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A Combinatorial Approach in Discrimination of Alcohol Use Disorders: Inclusion of Differentially Expressed Plasma Proteins*

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ABSTRACT

Alcohol use disorders are associated with a variety of medical, social, and economical problems worldwide. The diagnosis relies mainly on questionnaires, physical examination, clinical history, and biochemical markers. Alcohol or its metabolites may be responsible for changes in stability, structure and expression of proteins; therefore, proteomics may provide unique opportunities in the identification of novel biomarkers. The aim of this study was to compare the protein expression profile in plasma samples obtained from alcohol use disorder patients (n=30) with those of social drinkers (n=15) and nondrinkers (n=15) using two-dimensional gel electrophoresis, combined with mass spectrometric analysis to identify spots of interest and search for a potential combination of these proteins with some biochemical tests including carbohydrate deficient transferrin, total sialic acid, mean corpuscular volume, gamma-glutamyl transferase, alkaline phosphatase, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, and AST/ALT ratio. Discriminant analysis revealed that the combined use of all differentially expressed spots and biochemical tests revealed a sensitivity and specificity of 100%, while the combination of stepwise-selected 4 spots (identified as coagulation factor II, complement factor B, apolipoprotein E, latent human C1-inhibitor) and 5 known-markers (CDT, SA, MCV, GGT, AST/ALT) could classify 27 out of 30 alcohol use disorder patients correctly. This study shows that combining conventional markers with the spots of interest may be a strategy to increase sensitivity, since the conventional marker combination already has a specificity of 100%. Therefore, this combinatorial approach may be of use for classification studies; however further studies in larger samples are needed to test findings of this research.

Key words: Alcohol use disorders; proteomics; plasma protein expression; combinatorial biomarkers; two-dimensional gel electrophoresis; MALDI-TOF MS

Introduction

Alcohol use disorders (AUD) and associated medical, social, and economical problems remain as one of the major concerns worldwide. The related issues include cancer (oesophageal, liver), cirrhosis of the liver, epileptic seizures, homicide, and motor vehicle accidents. Diagnosis of AUD relies mainly on questionnaires, physical examination, clinical history and biochemical markers. Biomarkers to detect alcohol use disorders and to monitor the treatment progress have long been under investigation, since they provide objective information. There are several biomarkers including carbohydrate deficient transferrin (CDT), ethyl glucuronide(EtG), gamma-glutamyltransferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), mean corpuscular volume (MCV), and sialic acid (SA) [1].

Proteomics, the qualitative and quantitative comparison of proteome under different conditions to further unravel biological processes, has been one of the most promising tools in mining/hunting of biomarkers for certain diseases. Alcohol (or its metabolites) may alter protein expression, structure, and function, and these changes might be related to consequences of alcohol use disorders. The potential of proteomics in this field has been previously highlighted [2-4].

Two-dimensional gel electrophoresis (2-DE) combined with protein identification by mass

spectrometry continues as the workhorse for proteomics, although promising alternative/complementary technologies emerge [5]. Briefly, 2-DE attempts to purify or resolve proteins by differences in isoelectric points (pI) in the first dimension (isoelectric focusing, IEF), and then according to molecular weight (MW) in the second dimension (SDS-PAGE). Characterization of protein expression profile differences associated with disease conditions has been possible through recent advances in proteomics. Introduction of high resolution 2-DE of proteins by O'Farrell (1975) [6], enhanced resolution by the use of immobilized pH gradients(IPG) for IEF and last but not least the effective application of evolving/emerging mass spectrometry and databases, have been among the major factors in the advancement of proteomics.

The promising use of marker combinations in discriminating alcohol use disorder patients has been previously reported [7,8]. The objective of this study was to compare the protein expression profile in plasma samples obtained from alcohol use disorder (AUD) patients with those of social drinkers (SD) and nondrinkers (ND) using 2-DE, identify spots of interest through mass spectrometric analysis and search for a potential combination of some biochemical tests (CDT, SA, MCV, GGT, AST/ALT, LDH, ALP) with these identified proteins.

Materials and Methods

Sampling Procedures and Sample Preparation

The research protocol was approved by the Ethics Committee of Ankara Numune Hospital, studies were conducted according to the Helsinki Declaration of Human Experimentation, and informed consent was obtained from all participants. Individuals who were identified as positive for DSM-IV criteria were included in the analysis as the alcohol use disorder (AUD) patients. The patients were selected from individuals who applied for treatment in Alcohol and Drug Addiction Treatment and Research Center (AMATEM), Ankara, Turkey. The blood samples were collected from AUD patients (n=30), social drinkers (SD, n=15), and nondrinkers (ND, n=15) in evacuated K_2-EDTA blood tubes (for proteomics part) and in serum collection tubes (for CDT, SA, and other tests), centrifuged at 2500xg (4°C) within 2 hours, and plasma/serum were aliquotted in cryogenic vials and stored at -80°C until use. A reference group which only included "never drinkers" named as "nondrinkers" was formed in order to avoid the potential of including former drinkers who have abstained from alcohol due to poor health (the "sick quitter effect") [9]. All individuals were seronegative for HBV and HCV antibodies. Daily alcohol intake was assessed using a detailed questionnaire which obtains data including the amount and frequency of intake. The interview was uniformly conducted during whole study for all subjects. For the removal of top-20 most abundant proteins (albumin, IgG, IgA, IgM, IgD, transferrin, fibrinogen, α_2-macroglobulin, α_1-antitrypsin, haptoglobin, α_1-acid glycoprotein, ceruloplasmin, plasminogen, prealbumin, apolipoproteins A-I, A-II, B, complements C1q, C3, C4), a commercial depletion kit was used (PROT20, SIGMA). Each depleted plasma sample was collected in a centrifugal filter (Vivaspin 500, 3kDa MW cut off) for concentration and buffer exchange (with 50mM ammonium bicarbonate). The total protein concentrations of samples were estimated using BCA assay (BCA kit, SIGMA).

2D Gel Electrophoresis

All chemicals were "for proteomics use" where possible and in other cases, were of the highest grade commercially available. Expression difference analysis of depleted samples was performed using individual patient samples. The rehydration buffer (7M urea, 2M thiourea, 0.2%(v/v)ampholytes, 4%(w/v)CHAPS, 1%(v/v)HED, 1%(w/v) dithiothreitol and bromophenol blue) was prepared as described previously [10] except with a modification in concentration of urea. For each sample, the depleted, buffer-exchanged plasma sample (100µg protein per gel) and the rehydration buffer was mixed, sonicated for 5-10 seconds to increase solubility and then centrifuged. This mixture of sample and rehydration buffer was pipetted to focusing tray and active rehydration (50V for 12hrs, 20°C) of the IPG strips were performed in the IEF unit (PROTEAN IEF Cell, Bio-Rad). The first dimension (IEF) was run as recommended by the manufacturer (S1: 0-250V for 15 minutes, S2: 250-10000V for 2 hrs, S3:10000V for 4 ½ hrs). The electrode wicks were changed before the S3 step. For each sample, two different ranges of 17 cm IPG strips were used (pH 3-10 and pH 4-7, linear gradient). As soon as the IEF ended, the IPG strips were transferred to a clean rehydration tray and stored at -80°C. Before SDS-PAGE, the strips were equilibrated in the freshly prepared equilibration buffer (50mM TrisHCl pH8.8, 6M urea, 30% glycerol, 2% SDS) containing 1% dithiothreitol, and then in equilibration buffer containing 2.5% iodoacetamide. Both equilibration steps were performed for 15 minutes with gentle agitation on an orbital shaker at room temperature. After rinsing in SDS-PAGE electrophoresis buffer, each

IPG strip was carefully placed on top of the 1mm-thick 12.5% homogenous polyacrylamide gel cast in EttanDALT Gel Caster and sealed with 0.5% agarose solution. The SDS-PAGE was run using a vertical electrophoresis system (EttanDALTsix, GE Healthcare). The electrophoretic separation lasted for nearly 6 hrs at 20°C (1W/gel for 1hr, and then 13W/gel until the bromophenol blue reached the bottom of the gel). The Precision Plus Protein Standard unstained plugs (Bio-Rad) were used. As soon as the run ended, the 2D gels were removed from the gel cassettes, washed in 10%methanol, 7%acetic acid fixing solution for 30 minutes and stained overnight in Sypro Ruby protein gel stain. In order to decrease background fluorescence, the gels were washed in 10%methanol, 7%acetic acid solution for 30 minutes. Each step of the staining procedure was performed at room temperature, with gentle agitation and in plastic containers shielded from light. Digital images of ultrapure water-washed gels were captured using a 16-bit, cooled CCD camera (ImageQuant 350, GE Healthcare), and detailed automated gel image analysis was performed with a software (SameSpots, Nonlinear Dynamics, UK). The spots of interest were excised manually using clean pipette tips on a blue-light transilluminator (DarkReader Transilluminator, Clare Chemical Research).

Protein Identification by MALDI-TOF MS and MALDI-TOF/TOF tandem MS/MS

Protein identification by Mass Spectrometry was performed by Applied Biomics, Inc (Hayward, CA). Briefly, the gel spots were washed a few times then digested in-gel with modified porcine trypsin protease (Trypsin Gold, Promega). The tryptic peptides were desalted by Zip-tip C18 (Millipore) and were eluted from the Zip-tip with 0.5 µL of matrix solution (α-cyano-4-hydroxycinnamic acid (5 mg/mL in 50% acetonitrile, 0.1% trifluoroacetic acid, 25 mM ammonium bicarbonate) then spotted on the MALDI plate (model ABI 01-192-6-AB). The MALDI-TOF MS and TOF/TOF tandem MS/MS were performed on an ABI 4700 mass spectrometer (Applied Biosystems, Framingham, MA). MALDI-TOF mass spectra were acquired in reflectron positive ion mode, averaging 4000 laser shots per spectrum. TOF/TOF tandem MS fragmentation spectra were acquired for each sample, averaging 4000 laser shots per fragmentation spectrum on each of the 5 most abundant ions present in each sample (excluding trypsin autolytic peptides and other known background ions). Both of the resulting peptide mass and the associated fragmentation spectra were submitted to GPS Explorer workstation equipped with MASCOT search engine (Matrix science) to search the database of National Center for Biotechnology Information non-redundant (NCBInr). Searches were performed without constraining protein MW or pI, with variable carbamidomethylation of cysteine and oxidation of methionine residues, and with one missed cleavage also allowed in the search parameters. Candidates with either protein score C.I.% or Ion C.I.% greater than 95% were considered with high confidence.

Serum %CDT, Total Sialic Acid, and Other Tests

Separation of transferrin-isoforms in serum samples was performed using a commercially available HPLC kit (%CDT, Bio-Rad) as described by the manufacturer and an HPLC system (Agilent 1100 LC) equipped with a quaternary pump, degasser, column compartment (set at 35°C for analysis), automated sample injector, and a multiple wavelength detector. Relative amounts of each transferrin isoform to total transferrin were calculated as the percentage of peak area. Serum total sialic acid levels were determined using a commercially available colorimetric assay kit (Roche, Germany) and a microplate reader (SpectraMax190, Molecular Devices). Serum MCV, GGT, AST, ALT, LDH, and ALP were analyzed in an accredited laboratory (Düzen Laboratories, Ankara, Turkey).

Statistical Analysis

The normalized spot volumes were compared using SameSpots (Nonlinear Dynamics, UK) and the spots with a fold-change equal to or greater than 1.5 and statistically significant ($p<0.05$) ones were selected. All other statistical analyses were performed using SPSS 16.0 and results are expressed as mean±standard deviation. Values of the three groups (AUD, social drinkers and nondrinkers) were compared by ANOVA followed by post-hoc test (LSD or Dunnett's T). P-values less than 0.05 were considered to be statistically significant. Discriminant analysis was used as a classification technique. In a randomly selected set, the data were subjected to a stepwise discriminant analysis (Wilks' lambda) procedure to identify the variables which are capable of separating the AUD patients from ND and SD groups.

Results

Table 1 lists the characteristics of the patients. The age, BMI, and protein levels did not differ between groups, although a very slight gradual elevation was

Table 1. Demographic and clinical characteristics of study groups

Parameters	Groups		
	NonDrinkers (ND) (n=15)	Social Drinkers (SD) (n=15)	Alcohol Use Disorder (AUD) (n=30)
Age (years)	42.5±8	43.5±9	44.5±9
BMI (kg/m^2)	27.35±3.04	27.38±1.86	25.06±3.94
EDI (g alcohol/day)	-	28.3±8.4	216.5±96.6
Protein (g/L)	73.47±2.90	74.07±4.93	75.90±5.63
Carbohydrate deficient transferrin (CDT) (%)	1.08±0.38	1.26±0.54	6.66±6.35 ef
Total sialic acid (SA) (mg/dL)	64.27±2.03	65.32±1.76	71.44±4.26 ef
Mean corpuscular volume (MCV) (fL)	85.43±2.64	85.68±6.09	92.00±5.86 cd
γ-Glutamyl transferase (GGT) (IU/L)	28.00±17.46	25.07±5.95	217.67±235.16 cd
Aspartate aminotransferase (AST) (IU/L)	21.40±7.86	20.60±2.97	67.70±66.97 ab
Alanine aminotransferase (ALT) (IU/L)	28.73±20.12	26.73±10.34	44.93±34.54
AST/ALT	0.88±0.31	0.82±0.18	1.47±0.52 ef
Lactate dehydrogenase (LDH) (IU/L)	165.80±15.30	167.93±24.39	217.47±63.19 cd
Alkaline phosphatase (ALP) (IU/L)	68.00±14.82	73.80±15.70	84.57±24.64 a

Data are shown as mean±standard deviation.
[a] p<0.05 vs ND, [b] p<0.05 vs SD, [c] p<0.005 vs ND, [d] p<0.005 vs SD, [e] p<0.001 vs ND, [f] p<0.001 vs SD.

observed in the protein levels in the order of ND, SD, and AUD groups. The mean alcohol consumption in AUD group was 216.5±96.6 g alcohol/day, while the social drinkers consumed 28.3±8.4 g alcohol/day. Serum total sialic acid level, CDT, and AST/ALT ratio in AUD group were significantly elevated (p<0.001). Besides, GGT and LDH activities as well as MCV levels (p<0.005) and AST activity (p<0.05) were increased as compared with the reference groups. On the other hand, ALP activity was elevated as compared with only ND group (p<0.05). The ALT activity was increased in AUD patients as expected, however without any statistical significance. The range of AST/ALT ratio was between 0.62-2.48 in AUD group.

Figure 1 and Figure 2 illustrates the differentially expressed spots. The log-normalized volumes of three spots (spot#896,1000,1146) have been found to be lower in the AUD group as compared to ND and SD groups while two spots (spot#1021 and 1059) were up-regulated (Figure 2). Table 2 summarizes the identities of differentially-expressed spots as determined by mass spectrometric and database search as well as experimental pI/MW.

Discriminant analysis was conducted to determine if combinatorial use of differentially-expressed proteins and laboratory tests could discriminate the AUD patients from ND and SD groups. The fewest number of parameters required to obtain a reasonable classification matrix for classifying AUD patients correctly, a stepwise discriminant analysis (Wilks' Lambda) was performed and the following variables were selected: 1000, 1021, 1059, 1146, CDT, SA, MCV, GGT, AST/ALT. This combination of these selected parameters could classify 27 out of AUD patients correctly with a sensitivity of 90% (Table 3a), while the combined use of all parameters (896, 1000, 1021, 1059, 1146 + CDT, SA, MCV, GGT, AST/ALT, LDH, ALP) revealed a sensitivity and specificity of 100%, classifying all AUD patients correctly (Table 3b).

Discussion

Alcohol-related issues represent a dual character, being either beneficial through light/moderate consumption (e.g. in cardiovascular system) or detrimental through excessive, uncontrolled drinking behavior. Alcohol (and/or its metabolites) may be responsible for changes in protein structure, stability, and expression level. It has long been known that protein metabolism is susceptible to the impact of alcohol both at the tissue-specific and whole body

Table 2. Differentially-expressed proteins identified using mass spectrometry

Spot no.	Top Ranked Protein Name	NCBI Accession No.	Expected	Experimental*	Peptide Count	Protein Score (C.I. %)**	Total Ion Score (C.I. %)
			pI /MW (kDa)	pI /MW (kDa)			
896	hemopexin precursor	gi|386789	6.57/51.5122	6.65/51	12	114 (100)	26 (-)
1000	coagulation factor II (thrombin), isoform CRA_a	gi|119588383	5.64/69.2087	5.11/95	21	272 (100)	108 (100)
1021	Chain A, Human Complement Factor B	gi|134105218	6.81/84.2718	6.68/125	15	157 (100)	76 (100)
1059	apolipoprotein E	gi|178853	5.81/36.1849	5.57/36	18	500 (100)	303 (100)
1146	Chain A, Crystal Structure Of Latent Human C1-Inhibitor	gi|146386605	7.40/43.8226	5.79/108	13	204 (100)	103 (100)

* Experimental pI and MW were estimated from 2D-gel analyses by the software (SameSpots).
** Proteins identified with a protein score C.I.% or Ion C.I.% greater than 95%, details as depicted in the Materials and Methods section.

levels [11]. Proteomics- a rapidly evolving and challenging area, dealing with systematic, large scale analysis of proteins [12] may provide unique opportunities in this field. The protein expression differences related to alcohol use and alcohol use disorders have been investigated in several biological samples, including human serum [13-16], non-human primate (monkey) plasma [17], human brain [18], mouse brain [19], and rat liver samples [20].

The present study has focused on the comparison

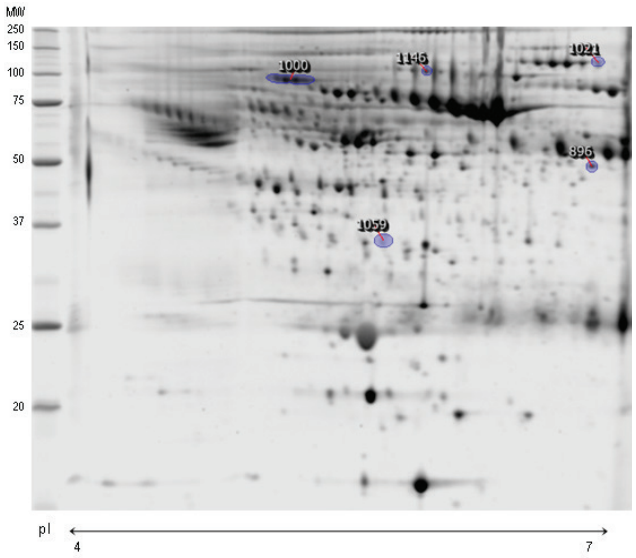

Figure 1. Representative 2D-gel image of a depleted human plasma sample. The IPG strip (pH 4-7, 17cm, linear) was loaded 100µg of protein. Following IEF (IEF Cell, Bio-Rad), and SDS-PAGE separation on a 12.5% homogenous gel (EttanDaltSix, GE Healthcare), Sypro Ruby stained gels were analyzed using SameSpots (Nonlinear Dynamics, UK). The numbers shown are automatically assigned spot numbers by the gel analysis software. Other experimental details are given in Materials and Methods section.

of the protein expression profile in plasma samples obtained from alcohol use disorder patients (n=30) with those of nondrinkers (n=15) and social drinkers (n=15) using two-dimensional gel electrophoresis (Figure 1), combined with mass spectrometric analysis to identify spots of interest (Table 2) and search for a potential combination of these proteins with some biochemical tests (Table 3). Blood plasma contains high abundance proteins that perform various housekeeping functions as well as secreted or shed low abundance proteins critical for signaling cascades and regulatory events; the presence of these components in blood reinforces the benefits of using a proteomic approach for identifying biomarkers [21]. Depletion of high abundance proteins to reveal the low abundant ones has become routine; however it must be borne in mind that this advantage may be at the expense of loss or under-representation of potential markers as a consequence of non-specific binding to depletion medium or through the possible "sponge" effect of high abundance proteins [22]. Parallel use of both depleted and undepleted samples may be of use in this regard. Expression profiles of the undepleted plasma were compared in two separate pools in our laboratory: AUD samples were pooled and named as "pool AUD", while samples obtained from nondrinkers and social drinkers were pooled in the group "pool SC". Each pool was run in triplicate and expression profiles were compared. The pool AUD patients differed from the pool SC, as evident with an increase in spot volumes of some high abundant proteins including haptoglobin, transthyretin, alpha-1-antitrypsin, alpha-1-acid-glycoprotein, and Apo AIV while a fragment of C1-inhitor (C1-INH) was down-regulated (unpublished results). One of the earliest studies regarding

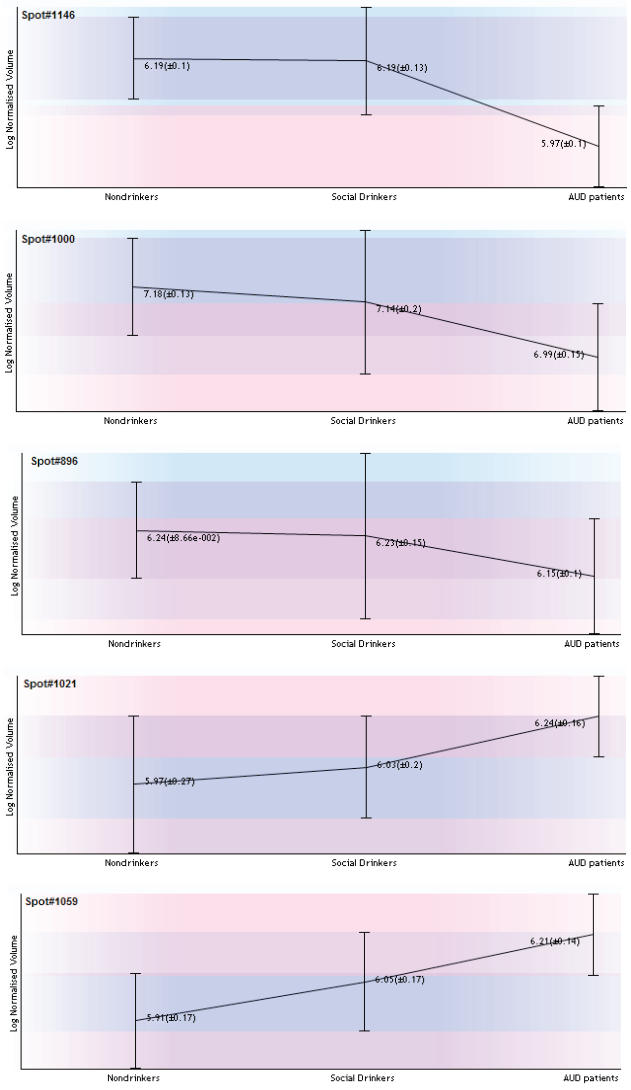

Figure 2. Normalised volumes of differentially-expressed spots of ND, SD, and AUD groups. The numbers given at the upper left side are the automatically assigned spot numbers by the gel analysis software (SameSpots, Nonlinear Dynamics, UK) and the identities of the spots are shown in Table 2.

application of 2-DE on alcohol-associated changes in human serum protein patterns reported by Marshall et al. (1984) [23] has revealed consistently elevated levels of alpha-1-acid glycoprotein, while in most of the samples, increase of IgA, alpha-1-antichymotrypsin, haptoglobins and apo A-I as well as decrease in the levels of antithrombin III have been observed. Results of an experimental design using difference gel electrophoresis has been reported by Nomura et al. (2007), and 8-spots were found to be differentially expressed before and after abstinence [4]: The proteins of decreased expression after abstinence were reported as complement component I inhibitor precursor, alpha-1-antichymotrypsin, alpha-2-HS-glycoprotein, alpha-1-acid glycoprotein-1 precursor, alpha-1-microglobulin/bikunin precursor, and Ig kappa chain C region whereas the expression of two proteins (ApoJ and haptoglobin) were increased after abstinence.

In the depleted individual plasma samples, as shown in Figure 2, the spot volumes of #896, #1000, and #1146 were decreased in AUD group. Decrease in the expression of hemopexin (Spot#896), a glycoprotein well-known for its heme-binding properties may reflect a recent release of heme compounds in the extracellular compartment [24]. A confirming study by Lai et al. (2009) has reported a significant increase of hemopexin after 6-weeks of abstinence [15]. The normalized spot volume of spot#1000 (coagulation factor II) was also significantly reduced in AUD group, as compared with ND and SD. Effect of chronic heavy alcohol use on coagulation appears to be complex: On the one hand, bleeding complications are more frequent among AUD patients, and these individuals may become susceptible to peri-/post-operative complications e.g. prolonged bleeding time [25, 26], on the other hand alcohol withdrawal in AUD may result in a rebound effect, increased response to aggregation especially induced by thrombin which may explain sudden cardiovascular events [27]. It is known that uncontrollable hemorrhage due to inadequate synthesis of clotting factors is a fatal complication of alcoholic cirrhosis [28]. Beside severe alcoholic liver disease resulting in impaired synthesis of hemostatic factors, thrombocyte function and thrombopoiesis are also affected [26]. Another down-regulated spot was spot#1146 (identified as C1-INH) which is a serine protease inhibitor which inactivates several different proteases, through binding and blocking the activity of its target by the suicide mechanism [29]. The observed molecular weight of this spot has been 108kDa (Table 2), in previous reports it has been reported that C1-INH has been found in SDS-PAGE as a glycosylated popypeptide of apparent Mr 105-115 kDa [30], usually as 105kDa [31]. C1-INH controls the complement system through the classical pathway by binding to C1 complex and iniating the diffusion of the fragments, C1r and C1s or through lectin pathway by inactivation of MASP-1 and MASP-2 [32, 33]. Other physiologically relevant targets include the contact system elements (factor XII and kallikrein), although no changes in plasma levels of the components of this system are detectable in deficient patients, signs of hyperactivation are clearly present when these patients develop angioedema. In

addition to the control of complement and contact systems activation, C1-INH inhibits factor XI, plasmin and tissue plasminogen activator [34]. It plays important roles in the regulation of vascular permeability and in the suppression of inflammation [35], and can be reduced in severe inflammation [36].

The spot volumes of #1021 and #1059 have been found higher in AUD patients (Figure 2). It is of interest that while spot #1021 identified as human complement factor B was increased in patients, adjacent isoforms did not reveal a significant change (data not shown). Bykov et al. (2007) [37] have reported an increase in the relative mRNA expression of some complement factors including Complement Factor B in livers of C3-deficient and wild-type mice as a result of chronic alcohol feeding. In an experimental model including C1q-deficient and wild-type mice, it has been shown that alcohol activates the classical complement pathway via C1q binding to apoptotic cells in liver tissue and suggested that C1q contributes to the pathogenesis of alcohol-induced liver injury [38]. However, the complement system has been implicated in several liver diseases [39]. Another spot with an elevated expression has been identified as ApoE (#1059) which revealed a gradual increase, in the order of ND, SD and AUD groups (Figure 2). Liappas et al., 2007 suggested ApoE has been as a marker of prolonged alcohol consumption, based on the findings of reduced levels of this apolipoprotein during treatment period, and the correlation between alcohol consumption and ApoE on admission and discharge [40]. On the other hand, it has been found that the serum high-density lipoprotein (HDL) particles of alcohol-fed rats were deficient in apolipoprotein E [41], and desialylation of apoE decreases its binding to HDL, leading to an impaired reverse cholesterol transport [42]. However, contradictory results on the alcohol-related change of serum ApoE also exists [43].

The transferrin isoforms (glycoforms) collectively referred as CDT, include the isoforms asialo-, monosialo-, and disialo-transferrin [44]. In the present study, %CDT was determined using an HPLC test evaluated previously [45], and it has been found significantly higher in AUD group as compared with the SD and ND groups (Table 1), in accordance with Schellenberg et al. (2008) [46]. Glycosylated proteins which may be specifically prone to the chronic effects of alcohol, as in the example of transferrin, may be potential targets in search of biomarkers. Spots #1021 and #1059 are glycoproteins rich-in sialic acid, and it may be relevant to investigate the expression profile of their isoforms. In this context, Finehout et al. (2005) investigated the expression levels of some complement proteins, including complement factor B, in cerebrospinal fluid as possible biomarkers for neurodegenerative diseases, and suggested that individual protein isoforms may offer potential targets to gain further insight on disease biomarkers [47]. Chrostek et al. (2007) found that the serum level of total sialic acid was correlated with the concentration of some sialylated glycoproteins (α1-antitrypsin, α1-acid glycoprotein, haptoglobin, ceruloplasmin, complement C3, and fibrinogen) in the sera of alcoholics [48]. Sialic acids occur mainly at terminal positions of glycoprotein and glycolipid oligosaccharide side-chains [49]. As shown in Table 1, serum total sialic acid levels were elevated in AUD group as compared with SD, and ND groups in line with previous reports [49, 50]. However, elevated SA concentrations may also be associated with some other conditions (e.g. cardiovascular diseases, inflammation, and cancer) which hamper the clinical use as a marker of alcohol use disorders.

The potential use of marker combinations in discriminating alcohol use disorder patients has been previously described [7, 51], which becomes particularly important when self-report/screening questionnaires may be misleading or a single test may be limited. Several combinations including CDT-GGT [8, 52], CDT-MCV-GGT [53] have been investigated. In the present study, the parameters including CDT, SA, MCV, GGT, AST/ALT, LDH, and ALP along with 5 spots of interest (#896, 1000, 1021, 1059, 1146) were evaluated using the discriminant analysis which allows building a predictive model for group membership. The combination of stepwise-selected spots (#1000, 1021, 1059, and 1146) and conventional markers (CDT, SA, MCV, GGT, AST/ALT) could classify 27 out of 30 AUD patients correctly with 90%sensitivity (Table 3), while of all cases 83.3% (50 out of 60) were correctly classified. The combination of all spots (896, 1000, 1021, 1059, 1146) and other parameters (CDT, SA, MCV, GGT, AST/ALT, LDH, ALP) could classify all patients correctly with a sensitivity and specificity of 100% (Table 3). A possible explanation may be the complementary properties of the parameters. Classifier performance summary of two representative combinations are shown in Table 3a and 3b; none of the nondrinkers and social drinkers were classified as AUD patients, on the other hand, 3 individuals from ND group were

Table 3. Classifier performance summary of two representative combinations
(a)(1000, 1021, 1059, 1146 + CDT, SA, MCV, GGT, AST/ALT),
(b) (896, 1000, 1021, 1059, 1146 + CDT, SA, MCV, GGT, AST/ALT, LDH, ALP)

(a)		Predicted Group Membership			
		ND	SD	AUD	Total
Original Class	ND	12	3	0	15
	SD	4	11	0	15
	AUD	1	2	27	30

(b)		Predicted Group Membership			
		ND	SD	AUD	Total
Original Class	ND	12	3	0	15
	SD	4	11	0	15
	AUD	0	0	30	30

misclassified as SD and 4 SD were erranously predicted as ND. In previous reports in this field, a 5.9kDa peak identified as a fragment of fibrinogen has been suggested as a potential marker [4, 13, 16], and it has been shown that a combination of 5.9kDa peak with GGT revealed a sensitivity of 96.8% and specificity of 82.8% [54]. More recently, Freeman et al. (2010) have reported in a non-human primate model system, that a three-protein biomarker set for detection of alcohol abuse (100% sensitivity, 92% accuracy) and a 14-protein panel for detection of alcohol-use (95% sensitivity, 88% accuracy) performed well, and suggested that a combined set of 17-plasma protein panel correctly classified abusive drinking with 100% sensitivity, differentiating any level of drinking from alcohol abstinence with 88% accuracy [17]. Another point to be discussed is the inclusion of spot #896 (hemopexin) in the discriminant analysis. When this spot is included to the analysis panel, the total number of correctly classified cases are 51 resulting in a slight increase of sensitivity in AUD group (from 90% to 93.3%, data not shown).

In the gel-based proteomics practices, no "one-size-fits-all" recipe for either IEF or SDS-PAGE exists yet, therefore almost every step needs to be tested and optimized for each specific sample type. There are limitations to our study that warrant discussion, including the sample size; however, the study was designed mainly for comparison of the alcohol use disorder patients with social drinkers and non-drinking controls on the basis of plasma protein expression profile comparison as an initial part of a broader project. Additional studies in larger samples are needed to test/replicate the findings of this research especially in which gender differences are taken into account. Additionally, it would be a viable strategy to re-evaluate items using stepwise discriminant analysis for the optimal combination of markers. The present spots of interest should be investigated in detail especially with regard to isoforms. Our future work is designed to focus on these issues as well as western blotting. This study shows that combining conventional markers with the spots of interest may be a strategy to improve the sensitivity, since the conventional marker combination already has a specificity of 100%. It should also be emphasized that this combinatorial approach did not result in loss of the specificity. These approaches, aside from satisfying scientific curiosity, may provide greater insight into some of the processes at least in part and open the door for future discoveries. The application of proteomics, despite laborious work requirement, holds great potential in understanding of the mechanisms underlying substance use disorders, and -omics including proteomics offer opportunities to discover a new generation of biomarkers. Numerous studies have been conducted in experimental models as well as in human; however it seems that there is way to discover pieces of the "alcohol use disorders" puzzle, more to explore the whole scene and interactions therein, and help to build a reliable test panel to be used in conjunction with clinical interventions.

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