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ORIGINAL ARTICLE

Heavy marijuana users show increased serum apolipoprotein C-III levels: evidence from proteomic analyses

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Marijuana (MJ) is the most commonly used illicit drug in the United States. Its abuse is associated with cognitive dysfunctions and increased resistance to blood flow in the cerebral vasculature. In addition, MJ abuse is associated with increased risks of potentially serious cardiovascular disorders. In the present study, we used the protein chip platform based on surface-enhanced laser desorption/ionization time-of-flight mass spectroscopy (SELDI-TOF-MS) to test the possibility that MJ abuse might be associated with changes in serum protein levels. Indeed, MJ users showed significant increases in three protein peaks, which were identified as three isoforms of apolipoprotein (apo) C-III. Immunoprecipitation using an apoC-III antibody also validated the identification of the proteins. Marijuana-induced increases in apoC-III levels might occur through chronic stimulation of hepatic cannabinoid receptors (CB1 and/or CB2) by its active ingredient, Δ^9tetrahydrocannibol (THC). Thus, chronic MJ abuse might cause increased transcription and/or translation of apoC-III in the liver with corresponding changes reflected in the plasma of these patients. In any case, because apoC-III is a cardiovascular risk factor, the increased levels observed in MJ users might explain, in part, the cardiac and cerebral abnormalities reported in these patients.
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Introduction

Marijuana (MJ) is the most commonly used illicit drug in the United States, with 4.3% of young adults reporting daily MJ use.[1] Acute adverse effects of MJ administration include tachycardia,[2] orthostatic hypotension,[3] supine hypertension,[4] dose-dependent increase in resting heart rate,[5] increase in myocardial oxygen demand,[6] ventricular tachycardia, acute myocardial infarction and psychosis.[7] Long-term abuse of MJ is also associated with cognitive deficits that included impaired learning, poor retention and retrieval and perceptual abnormalities.[8–11] Chronic MJ abusers can also experience cerebrovascular accidents including transient ischemic attacks and strokes that have been reported in young adults with history of heavy drug use.[12–15]

The physiologic effects of MJ are secondary to the effects by Δ^9tetrahydrocannibol (THC), the active ingredient in MJ.[16] THC exerts its effects by binding to cannabinoid (CB) receptors located on several cell types in various organs.[17] The CB receptors include CB1 and CB2 receptors, which are differentially expressed in the brain and periphery.[18] In the periphery, CB receptors are found in heart, liver, kidney, spleen, small intestine, testis or ovary.[17] Thus, it was not far-fetched to suggest that chronic MJ abuse might be associated with changes in the expression of proteins that would be reflected in the serum. It is also possible that some of these proteins might be responsible, in part, for some of the adverse effects reported as a consequence of prolonged MJ abuse.

As a first step toward testing these ideas, we decided to make use of a proteomic approach that has been adapted for analysis and characterization of overall protein expression in the plasma, other body fluids and various cell extracts.[19,20] The surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) ProteinChip strategies consist of arrays on which a chromatographic phase is grafted to bind different serum proteins, according to their biochemical properties. The proteins selectively retained on the surface are then analyzed using a SELDI-TOF mass spectrometer (MS). That approach has allowed researchers to successfully identify unique protein biomarkers for cancers of the ovary,[21] prostate,[22,23] mammary glands[24] and for the pathophysiology of

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schizophrenia.[25] Similar high-throughput proteomic approaches are being used to study toxic effects of exposure to methamphetamine and methylenedioxymethamphetamine.[26] Thus, the purpose of the present paper is to report the identification of apolipoprotein (apo) C-III, as an apolipoprotein that is significantly increased in the serum of MJ abusers.

Materials and methods

Subjects

Eighteen MJ users and 24 control subjects were used in the present study. The demographic data for these volunteers are given in Table 1. All volunteers had undergone medical, neurological, psychological and laboratory evaluations. Exclusion criteria that applied to all subjects include: (1) major medical and psychiatric illnesses including history of hypertension, (2) head injuries with loss of consciousness for greater than 5 min, (3) evidence of any neurological abnormalities by history or examination, (4) HIV seropositivity and (5) drug (cocaine, heroin and so on) or excessive alcohol use by DSM-IV criteria for alcohol abuse or dependence. In addition to self report, the use of other illicit drugs was also ruled out by obtaining at least two observed urine samples for toxicological examinations during screening visits. These standard toxicological examinations did not document the presence of any illicit drugs (that is, amphetamine, barbiturates, benzodiazepines, CBs, cocaine metabolites, methadone, opiates and phencyclidine) in any study participant, except for CBs for the MJ group. The detection threshold for a positive screen for CBs was 50 mg per 100 ml. The research protocol was approved by the National Institute on Drug Abuse and Johns Hopkins Bayview Medical Center Institutional Review Boards for Human Research and was carried out in accordance with the Declaration of Helsinki. Informed consent was obtained from all subjects. These patients have been the subject of previous papers from this laboratory.[28]

In addition to the medical history, demographic information and drug use history information were obtained from the Addiction Severity Index.[27] The metric, joints per week, was determined from the subjects self-reported drug history. The heavy users smoked the equivalent of 130.8 ± 73.0 joints per week ($n = 20$; range 78–350). Blood samples were obtained in 7 ml Kendal vacutainer glass purple top tubes (BD 311743) following venous blood sample procedures. The clotted samples were centrifuged (10 min, 4 °C, 3500 × *g*), aliquoted in eppendorf tubes and frozen at −80 °C within 2 h after venous puncture.

Serum sample preparation: subcellular pre-fractionation

Serum samples were pre-fractionated using pH gradients prior to analysis. Pre-fractionation has been shown to increase the number of detected peaks[29] and, thus, can increase the probability of identifying peptides or proteins that are differentially expressed.[30] Serum fractionation employing 96-well filtration plate filled with dehydrated Q Ceramic HyperD F sorbent (Expression Difference mapping kit from Ciphergen Biosystems Inc., CA, USA) was used as a first purification step. A volume of 100 µl of the serum was diluted in 150 µl of U9 and incubated for half an hour at room temperature. The samples were diluted twice in binding buffer, and loaded on the spin column and incubated under shaking for half an hour at room temperature. The column was centrifuged at low speed (1000 r.p.m. for 1 min), to obtain

Table 1 Demographic measures and drug history

Demographic measures	*Controls* (n = *24*) *mean* ± *s.d.*	*Marijuana users* (n = *18*) *mean* ± *s.d.*
Age: years	21.8 + 6.3	21.4 + 2.7
Education: years	12.2 + 1.3	11.5 + 1.2
Shipley: IQ	102.2 + 10.2	95.2 + 9.6
Women[a]	72%	47.4%
African Americans[a]	68%	94.7%
Drug history measures		
Alcohol: days per 30 days[b]	0.73 + 1.8	5.6 + 6.4*
Alcohol: years[c]	0.48 + 2.0	2.4 + 3.8
Marijuana: days per 30 days		29.1 + 1.7
Marijuana: years[c]		6.2 + 1.6
Cigarettes per day	2.0 + 5.7	5.79 + 6.99*
Cigarettes: years	0.52 + 1.6	6.37 + 15.6

Data are shown as mean ± s.d.
[a]Data are shown as percentage.
[b]The number of days of substance use in the last 30 days from the ASI.[27]
[c]Years of substance use calculated from ASI.[27]
*Mean for MJ users greater than controls at $P < 0.05$ using Student's *t*-test.

the flow through fractions. Then, a sequential fractionation of the column with a decreasing buffer pH (pH 9; pH 7; pH 5; pH 4; pH 3; organic solvent) was performed. Each solution was incubated for 20 min on the column. Fractionated serum samples were then applied to Cu II IMAC30 (immobilized metal affinity capture) chips in triplicate using the Ciphergen Bioprocessor (96-well plate). IMAC30 ProteinChip Arrays reversibly bind proteins to the surface, through a co-ordinated metal (Cu^{2+}) interaction. This spotting allows the visualization of the different proteins in each fraction to localize the proteins of interest. Reference serum samples were also spotted along with the subject's fractionated serum samples.

IMAC30 ProteinChip processing

The IMAC30 chips were placed in a ProteinChip bioprocessor and 50 µl of 0.1 M copper sulfate was incubated on the chips for 5 min with vigorous shaking. The chips were then rinsed with deionized water and neutralized for 5 min with 50 µl of 0.1 M sodium acetate, pH 4.0. After another rinse with deionized water, the chips were equilibrated by washing twice with 150 µl of binding buffer (0.1 M sodium phosphate and 0.5 M sodium chloride, pH 7.0). Next, the chips were loaded with 90 µl of binding buffer to which 10 µl of the fractionated serum (Fraction 6/organic fraction) was added. The serum was incubated with the chips for 1 h with vigorous shaking. The serum was removed after an hour, and the chips were washed three times with 150 µl of binding buffer. A final rinse with deionized water was performed, the bioprocessor was removed and the chips were allowed to air dry for 30 min. Once the chips were dry, 1 µl of a 50% solution of SPA (sinnapinic acid) diluted in 50% (v/v) acetonitrile and 0.5% TFA (v/v) was applied to each spot. The SPA solution was allowed to dry completely (15 min interval) before it was added for a second time.

SELDI-TOF mass spectrometric analysis: data acquisition and processing

After the addition of the matrix (SPA), protein chips were read by a Protein Biology System II (PBS II) instrument equipped with ProteinChip Software Version 3.2 (Ciphergen Biosystems). ProteinChip Reader calibration in the low to high mass range was performed using All-in-1 protein standard II prior to obtain the most accurate mass readings possible. Spectra were obtained at laser intensities: low intensities (185 and 195, arbitrary units) were used for low mass proteins (<20 kDa) and high intensities (200 and 215, arbitrary units) were used for high mass proteins (>20 kDa). For both ranges, data were collected (with detector sensitivity = 9) by averaging at least 200 laser shots/spectra collected in the positive mode from each spot.

The raw data were transferred to ProteinChip Software Version 3.1 (Ciphergen Biosystems) for analysis. Spectra were calibrated with the same external calibrants (IgG; albumin; enolase; carbonic anhydrase; myoglobin; Cytochrome c; hirudine—All-in-1 peptide standard II; Ciphergen Biosystems) used for instrument calibration. Peak intensities were normalized to total ion current, excluding the mass range below 3000 Da, which is composed of strong signals from the matrix. In this study, the mass range was narrowed to 3000–10 000 Da based on visual inspections of spectra. Each spectrum was rescaled by a normalization factor, which was based on the average total ion current. Spectra were deleted if the normalization factor was more than two times higher or lower than the mean normalization factor. Baseline was subtracted and 'detected' peaks were identified as those with a peak height $3\times$ noise and $6\times$ the valley depth. The signal-to-noise cutoff was set at 3 for the first pass of peak detection (to pick up obvious and well-defined peaks) and 1.5 for the second pass (to detect smaller peaks) and to ensure one does not miss a potential significant peak. Detected peaks were labeled by m/z and the total number of detectable peaks was recorded.

After the above processing, mass spectral data were used for expression difference mapping using the Ciphergen ProteinChip software system (Ciphergen Biosystems), which provides rapid statistical analysis of multiple samples to identify peaks with differential intensities. The first step in the statistical analysis of peak intensity was to carry out biomarker wizard analysis on calibrated and normalized data. To average the triplicate data export the biomarker wizard analysis as a .csv file and create a pivot table to average the triplicate with Excel. The program grouped peaks or 'clusters' within a designated mass range (for example, 3000–20 000 kDa) that were present across multiple spectra at the same (that is, within a narrow window, such as 0.3%) m/z value. Each cluster then was treated as a single protein or peptide fragment. Intensities of clustered peaks were analyzed by Student's t-test between two sample groups. Differences were considered significant when $P<0.05$. The experiments were performed blind to diagnosis.

Reproducibility of SELDI-TOF MS spectra

The reproducibility of spectra generated by SELDI-TOF MS was evaluated in two experiments. The goal of the first experiment was to investigate whether peaks of the same m/z appeared in the duplicate spectra measured under the identical protocol (that is, two spots on the same 96-well plate) and the goal of the second experiment was to determine the variability of spectra across chips and over time (experiment done in triplicate). Mass spectra were generated, calibrated by external calibrants (All-in-1 protein standard II, Ciphergen Biosystems) and normalized by total ion current. Three peaks from the organic fraction spectra ($P<0.05$) were chosen to analyze the variation of m/z and relative peak intensity.

Enrichment of the biomarker protein by reverse phase beads

Among the three peaks that were identified, we enriched the peak (9.4 kDa) that showed the highest levels of significance ($P < 0.002$, Table 2). Organic fractions containing the proteins of interest were equilibrated with 50 µl of RPC Poly-Bio beads (Biosepra, Cergy, France) with 10% ACN/0.1% TFA. The sample solution was adjusted to a final concentration of 10% ACN/0.5% TFA, mixed with 50 µl of equilibrated RPC beads and incubated for 30 min at room temperature on the rotating shaker. Samples were then centrifuged for 1 min at 5000 r.p.m. The supernatant was transferred to a fresh tube and bound proteins were eluted using 10, 20, 30, 40 and 50% ACN in 0.1% TFA. Proteins in the eluted fractions were analyzed by profiling aliquots of each fraction on to a NP20 ProteinChip Array. The fractions of interest were observed in the 50% acetonitrile fraction. This fraction was further concentrated by vacuum centrifugation and dissolved in sample buffer for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Purification by SDS-PAGE gel electrophoresis

The dried fractions were dissolved in a loading buffer (2 × Tris-glycine SDS sample buffer, Invitrogen, Carlsbad, CA, USA), and loaded onto a 18% Tris Glycine polyacrylamide gel (Invitrogen). This was stained overnight using colloidal Coomassie blue (Safe-stain, Invitrogen) and then destained with deionized water. The gel spot was extracted and used for passive elution or for in-gel digestion.

Passive elution of proteins from SDS-PAGE and protein in-gel digestion

The gel pieces were dehydrated in 100% acetonitrile (15 min). After removal of acetonitrile, the gel pieces were covered with 70 µl of 50% formic acid, 25% acetonitrile, 15% isopropanol, 10% H_2O and incubated for 2 h at room temperature under vigorous shaking. Aliquots of the samples were analyzed directly on a NP20 array. This step allows for the comparison of the molecular mass of the passively eluted protein with the original SELDI-TOF MS spectra to ensure the digestion of the correct marker protein.[31]

For in gel digestion, aliquots of 50% acetonitrile and of 100 mM ammonium bicarbonate (pH 8) solutions were added to the gels. This was followed by shaking for 1 h, followed by the addition of 100% acetonitrile and further shaking. After removal of acetonitrile, the gel pieces were dried in a 70 °C heat bath (dry) for 5 min. The gel pieces were rehydrated with 50 mM ammonium bicarbonate (pH 8) containing 10–20 ng µl^{-1} modified Trypsin (Roche Applied Science, Indianapolis, IN, USA). The samples were then incubated for 16 h at 37 °C. These samples are tested further with QSTAR mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a PCI-1000 ProteinChip Interface.

Protein identification by peptide fragmentation using a QSTAR mass spectrometer (ABI/Sciex) equipped with a PCI-1000 ProteinChip interface

A portion of the tryptic digests was analyzed on a Q-TOF Tandem mass spectrometer (QStar Pulsar, ABI Applied Biosystems, Darmstadt, Germany) equipped with a SELDI source: PCI 1000 ProteinChip Interface (Ciphergen Biosystems). The system was externally calibrated using human angiotensin I (m/z 1296.6853) and porcine dynorphin A (m/z 2147.1990). Raw data were analyzed using Analyst Software (ABI, Foster City, CA, USA). After reviewing the spectra, we selected specific ions for CID fragmentation and submitted the CID spectral data to the database-mining tools Mascot (Matrix Science, London, UK) for identification.

Confirmation of apoC-III protein identification using Immunocapture

To confirm the identity of the biomarker, we used immunocapture assays. The affinity-purified anti-human apoC-III antibody (1:4 dilution; Academy Bio-medical Company, Inc., Houston, TX, USA) and control rabbit IgG (host species) were added to protein A ceramic HyperD beads (Biosepra). After vigorous shaking, the beads were washed 3 times with 1 × PBS. The diluted serum sample (1:10 dilution with 1 × PBS) was then added and incubated for 90 min.

Table 2 Biomarkers from univariate cross-site sample comparison of marijuana users vs control individuals after controlling for gender, ethnicity, daily tobacco use and monthly alcohol use showing m/z ratio, direction of change and P-value for each peak

m/z[a]	*Protein chip array type*	*Fraction used*	*Direction of change in marijuana users*	P-*values*[b]
8764	IMAC30 Cu	Fraction 6	↑	< 0.014
9447	**IMAC30 Cu**	**Fraction 6**	↑	**< 0.002**
9750	IMAC30 Cu	Fraction 6	↑	< 0.004

The spectra were calibrated with the protein low calibration on the NP-20 array.
[a]m/z mass/charge.
[b]P-values calculated from Student's t-test.
The peak information presented in bold was the peak selected for further enrichment.

The beads were washed according to the following procedure: once with 1 × PBS; 5 times with 50 mM Tris-HCl (pH 8.0), 1 M urea, 0.2% CHAPS, 500 mM NaCl; 3 times with 1 × PBS and once with deionized water. The washes were followed by addition of 100 mM acetic acid. An aliquot of the sample was then profiled onto a NP20 Chip array and the peaks were compared to an apoC-III peak.

Assays of serum lipid and protein parameters
Triglycerides, cholesterol, α-1-globulin and albumin were determined using commercial kits (Roche, Basel, Switzerland) and Hitachi 917 autoanalyzer from Roche Company.

Statistical analyses
Potential differences between the controls and the MJ users were assessed using analysis of covariance (ANCOVA) with gender, ethnicity, monthly alcohol use and daily tobacco use as co-variates. These covariates were used to statistically control for small ethnic and gender sampling differences observed between the two groups. They were used to control for slightly heavier use of alcohol and tobacco by the MJ users. *Post-hoc* analyses were done using Student's *t*-test. Correlations were evaluated by calculating Pearson correlation coefficients (using a two-tailed test; alpha = 0.05). Analyses were performed using SPSS (version 12.0) (SPSS Inc., Chicago, IL, USA). The null hypothesis was rejected at $P < 0.05$.

Results

MJ users show increased abundance of a protein peak at approximately 94 kDa m/z
In this study, we profiled the fractionated sera of 18 MJ users and of 24 controls using IMAC Cu^{2+} chromatographic surface. Profiles generated on IMAC Cu^{2+} arrays allowed the best discrimination between the control and the patient groups. To choose the optimal fraction and array, the number of significant *m/z* values and the cluster plots were evaluated for each condition using the Ciphergen Express software. We found several SELDI-TOF MS peaks that retained discriminatory values in each of the three data sets. By using Biomarkers Patterns, decision trees were made and the decision trees generated from the fraction 6 samples resulted in a superior classification. All data from fraction 6 were reproduced at least three times, thus, ensuring a good level of confidence in our observations. Table 2 shows the list of peaks that showed significantly different between the groups ($P < 0.05$) in the ANCOVA after controlling for gender, ethnicity, daily tobacco use and monthly alcohol use. Gender, ethnicity daily tobacco and monthly alcohol use did not contribute to the differences observed between the control and MJ group as these covariates were not significant in these analyses.

Figure 1a shows representative SELDI-MS spectra of MJ users and controls obtained on IMAC Cu^{2+} array, using SPA as matrix. Variation of intensity between patients and controls are visible at approximately 9.4 kDa *m/z*. The intensity values for the 9.4 kDa *m/z* peak were consistently higher in the serum of MJ users. As shown in the cluster plot in Figure 1b, the 9.4 kDa *m/z* was one of the three diagnostic peaks that displayed a clear discriminatory differential expression in MJ users in comparison to control subjects, with the MJ users showing significant higher expression of the identified peak.

Identification of apoC-III and its isoforms as the relevant proteins that are differentially expressed between the two groups
Isolation and enrichment of the identified 9.4kDa *m/z* peak was done as described above. Figure 2 shows that the fraction eluted by 50% acetonitrile/0.1% TFA fraction contained the 9.4 kDa *m/z* peak along with two other peaks (8.8 and 9.7 kDa) that were also observed in the profiling process (Table 2). The selected protein fraction was also subjected to SDS-PAGE and Coomassie staining for further isolation and purification. In each case, three protein bands with an apparent molecular weight between 8 and 10 kDa on the SDS-PAGE were found to be increased in sample of MJ users. The three bands are shown in Figure 3a. The three protein bands were excised and the proteins were passively eluted from the gel pieces, and their masses were verified by MS profiling (Figure 3b), which confirms the SELDI-TOF-protein profile results.

Identification of the 9.4 kDa *m/z* peak was done after excising this peak out of the SDS-PAGE gel and in-gel trypsin digestion. The protease digest was subjected to tandem mass spectrometry. The tryptic digest fragment of 1716 *m/z* (Figure 4a) was DALSSV-QESQVAQQAR. The CID spectral data was submitted to database-mining tools Mascot and were identified as apoC-III (P02656) with a probability Mascot score (probability based mowse score) of 128 (Figure 4b). Protein scores greater than 67 are significant ($P < 0.05$). The same protein purification and identification were repeated on four control and four MJ users serum samples, and each time the excised protein band was identified as apoC-III.

Although the purification technique and further identification using tandem MS analysis showed consistently that the putative protein marker was apoC-III, we chose to further confirm its identity by specific immunoaffinity capture on ProteinChip arrays containing polyclonal anti-sera recognizing apoC-III (Figure 5). The peak at 9420 Da was specifically captured by anti-apoC-III from both controls and MJ users serum samples (Figure 5). Isoforms of apoC-III (8751 and 9751 Da) were also detected.

Effect of MJ on serum triglyceride levels
Because apoC-III and triglyceride levels are known to show positive correlations, we decided to measure

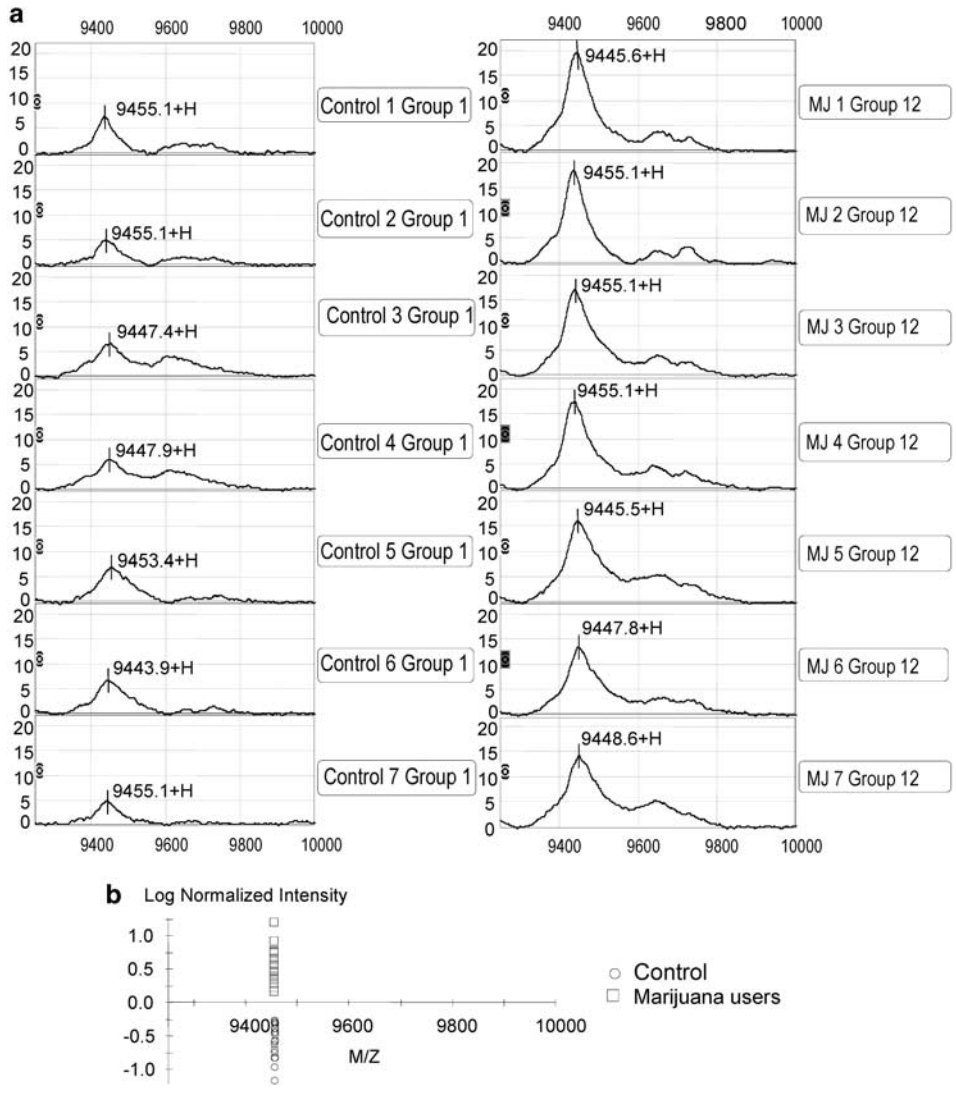

Figure 1 SELDI-TOF-MS protein spectra of marker of *m/z* value around 9.4 kDa. (**a**) Representative protein spectra of seven control subjects and seven MJ (marijuana) users. The marker is indicated with an arrow. The mass is given as *m/z* values on the *x*-axis and the intensity is displayed along the *y*-axis. Note the different sizes of the peaks in the control and MJ groups. The bands at 9.4 kDa were significantly increased in the MJ patients ($P < 0.002$, Student *t*-test). (**b**) Range of values for individual MJ users (on top of line) and controls (bottom of line). Note the lack of overlap between the two groups, with the MJ group showing higher values.

triglyceride levels in the serum collected from these participants. Table 3 shows that the triglyceride levels showed non-significant increases in the serum of MJ users. Nevertheless, Figure 6a shows that there were significant positive relationships between serum levels of apoC-III and the concentration of serum triglycerides ($r = 0.507$, $P < 0.001$). The levels of apoC-III also correlated with those of α-1-globulin and cholesterol (Figures 6b and c) but not with those of serum albumin (Figure 6d). Table 3 also shows values for the levels of α-1-globulin and albumin measured in the same serum samples. Gender, ethnicity, daily tobacco and monthly alcohol use did not contribute to these findings as these covariates were not significant in these analyses.

Discussion

The main finding of the present study is that chronic MJ abuse is associated with significant increases in serum apoC-III levels. In addition, there were significant correlations between apoC-III and triglyceride levels. Because of the relationship between apoC-III levels and vascular risk factors, the observations in MJ abusers will be discussed in terms of the potential relevance to MJ-induced adverse vascular events, which include coronary abnormalities.[32] These vascular dysfunctions are thought to be secondary to complex interactions between the various metabolic and physiologic effects of chronic MJ use. The present study, which profiled proteins in

the serum of these patients using SELDI-TOF-MS, has now identified ApoC-III as a possible agent in this complex network of MJ-induced effects in humans.

ApoC-III is a 79 amino-acid glycoprotein that contains galactose, as its sugar moiety.[33] ApoC-III resides on the surface of very low density lipoprotein and low density lipoprotein and influences their metabolism.[34] There are three isoforms of the protein, designated by the number of sialic acid residues are called C-III$_0$, C-III$_1$ and C-III$_2$.[35,36] ApoC-III plays a regulatory role in the catabolism of triglyceride rich lipoproteins by inhibiting the activity of lipoprotein lipase, which hydrolyzes lipids in lipoproteins.[37] It also blocks the activity of hepatic triglyceride lipase, which metabolizes triglycerides[38] that are independent risk factors for cardiac vascular disorders.[39] Several lines of evidence have documented positive correlations between plasma apoC-III levels and elevated levels of plasma triglycerides.[40–42] Animals studies have indeed shown that overexpression of human apoC-III in mice causes severe hypertriglyceridemia,[43] whereas disruption of the endogenous apoC-III gene prevents postprandial hypertriglyceridemia in mice.[44] In addition to its effects on triglycerides, apoC-III can contribute to atherosclerosis by recruiting monocytes onto vascular endothelium.[45]

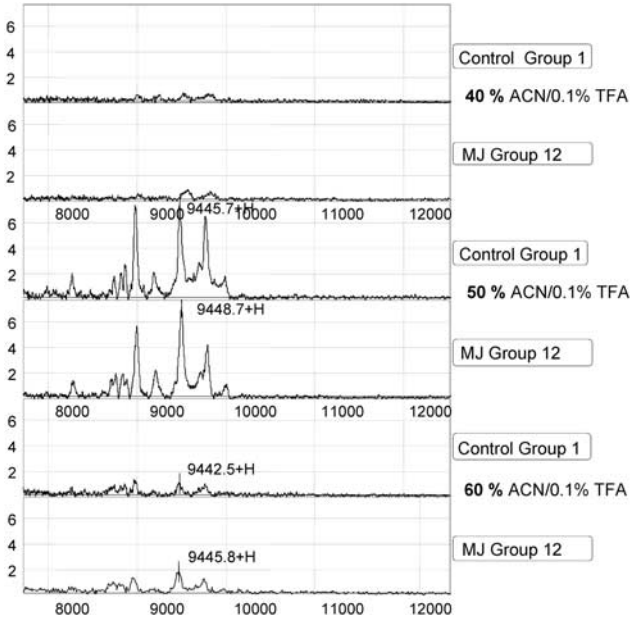

Figure 2 The flow through from the Q fractionation was bound to the reverse phase beads and then eluted with increasing concentrations of ACN/0.1% TFA. The biomarker was found in the 50% ACN/0.1% TFA fraction.

The increased serum apoC-III levels in MJ users and the positive correlations between serum apoC-III and triglyceride levels observed in the present study are consistent with the findings of a previous paper that had reported significant increases in serum HDL-triglyceride concentrations in MJ users in comparison to controls.[46] Thus, when taken together with apoC-III-mediated decreases in the rate of mitochondrial respiration,[47] the observed increases in apoC-III in the MJ users hint to the possibility that chronic MJ abuse could lead to impairments of cellular energetics and mitochondrial function, which are critical events associated to myocardial infarction, stroke and ischemic/reperfusion damage.[48] This discussion might

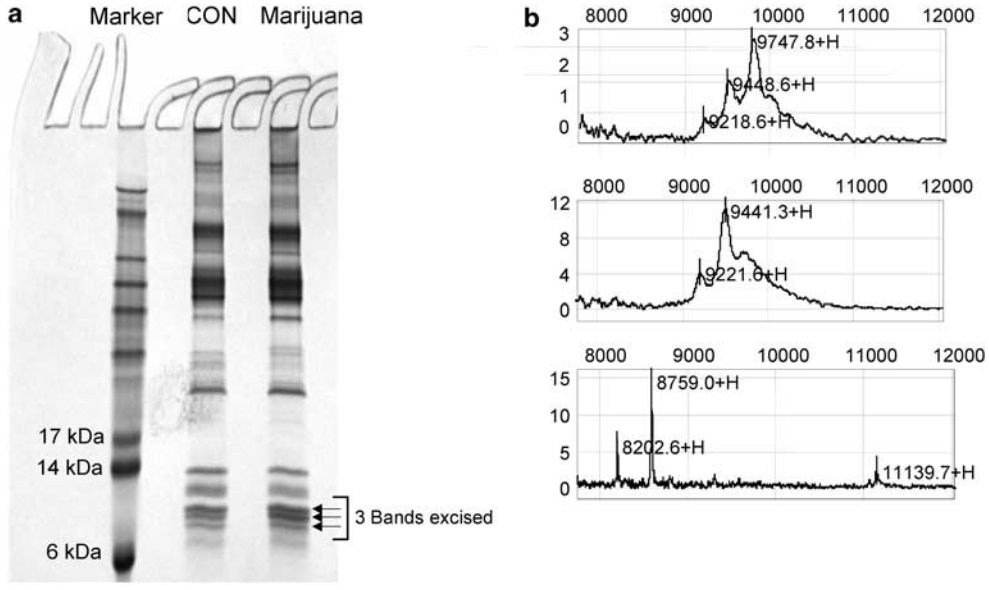

Figure 3 Confirmation of the identity of the 9.4 kDa putative biomarker. (**a**) Coomassie blue stained band pattern from samples of one healthy control sample and one of MJ patients separated by SDS-PAGE. The left lane shows the marker, the middle lane shows the control sample and the right lane shows the MJ sample. The bands which were excised are indicated by arrows. (**b**) Profile of the biomarker on a NP-20 array after elution in formic acid, acetonitrile and 2-propanol. These results confirm the identity of the excised proteins. SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

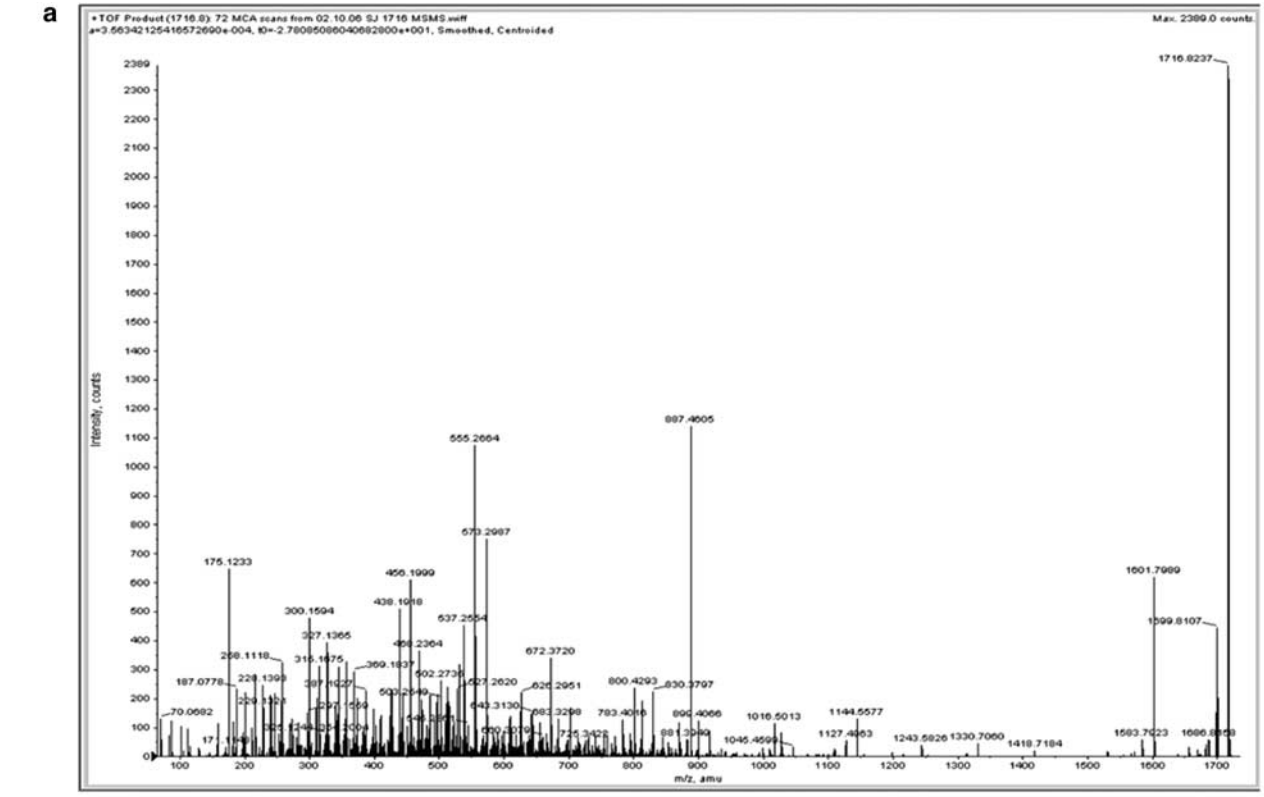

Figure 4 The sequence of the most abundant discriminating peptide (*m/z* 1716 Da) was defined by TOF/TOF sequencing as DALSSVQESQVAQQAR, which matches with high probability (MASCOT score 128) the sequence of apolipoprotein C-III.

offer a partial explanation for the chronic effects of the drug on cardiac and cerebrovascular systems.[49–51]

The manner by which chronic MJ smoking might cause these increases in serum apoC-III levels remains to be clarified. However, recent clinical data have demonstrated significant effects of CB1 receptor signaling on lipid parameters. Specifically, treatment of obese patients with the CB1 receptor blocker, rimonabant, caused significant improvements in the levels of triglycerides.[52,53] These results provide

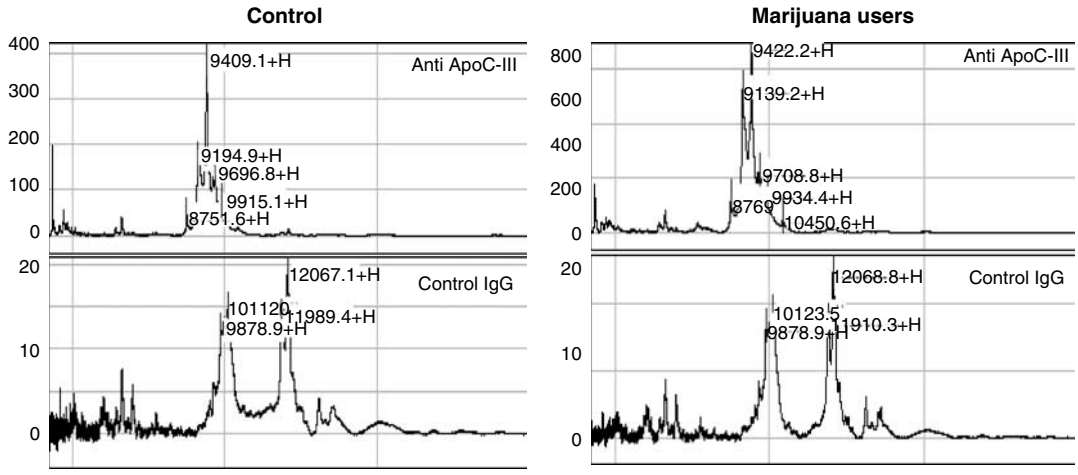

Figure 5 Confirmation of biomarker using anti-apoC-III antibody. Protein A Hyper D beads were incubated with rabbit anti-human antibody and control rabbit IgG. After washing, serum was incubated with serum diluted in PBS. The beads were washed followed by elution with 12–15 µl of 0.1 M acetic acid. Eluted fractions were profiled on NP20 arrays. Note the differences in the scales representing the control and MJ (marijuana) groups. In a fashion similar to the observation in Figure 1, the peaks in the MJ group were higher than those of the control group.

Table 3 Serum concentration of triglycerides, cholesterol, α-1-globulin and albumin in controls and heavy marijuana users

	Controls (n = *24*)	*Marijuana users* (n = *18*)	P–*value*
Triglycerides	91.17 ± 12.49	122.00 ± 14.43	0.09
Cholesterol	175.33 ± 5.75	167.33 ± 6.64	0.36
α-1-globulin	0.213 ± 0.021	0.233 ± 0.024	0.51
Albumin	4.258 ± 0.147	4.25 ± 0.17	0.97
BMI	25.711 ± 1.255	25.863 ± 1.449	0.94

Abbreviation: BMI, body mass index.
Triglyceride and cholesterol concentrations are expressed in milligram per 100 ml and represent mean ± s.e.
α-1-globulin and albumin concentrations are expressed in gram per 100 ml and represent mean ± s.e.

further support for a role of CB receptors in the mediation of the cardiovascular adverse effects observed during chronic MJ consumption,[49–51] which are mediated through actions of the actions of Δ^9-THC, the major psychoactive compound in MJ. After inhalation, Δ^9-THC is rapidly absorbed and distributed throughout the circulation; it is then metabolized, in the lungs and liver, to the more potent, 11-hydroxy- THC, which crosses the hematoencephalic barrier very easily.[54] In the periphery, THC exerts its effects by binding to CB receptors located in many peripheral tissues, including adipose tissue and liver, which are key organs involved in carbohydrate and lipid metabolism. The possibility that MJ exerts its actions via stimulation of CB1 receptors is supported by experiments documenting promotion of lipogenesis in the rodent liver by the CB1 agonist, HU-210 and its reversal by pretreatment with the CB1 blocker, rimonabant.[55] Moreover, in adipose tissue, the CB1 agonist has been shown to influence activity of lipoprotein lipase,[56] an enzyme whose activity is also regulated by apoC-III.[37] A role for peripheral CB receptors in the actions of MJ is also supported by the report that the levels of CB1 receptor mRNA are significantly higher in peripheral mononuclear cells of MJ users than in those of control subjects.[57] Furthermore, molecular characterization of transcriptional regulatory elements in the apoC-III promoter has also revealed binding sites for the inducible transcription factor, NF-κB.[58,59] Because Δ^9-THC, the active ingredient in MJ can induce NF-κB activation via increased phosphorylation of I-κB,[60] chronic MJ abuse might also affect apoC-III expression via THC-mediated NF-κB-induced hepatic inflammation that is known to cause substantial changes in the production and utilization of triglyceride rich lipoproteins.[61] Thus, the collected evidence suggests that MJ abuse may lead to sustained changes in THC-induced signaling pathways that promote the increased apoC-III levels that we are now reporting in the serum of MJ users.

Conclusion

Marijuana is the most commonly abused drug in the USA and evidence exists that its abuse is a risk factor

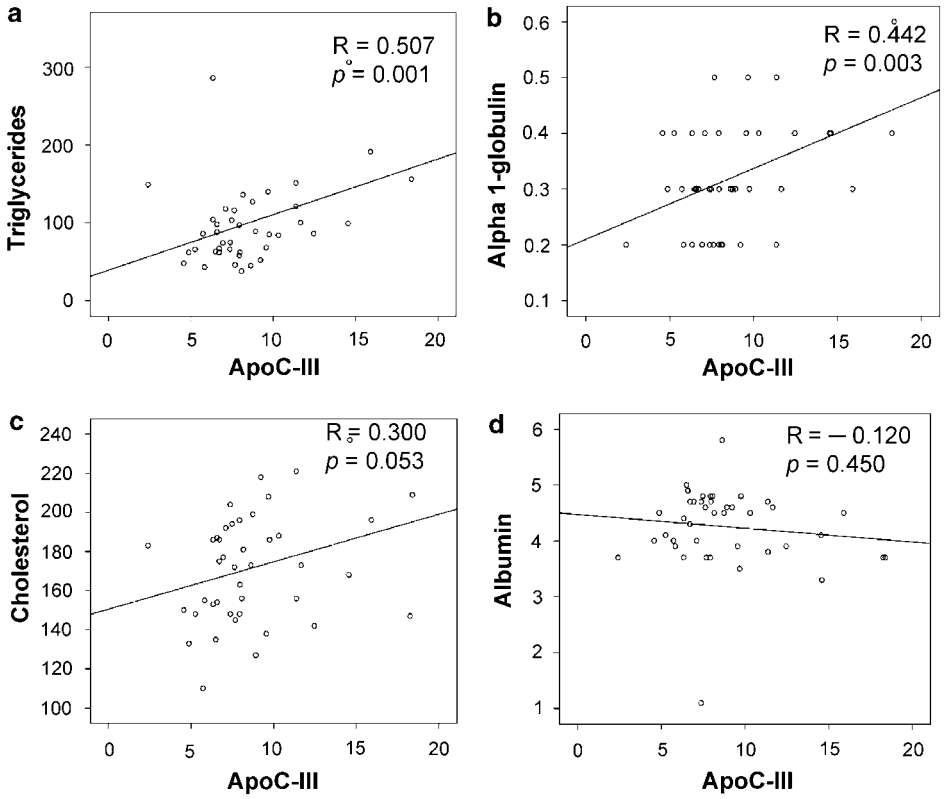

Figure 6 Correlation analyses of apoC-III levels with (**a**) triglycerides, (**b**) cholesterol, (**c**) α-1-globulin and (**d**) albumin in control subjects and MJ (marijuana) abusers.

for cardiovascular and cerebrovascular disorders. Our present observations that MJ abusers show significant increases in serum apoC-III levels suggest that THC-induced apoC-III upregulation might be a significant player in MJ-mediated vascular and neuroimaging abnormalities.[28,62–65] The idea of a possible involvement of an apolipoprotein in a neuropsychiatric disorder is consistent with the long history of the involvement of apolipoproteins (for example, ApoE) as etiological factors in neurodegenerative disorders such as Alzheimer's disease.[66,67] Verification of these ideas will await further studies that will seek to elucidate the role of apoC-III in mediating some of the long-term neuropsychiatric and neurological effects of MJ.

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