Serum Proteomic Profiles in Subjects with Heavy Alcohol Abuse

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Abstract

Objectives: The abuse of alcohol is a major public health problem, and the diagnosis and care of patients with alcohol abuse and dependence is hindered by the lack of tests that can detect dangerous levels of drinking or relapse during therapy. Gastroenterologists and other healthcare providers find it very challenging to obtain an accurate alcohol drinking history. We hypothesized that the effects of ethanol on numerous systems may well be reflected in changes in quantity or qualities of constituent or novel plasma proteins or protein fragments. Organ/tissue-specific proteins may be released into the blood stream when cells are injured by alcohol, or when systemic changes are induced by alcohol, and such proteins would be detected using a proteomic approach. The objective of this pilot study was to determine if there are plasma proteome profiles that correlate with heavy alcohol use.

Methods: Paired serum samples, before and after intensive alcohol treatment, were obtained from subjects who attended an outpatient alcohol treatment program. Serum proteomic profiles using MALDI –OTOF Mass Spectrometry were compared between pre- and post treatment samples.

Results: Of 16 subjects who enrolled in the study, 8 were females. The mean age of the study subjects was 49 yrs. The baseline laboratory data showed elevated AST (54 ± 37 IU/L), ALT (37 ± 19 IU/L), and MCV (99 ± 5 fl). Self-reported pre-treatment drinking levels for these subjects averaged 17 ± 7 drinks/day and 103 ± 37 drinks/week. Mass spectrometry analyses showed a novel 5.9 kDa protein, a fragment of alpha fibrinogen, isoform 1, that might be might be a new novel marker for abusive alcohol drinking.

Conclusions: We have shown in this pilot study that several potential protein markers have appeared in mass spectral profiles and that they may be useful clinically to determine the status of alcohol drinking by MALDI –OTOF mass spectrometry, especially a fragment of alpha fibrinogen, isoform 1. However, a large-scale study is needed to confirm and validate our current results.

Introduction

The abuse of alcohol is a major public health problem, and the diagnosis and care of patients with alcohol abuse and dependence is hindered by the lack of tests that can detect dangerous levels of drinking or relapse during therapy. Such tests would be valuable for screening for alcohol use disorders and for the testing of potential therapies. It is not uncommon that alcoholics typically underestimate their consumption or deny that they have a problem with alcohol (Ferguson et al., 2003). In a specific clinical setting such as pre-liver transplant evaluation, such tests would be even more

beneficial to as- certain that patients with alcoholic liver disease attain and continue their sobriety. In an era of organ shortage, use of liver transplants in patients with undetected or ongoing alcohol use may negatively affect the public attitude on transplantation and organ donation (Mathurin, 2005).

Several questionnaires have been developed and validated to screen for or establish the diagnosis of alcoholism. Traditionally, the CAGE questionnaire is the most commonly used for screening because it is fast and easy to remember (Ewing, 1984). However, as a screening tool, the CAGE questionnaire does not discern between current and past drinking patterns. Another tool, the Alcohol Use Disorder Identification Test (AUDIT), was developed to improve the sensitivity of identifying heavy drinking and active alcohol use (Saunders et al., 1993); however, its sensitivity to screen for alcohol use disorder, when using Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria as the gold standard, was only 76%. Direct measurement of alcohol concentration in blood or urine samples is not useful as it is only present for a short time after stopping drinking, and thus does not provide information beyond the recent past. The plasma levels of enzymes expressed in the liver (gamma glutamyl transpeptidase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and the mean corpuscular volume of erythrocytes (MCV) are among the commonly used markers used to identify chronic alcohol use. However, these measures have low sensitivity for recent excessive intake, and abnormal levels may result from several causes besides heavy drinking. For example, chronic HCV infection or obesity is common among alcoholic patients and increase the transaminases; GGT can be elevated by heart disease, concomitant medication use, and diabetes. Carbohydrate-deficient transferrin (CDT), forms of the serum iron carrying protein transferrin with altered carbohydrate composition, is a more specific marker for identifying excessive alcohol consumption and monitoring abstinence. However, none of these tests has the desired sensitivity and specificity. For example, in a group of 92 subjects entering treatment for alcoholism, only 63% had an abnormal %CDT result (Walter et al., 2001). Combination of CDT and GGT seems to increase sensitivity at the expense of reduced specificity (Salaspuro, 1999). Moreover, the results of these tests do not bear a linear relationship to quantity of alcohol consumed, and do not become abnormal quickly when patients relapse into drinking.

Since the metabolism of ethanol generates the highly reactive protein modifying reagent acetaldehyde, and acetaldehyde-protein adducts have been identified in many laboratories (Sillanaukee et al., 1991; Sillanaukee et al., 1991; Worrall et al., 1994; Niemela, 1999; Roy et al., 2002), there is good a priori reason to believe that alcohol use will cause reproducible changes in plasma proteins. In fact, such adducts (e.g., of hemoglobin, albumin, or other proteins) have been found in human plasma (Worrall et al., 1994; Worrall et al., 1998; Gross et al., 1992). The objective of this pilot study was to determine if there are plasma proteome profiles that correlate with heavy alcohol use (defined by the DSM-IV diagnosis of alcoholism). We hypothesized that the effects of ethanol on numerous systems may well be reflected in changes in quantity or qualities of constituent or novel plasma proteins or protein fragments. Organ/tissue-specific proteins may be released into the blood stream when cells are injured by alcohol, or when systemic changes are induced by alcohol, and such proteins would be detected using a proteomic approach.

Patients and Methods

Patients

The study was approved by the Indiana University Purdue University at Indianapolis and Institutional Review Board and by the Fairbanks Addiction Treatment Center. All subjects were recruited from Fairbanks, a non-profit addiction treatment facility located in Indianapolis, Indiana focused on treatment, education and research of alcohol and drug abuse and addiction. Subjects were those seeking treatment for alcohol abuse and dependence. At the first visit, subjects were seen by addiction psychiatrists when the full detailed interview, history taking and physical examination were performed. Inclusion criteria were subjects who met the DSM-IV Diagnostic Criteria for Alcohol Dependence/abuse (Hasin et al., 2006) and who had been actively drinking within 7 days of the first visit (because of our interest to identify the potential markers for recent alcohol use). Subjects were excluded if they had history of any localized or systemic infectious disease within 4 weeks prior to the study and had symptoms and signs of decompensated liver disease (jaundice, ascites or hepatic encephalopathy). Eligible subjects who met all the enrollment criteria were asked to answer questionnaires which included the following information: a) background information and demographics, b) alcohol consumption, c) alcohol abuse and dependence, d) previous history of alcohol treatment utilization, e) family history of alcoholism, f) tobacco use and dependence, g) medication use, h) past medical history, i) drug abuse and dependence, and j) family history of drug abuse. Seven mL of blood from each subject were obtained according to the protocol described in the proteomic analysis section. This set of samples is referred to as 'pre-intensive outpatient treatment samples or pre-treatment samples' in the remainder of the manuscript. Alcohol treatment at Fairbanks is an intensive 6-week daily outpatient program. During this 6-week period, all patients were followed closely and none

relapsed. At the end of the 6-week treatment program, another set of blood samples (post-intensive outpatient treatment samples or post-treatment samples) were drawn and serum proteomic profiles were compared between pre- and post treatment samples. During the study period, 75 subjects were screened; however, only 16 subjects met the established inclusion criteria and from whom both pre- and post-treatment samples were obtained.

Materials and Methods

Materials

Phosphate buffered saline (PBS), 1-propanol, guanidine hydrochloride solution (GuHCl), DL-dithiothreitol (DTT), trifluoroacetic acid (TFA), iodoacetic acid and α-cyano-4-hydroxycinnamic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN) and mass spectrometry-grade water were obtained from EMD Chemicals (Gibbstown, NJ, USA). Tris-(hydroxymethyl) aminomethane was purchased from VWR (West Chester, PA, USA). Hydrochloric acid (HCl) was obtained from Fisher Scientific (Pittsburgh, PA, USA). Oasis® HLB SPE plates were purchased from Waters Corporation (Milford, MA, USA). The ProXPRESSION™ Biomarker Manual Enrichment kits and MALDIchip™ plates were obtained from PerkinElmer (Wellesley, MA, USA).

Samples

These included 16 subjects who met the inclusion criteria and from whom post-treatment samples were also obtained. Seven mL of blood were drawn and centrifuged at 1500 x g for 10 min at room temperature, within 1–3 hour(s) after acquisition. Aliquots (0.5 mL) were placed in separate 2 mL cryovials and stored at -80ºC until analyses.

Enrichment of Biomarkers from Associated Carrier Proteins

Potential biomarkers associated with carrier proteins such as albumin were enriched using the ProXPRESSION™ Biomarker Manual Enrichment user guide. The spin columns contain Vivapure Blue, a Cibacron blue dye-based membrane with a high affinity for albumin. The spin column was equilibrated with 300 µL of 30% 1-propanol in PBS 3 times then 100% PBS 3 times by centrifuging at 500 × g for 3 min. The 500 µL of PBS diluted sample (100 µL plasma) was loaded onto the equilibrated spin column. The spin columns and collection tubes were then centrifuged at 12,000 × g for 5 min. The flow-through was collected and reapplied to the column, and then the 2nd flow-through was discarded. The spin column was then washed 3 times with 300 µL of PBS by centrifuging at 500 × g for 3 min. The

spin column was eluted with 100 µL of 10 mM DTT in 8 M guanidine hydrochloride (GuHCl) solution twice by centrifuging at 12,000 × g for 5 min. The elution collection was incubated at 37 °C for 30 min. Following incubation, 18 µL of 500 mM iodoacetic acid in 1 M Tris/HCl pH 8.0 was added to the elution, and the mixture was placed in the dark for 30 min. Then 10 µL of 1 M DTT, 75 µL of 10% TFA and 300 µL of 0.25% TFA were added into the mixture. These protein samples were subsequently referred to as "biomarker enriched samples". Although the protocol is known to capture only a portion of the albumin from the serum, this protocol delivers highly reproducible peptide profiles with intensity CVs typically 5%–10% (Lopez et al., 2005).

The purification of extracted peptides and protein fragments was conducted with an OASIS plate and vacuum. The plate was preconditioned by washing with 600 µL of 100% ACN twice followed by 600 µL of 0.25% TFA twice. The sample was loaded on the preconditioned plate and incubated for 1 min. The plate was washed with 600 µL of 5% ACN in 0.25% TFA twice. Then, the sample was eluted from the OASIS plate into a fresh collection plate with 25 µL of 70% ACN in 0.25% TFA twice. The total collected volume was 50 µL. The MALDI plate was immediately spotted with 1 µL of 2.5 µg/mL α-cyano-4-hydroxycinnamic acid matrix and 6 µL of sample for MALDI-OTOF analysis (see below).

MALDI –OTOF Mass Spectrometry

Mass spectra were acquired on a prOTOF™2000 MALDI orthogonal time-of-flight (OTOF) mass spectrometer interfaced with TOFWorks™ software (PerkinElmer/SCIEX). As a consequence of the mass spectrometer's orthogonal design, a single external mass calibrant was used to achieve >10 ppm mass accuracy over the entire sample plate. In this study, a two-point external calibration of the prOTOF instrument was performed before acquiring the spectra in a batch mode from 75 samples in triplicate across three target plates (225 total spectra). Typical resolution for peptides and proteins up to 10 kDa was approximately 10,000 (FWHM).

Processing and Analysis of MALDI Spectra

MALDI mass spectral data were processed and analyzed using Progenesis PG600™ software (NonLinear Dynamics) essentially as described by Lopez et al., (2007). Raw spectra were loaded directly into the PG600 program using the prOTOF loader program. The individual MALDI peak quantity was determined as the integral of the corrected intensity over the width of the peak envelope. The raw signal is filtered to remove noise and background signals by a dis-

crete wavelet transform. Peaks were then detected and the relationship between m/z value and the size (width and intensity) of the isotopic peak distribution determined by detecting the start and end of the peak envelope. The intensity of this envelope was then integrated over this range to give the Peak Quantity. The data were normalized by summing all spectral peaks, with the assumption that all spectra have approximately the same material in them. All sample spectra were processed in this manner, and analyses were performed on the 16 pre- and post treatment samples to find discriminant markers between the two groups. Parameters for biomarker selection were set within the PG600 program to include peaks with a mean quantity threshold of ≥50 and statistical confidence interval for treatment group differences set at P<0.05.

Results

Patient Data

Of 16 subjects whom paired serum samples were avail-able, 8 were females. They had an average age of 49 yrs, height 68 in, weight 159 lbs, and BMI 26 kg/m². All clinical data fell in the normal range with the exception of elevated AST (54 ± 37 IU/L), ALT (37 ± 19 IU/L), and MCV (99 ± 5 fl). Self-reported pre-treatment drinking levels for these subjects averaged 17 ± 7 drinks/day and 103 ± 37 drinks/week. Values for the entire group of 75 were nearly identical. We defined a "drink" as the following equivalents: 1.5 oz liquor (40% EtOH), 5 oz wine, or 12 oz beer. Many subjects self-reported drinking reflected a combination of these. Drinking onset was reported as 18 ± 3 yrs of age and 7 ± 7 drinks were consumed on the day of last use, which was reported to be from 0-144 hours prior to pre-treatment sampling. Selected physical and clinical values of the 16 subjects, segregated by gender, are listed in Table 1. Statistical comparisons of all pre-treatment sample data to MALDI profiles using Pearson correlation, regression, and Student t-test analyses detected no significant relationships. This was also true of drinking level, alcohol type, and other characteristics. Several MALDI profile changes

Group	Age (yrs)	use amount (drinks/day)	pattern (drinks/wk)	AST	ALT	GFR	RBC	MCV
8 Female Subjects	47 ± 10	13 ± 5	91 ± 38	72 ± 60	44 ± 29	104 ± 21	3.9 ± 0.3	101 ± 4
8 Male subjects	51 ± 6	20 ± 7	115 ± 38	37 ± 16	31 ± 11	99 ± 26	4.5 ± 0.2	97 ± 7
P	0.5	0.07	0.4	0.3	0.4	0.6	0.02	0.3
normal values	-	-	-	0-35	3-36	120-125	4.2-6.0	80-95

Table 1: Selected physical and clinical values of 16 subjects from whom pre- and post-treatment samples were analyzed, segregated by gender.
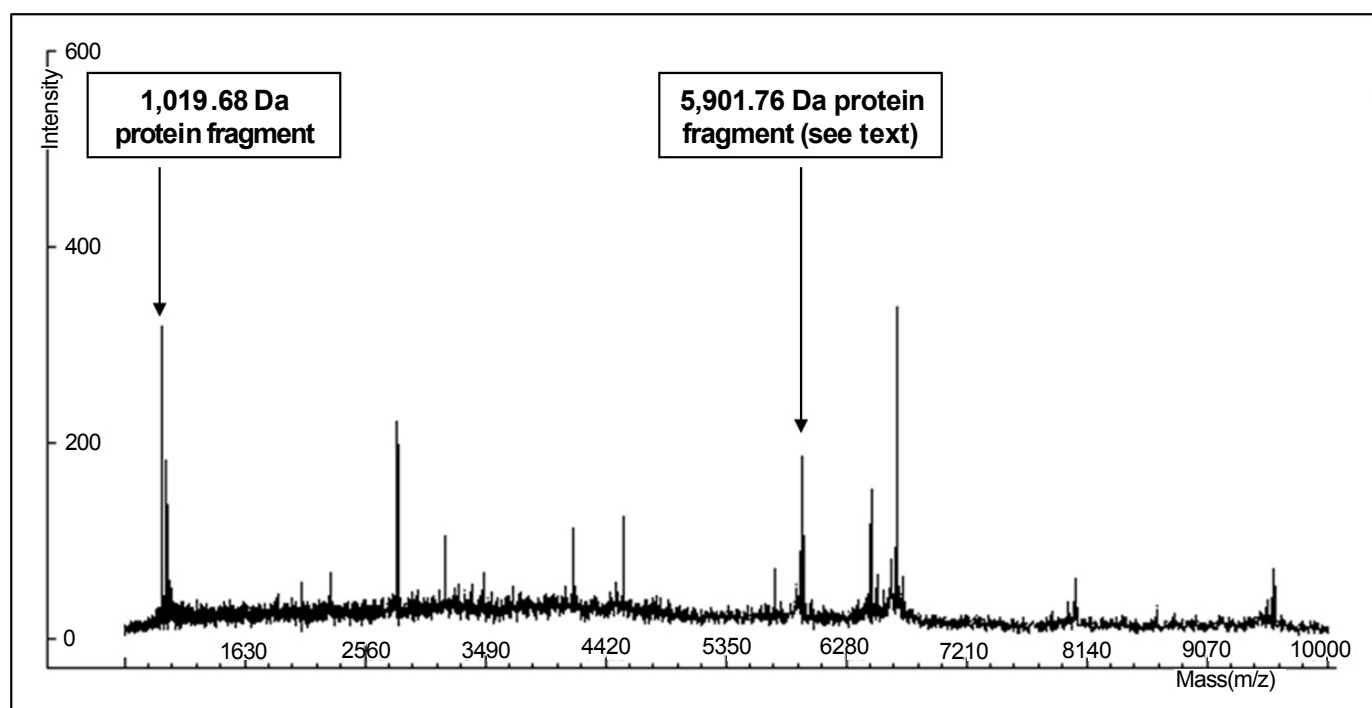

Figure 1: Matrix-assisted laser-desorption ionization orthogonal time-of-flight (MALDI-OTOF) mass spectrum of a typical enriched biomarker sample from a pre-treatment subject. Two peaks whose intensities were altered by abstinence are shown and the differences are depicted in Fig. 2.

showed positive or negative correlations to ALT and AST levels, but were statistically insignificant due to the limited size of the sample. However, both AST and ALT values were significantly correlated to self-reported drinks/week ($P<0.001$).

MALDI Profiles of Biomarker Enriched Samples

Potential biomarkers associated with carrier proteins such as albumin were enriched using ProXPRESSION™ Biomarker Manual Enrichment kits and resulted in the detection of 769 spectral peaks or features (from 703.38 – 9,488.02 Da) that were matched and compared pre-treatment *vs.* post-treatment across all 32 samples (representing the mean profile of technical triplicates, *i.e.* 96 total spectra). Using this protocol, Lopez et al., (2005) reported CV of 5-10% for peak intensity variation among technical replicates. In the current study the average CV across technical replicates was 12%. A typical MALDI profile is illustrated in Figure 1. Statistical comparisons conducted within the PG600 software determined that 31 of these were found to differ Pre- *vs.* Post-treatment ($P<0.05$); 16 features decreas-

ing with treatment (elevated by alcohol abuse) and 15 features increasing with treatment (depressed by alcohol abuse) (see Figure 2 and Table 2).

MALDI Profiles of Biomarker Enriched Samples – Analysis Stratified by Gender

It is known that women have a higher risk of developing cirrhosis than men, even at a lower daily alcohol intake and lower cumulative exposure to alcohol (Corrao et al., 1997). In men, the riskof alcoholic cirrhosis increases with daily alcohol consumption greater than 60-80 g/d, while women have a lower threshold and are considered to be at risk with daily alcohol intake greater than 20 g/day. Additionally, there is a more rapid development of liver disease in women who abuse alcohol as compared with men. Because of the gender-susceptibility on the effect of alcohol drinking and end organ damage, we analyzed the data to see whether there were gender-specific effects on the markers of interest. Table 1 demonstrates that significant group-wide alterations in spectral peak intensity observed between pre-treatment and post-treatment samples are largely a function of responses
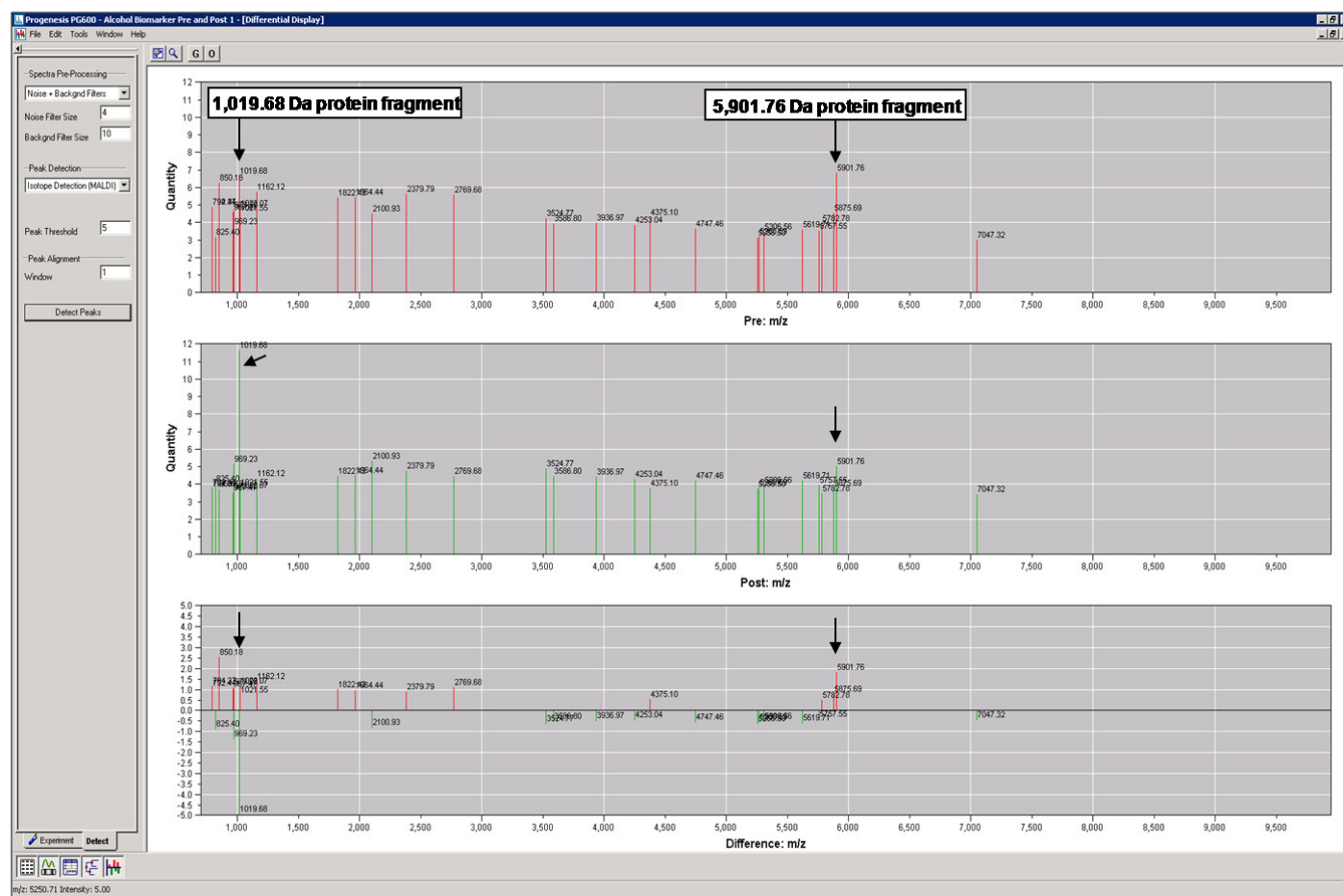

Figure 2: PG600™ Software comparison of 31 spectral peaks from all pre- and post-treatment samples that were detected, normalized, processed, and statistically compared. Upper panel shows mean pre-Tx peak intensities, middle panel mean post-Tx, the bottom panel shows the comparative difference of the 31 significantly different biomarker candidates. X-axes are mass/charge (m/z) and Y-axes are normalized peak intensity calculated from ion current.

Peak	m/z	PreTx Quantity	Post Tx Quantity	Fold Difference	Group P Value	Female P value	Male P value
53	792.44	4.86	3.81	1.3	0.04	0.004	0.9
54	794.37	4.88	3.72	1.3	0.02	0.09	0.08
73	825.4	3.17	4.04	-1.3	0.04	0.07	0.4
88	850.18	6.26	3.72	1.7	0.005	0.03	0.06
153	967.47	4.57	3.53	1.3	0.02	0.1	0.08
154	969.23	3.78	5.14	-1.4	0.01	0.0005	0.4
156	973.5	4.71	3.58	1.3	0.01	0.0004	0.6
177	1019.68	6.67	11.63	-1.7	0.03	0.2	0.08
178	1021.55	4.55	3.81	1.2	0.04	0.5	0.03
179	1023.07	4.78	3.61	1.3	0.009	0.03	0.2
226	1162.12	5.72	4.32	1.3	0.009	0.3	0.008
363	1822.43	5.42	4.44	1.2	0.03	0.3	0.02
382	1964.44	5.46	4.49	1.2	0.03	0.04	0.3
400	2100.93	4.5	5.31	-1.2	0.04	0.3	0.09
437	2379.79	5.62	4.74	1.2	0.04	0.4	0.03
484	2769.68	5.58	4.47	1.3	0.03	0.09	0.1
561	3524.77	4.24	4.87	-1.2	0.02	0.3	0.03
567	3586.8	3.92	4.46	-1.1	0.01	0.03	0.2
603	3936.97	3.96	4.43	-1.1	0.04	0.0003	0.8
634	4253.04	3.85	4.28	-1.1	0.04	0.03	0.5
645	4375.1	4.29	3.77	1.1	0.04	0.2	0.04
675	4747.46	3.62	4.18	-1.2	0.03	0.4	0.02
702	5256.5	3.12	3.74	-1.2	0.001	0.0007	0.2
703	5268.59	3.21	3.79	-1.2	0.008	0.0006	0.6
704	5306.56	3.44	3.96	-1.2	0.03	0.2	0.1
713	5619.71	3.57	4.17	-1.2	0.03	0.001	0.3
718	5757.55	3.49	3.92	-1.1	0.007	0.0003	0.6
720	5782.78	3.94	3.45	1.1	0.02	0.006	0.3
726	5875.69	4.54	3.77	1.2	0.007	0.02	0.1
728	5901.76	6.83	5.02	1.4	0.04	0.05	0.4
764	7047.32	2.99	3.43	-1.2	0.04	0.006	0.4

Table 2: Potential protein-bound and enriched biomarkers of abusive alcohol drinking detected by MALDI mass spectrometry in both pre- and post- treatment samples.

to abusive alcohol drinking in female subjects (n=8) compared to the males (n=8). Of the 31 peptides designated as potential biomarkers, 24 were not significantly different (P<0.05) in male samples, including the notable 5.9 kDa peptide. Additionally, 7 peptides were significantly different in males but not in females. This gender-related effect has been shown to be consistent across other analytical strategies applied to these samples, such as LC-MS/MS identification/quantitation of biomarker enriched samples (Lai et al., 2009), and LC-MS/MS identification/ quantitation of proteins in immunodepleted sera (unpublished data).

Discussion

The objective of this project is to detect a novel biomarker (or confined set of biomarkers) of heavy/abusive alcohol consumption in the plasma of human subjects. We hypothesized that alcohol consumption at high levels elicits cellular and molecular responses whose sequelae are revealed through the appearance of unique and low-abundance polypeptides or proteins in the plasma. These molecules may be derived from a variety of tissues and cells and are unrelated to conventional/traditional markers of alcohol-induced liver injury. As a result of the comprehensive mass spectrometry-based analysis used in this pilot study, we have identified several biomarker candidates whose presence in serum is altered in subjects who drink alcohol abusively compared to serum after 6 weeks of abstinence. Identification and relative quantitation of some of these proteins with respect to clinical data supports our hypothesis that the proteins are of heterogeneous origin and that they are not related to conventional or traditional indices abusive drinking and alcohol-induced liver injury.

The mass spectral profiles showing 31 differentially intense peaks, particularly those elevated prior to alcohol treatment program, are potential biomarkers. Above all, the peak at 5901.76 m/z is of interest, as it is lowered by abstinence and corresponds to a similar peak observed in a previous study by Nomura et al., (2004). In that investigation, a serum-

based surface-enhanced laser desorption ionization (SELDI) approach detected a 5.9 kDa peak that was identified as a fragment of alpha fibrinogen, isoform 1 (or alpha E) representing a fragment with residues. SSSYSKQFTSSTSYNRGDSTFESKSYKMADEAGSEAD HEGTHSTKRGHAKSRPV$_{576-629}$ (near the C-terminus) and was observed to be lowered roughly 4-fold in the serum by abusive drinking and raised after abstinence. If the 5.9 kDa fragment in our study is identical to the previous SELDI result, the data suggest that during abusive drinking, the fibrinogen fragment binds more readily to albumin and consequently is lower in serum in its unbound form, and cessation of drinking somehow reverses this effect. Because the Nomura study did not analyze protein-bound fibrinogen degradation products (e.g. to albumin etc.), their results were unable to account for it. It is also possible that there is a change in protein/peptide sialylation (e.g. less sialic acid during heavy drinking, such as desialo-transferrin *aka* carbohydrate-deficient transferrin (CDT) (Takase et al., 1985; Behrens et al., 1988)), enabling greater binding to albumin and other prominent serum carrier-proteins. This is currently being investigated. Nevertheless, our observations are consistent with those of Nomura et al., (2004) and suggest that its differential detection may be the result of a change in compartment (bound vs. un-bound), and that the 5.9 kDa peptide indeed may be a potential marker of abusive alcohol drinking. Another reason this 54 amino acid polypeptide has potential as a biomarker is related to its apparent uniqueness. Twenty-one shorter length versions of it have been observed in human sera and are reported in the Peptide Atlas Database (Deutsch et al., 2008) (http://www.peptideatlas.org/), several in mass spectrometric assays of human serum (http://www.asms.org/asms05pdf/A051987.pdf) and cancer biomarker patent applications. Only a few references to the full-length form can be found in serum peptide literature, such as its down-regulation in the sera of thyroid cancer patients (Villanueva et al., 2006), in normal sera detected as a prominent 5.9 kDa peak and identified via MS/MS using a SELDI QSTAR approach (Peng et al., 2009), and in sera analyzed in parallel by SELDI and nanoscale LC-MS/MS (Davis et al., 2007). It is unlikely that the 5.9 kDa polypeptide is a fragment produced by fibrinolysis, as its sequence is inconsistent with known plasmin cleavage points in fibrinogen α-chains (Manabe et al., 2007). It is thus likely to be a unique degradation product of fibrin or fibrinogen. Because the Nomura study, like the present analysis, relied on data from only 16 subjects, further investigation using a larger sampling is warranted to corroborate these results.

Another interesting finding is that the alteration in the peaks of these biomarkers of interest is gender-specific. The implication of this finding is not yet known. Because women are more susceptible to alcohol-induced end organ damage, it is possible that organ/tissue-specific proteins may be released more into the blood stream in female when cells are injured by alcohol than in male counterparts.

In summary, we have shown in this pilot study that several potential protein markers have appeared in mass spectral profiles and that they may be useful clinically to determine the status of alcohol drinking by MALDI –OTOF mass spectrometry. However, a large-scale study is needed to confirm and validate our current results.

Acknowledgment

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