between HCV infection and lipoprotein metabolism that could be dependent on other factors associated with HCV entry and the early innate immune response.

In conclusion, we have identified ApoC-III as a potential low-molecular weight plasma biomarker associated with the resolution of HCV infection. This work requires further investigation, in a large cohort of unexposed donors or donors with HCV clearance or chronic infection, to study the predictive value of this candidate biomarker and to determine if ApoC-III is an actor or a marker of differential HCV infection evolution. The proportion of the three isoforms of ApoC-III will be evaluated in the three groups. It is likely that lipoproteins analyses in this large cohort of patients will be helpful in furthering the understanding of the basic processes promoting spontaneous HCV clearance.

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