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ORIGINAL ARTICLE

Differentially Altered Plasma Proteins in Patients diagnosed with Alcoholic and Nonalcoholic Fatty Liver Disease

[1]Kamlesh K Bhopale, [1]Dhananjaya Nauduri, [2]Kizhake V Soman, [3]Gagan K Sood, [1]Anthony Okorodudu
[1]GAS Ansari, [1]Bhupendra S Kaphalia

[1]Department of Pathology, The University of Texas Medical Branch, Galveston, Texas, USA
[2]Department of Biochemistry and Molecular Biology, UTMB NHBLI Proteomic Center
The University of Texas Medical Branch, Galveston, Texas, USA
[3]Baylor College of Medicine, Houston, Texas, USA

Correspondence: Bhupendra S Kaphalia, Professor, Department of Pathology, The University of Texas Medical Branch, Galveston Texas-77555-0438, USA, Phone: 409-772-4995, Fax: 409-747-1763, e-mail: bkaphali@utmb.edu

ABSTRACT

Alcoholic liver disease (ALD) and nonalcoholic fatty liver disease (NAFLD) are major health problems. Although both diseases follow a similar course of disease progression from an early reversible fatty liver stage to severe necroinflammation with or without fibrosis and cirrhosis, the mechanisms of ALD and NAFLD are not well understood. This preliminary study was conducted to determine blood alcohol concentration (BAC) and altered proteins and fatty acid ethyl esters (FAEEs, nonoxidative metabolites of ethanol) in the plasma of patients diagnosed with ALD or NAFLD. Differentially altered proteins were identified by two-dimensional gel electrophoresis and mass spectrometry, FAEEs by gas chromatography and BAC by an enzymatic method. Precursors of complement C3, complement C6 and serum amyloid A-4 protein, and Ig gamma-3 chain C region were significantly decreased in ALD *vs* controls (group 1). Haptoglobin precursor was the only altered protein (increased) in NAFLD *vs* controls (group 2). However, significantly increased levels of precursors of complements C3, C4-B and C6 and decreased levels of immunoglobulin J chain and Leucine-rich alpha-2 glycoprotein precursor were observed in NAFLD *vs* ALD (group 3). These results indicate potential of precursors of complements C3 and C6 and haptoglobin for the differential diagnosis of ALD and NAFLD. Although high BAC and plasma FAEEs levels in the ALD group appear to be associated with chronic alcohol abuse, biological significance of endogenous biosynthesis of FAEEs yet to be established. Therefore, a detailed proteomic and lipidomic study need to be conducted in larger cohorts to understand the etiopathogenesis of ALD and NAFLD.

Keywords: Alcoholic liver disease, Nonalcoholic fatty liver disease, Fatty acid ethyl esters, Complements.

INTRODUCTION

Alcoholic liver disease (ALD) and nonalcoholic fatty liver disease (NAFLD) both causing high morbidity and mortality are major health problems.[1-4] An entire pathological spectrum of the fatty liver disease encompasses from an early steatosis stage, which is considered to be a reversible condition to necroinflammation with or without progressive fibrosis and cirrhosis.[2,3] The risk of developing cirrhosis has been shown to increase stepwise with degree of fatty liver (steatosis).[5] However, irrespective of different etiologies, ALD and NAFLD follow a same course of disease progression—fatty liver→inflammation→fibrosis → cirrhosis. It is, therefore, important to determine the molecular basis of ALD and NAFLD and identify biomarkers for early diagnosis and develop preventive measures.

The etiologies of ALD and NAFLD are distinguishable as former is caused by chronic alcohol abuse and later one by insulin resistance and/or metabolic syndrome.[1,2] An accumulation of lipids in the livers resulting into progressive NAFLD ranges from 17 to 33% in Western population.[1] Obesity contributes to diabetes and high blood cholesterol and has dramatically increased in the US during the past 20 years. Such condition can further complicate NAFLD; a more common disease due to growing number of obese children worldwide. In the last 10 years, the rate of obesity has doubled in adults and tripled in children population.[6] Colorado was the only state with < 20% obesity prevalence in the US. An obesity prevalence of ≥ 25% is reported in 32 states with six of the states (Alabama, Mississippi, Oklahoma, South Carolina, Tennessee and West Virginia) had equal to or greater than 30% prevalence (http://www.cdc.gov/obesity/data/trends.html).

The plasma proteome and metabolome, which reflect metabolic changes and toxic insults occurring in the livers, can be utilized to identify potential biomarkers and toxic pathways involved in ALD and NAFLD. Blood alcohol concentration (BAC) and lipid metabolites of ethanol, such as fatty acid ethyl esters (FAEEs; nonoxidative metabolites

of ethanol) can be used to diagnose acute *vs* chronic alcohol abuse.[7] We have also demonstrated metabolic basis of ethanol-induced liver and pancreatic injury in hepatic alcohol dehydrogenase-deficient deer mice fed ethanol via Lieber-DeCarli liquid diet.[8,9] Recently, we and others have investigated whole hepatic lipidome and proteome associated with progression of ethanol-induced fatty liver using lipidomic and proteomic approaches.[10,11] In order to understand the pathogenesis and identify potential biomarker(s) of ALD and NAFLD, we determined differentially altered proteins and measured various FAEEs in the plasma of patients diagnosed with ALD or NAFLD.

MATERIALS AND METHODS

Chemicals and Reagents

Unless indicated, all chemicals and reagents and fatty acid ethyl ester (FAEE) standards were obtained from Sigma-Aldrich Company (St Louis, MO).

Sample Collection

Blood was collected in plasma preparation tubes from adult patients clinically diagnosed with ALD or NAFLD at UTMB's Liver Transplant Center. ALD and NAFLD were diagnosed based on abnormal liver enzymes, negative markers of viral hepatitis B and C, autoimmune hepatitis, Wilson disease, hemochromatosis and α-1 antitrypsin deficiency and ultrasound or histopathological evidence of fatty liver along with a history of > 40 g/day alcohol intake for ALD and < 20 gm/week for NAFLD. Based upon the above diagnostic parameters, patients selected for the analysis of differentially altered plasma proteins fell in the category of fatty liver with necroinflammation. However, this is a pilot study to identify plasma proteins and lipid metabolite biomarkers in patients diagnosed with ALD or NAFLD. The age and gender of the patients diagnosed with ALD or NAFLD and healthy controls, and BAC are summarized in Table 1. In this pilot study, we identified differentially altered plasma proteins, BAC and total plasma lipids including FAEEs in a limited number of patients diagnosed with ALD or NAFLD. Plasma of healthy subjects without any known disease including ALD or NAFLD was used as control.

Proteomic Studies

Proteomics, a high throughput technique allowing simultaneous identification of several proteins, was performed by two-dimensional gel electrophoresis (2-DGE) and mass spectrometry (MALDI TOF/TOF), at UTMB's NHBLI Proteomics Center. A major problem in separation and identification of low abundant proteins by 2-DGE is the presence of high concentration of albumin and IgG in the plasma/serum.[12] In order to enhance the detection of low abundant plasma proteins and increase the sensitivity of the method, albumin and IgG were removed from the plasma by ProteoExtract™ Albumin/IgG removal kit (Cat. No.122642, Calbiochem, San Diego, CA) as per manufacturer's instructions. The proteins were precipitated using ProteoExtract™ Protein Precipitation kit (Cat. No. 539180, Calbiochem), concentrated to a desired volume and subjected to 2-DGE.[13] In brief, isoelectric focusing (IEF) of the plasma proteins was performed with 11 cm long precast IPG strip (pH 3 to 10). A 200 µl samples (200 µg protein) was loaded on to the gel and IEF performed. The strip was placed at the bottom of 11 × 10 cm gel for the second dimension. After completing the run, the gel was fixed (10% methanol, 7% acetic acid in double distilled water). The gel was stained with Sypro-Ruby (Bio-Rad) followed by destaining in the fixing buffer for imaging.

The images were analyzed with Progenesis Discovery software version 2.0 (now known as Samespots; Nonlinear Dynamics, UK). One of the images in the analysis was selected by the program as the reference gel and the other images were aligned to it. The program then performed spot detection on the aligned set of gels and background subtraction. Spot volumes on all gels were normalized to those of the reference gel to enable comparison between sample groups (control, ALD and NAFLD). Differentially expressed ($p \leq 0.05$ and fold-change ≥ 2.0) proteins were

Table 1: BAC in controls and patients diagnosed with ALD and NAFLD [M-male; F-female, age (yrs)]

Groups	*Sex/age*	*BAC (mg%)*
Control	M/48	—
Control	M/32	—
Control	M/53	—
Control	M/41	—
Control	M/45	—
Control	M/55	—
ALD	M/66	309
ALD	M/43	192
ALD	M/45	44
ALD	M/40	191
ALD	M/29	283
ALD	F/61	263
ALD	F/36	488
NAFLD	M/29	<15
NAFLD	M/28	<15
NAFLD	M/35	<15
NAFLD	M/34	<15
NAFLD	M/62	<15
NAFLD	M/52	<15

digested by trypsin and analyzed by mass spectrometry (MALDI TOF/TOF, Applied Biosystems Voyager Model DE STR) using peptide mass fingerprinting followed by database search (MASCOT/SwissProt). Proteins matching the tryptic digestion patterns were assigned an expectation score, E, representing the probability that the match has not occurred by chance. Lists of proteins with significant expectation scores (i.e. E < 0.001) were compiled and tabulated along with their calculated isoelectric points, molecular weights and percent protein coverage (Tables 2 to 4).

BAC

An enzymatic method was used to analyze BAC as described earlier using Vitros 5, 1 FS Chemistry System (Ortho Clinical Diagnostics, Johnson and Johnson Co, Rochester, NY) in UTMB's Clinical Chemistry Division.[7] This reflectance spectrometry method measured the reduced form of nicotinamide adenine dinucleotide formed during oxidation of ethanol to acetaldehyde by alcohol dehydrogenase.

Plasma Lipids and FAEEs

Plasma lipids and FAEEs were determined as described earlier.[7,8] In brief, total lipids were extracted from 1 ml plasma, dried under gentle stream of nitrogen and the dry weight determined. The extracted lipids were dissolved in small amount of chloroform (0.1 ml) and subjected to solid phase extraction using silica gel SepPak cartridge to obtain the neutral and phospholipids fractions. The neutral lipid fraction was separated by thin layer chromatography. The ester fraction was analyzed by gas chromatography (GC) and GC-mass spectrometry (GC-MS). The data for individual FAEE are corrected for the percent recovery.

Statistical Analysis

The data sets were analyzed for statistical significance using Tukey-Kramer multiple comparison and one way ANOVA. The results are expressed as mean ± SEM (standard error of mean).

RESULTS

Key findings in this study are decreased levels of precursors of C3 and C6 complements in ALD (group 1) and increased levels of haptoglobin precursor in NAFLD (group 2).

Table 2: Differentially altered proteins in the plasma of patients diagnosed with ALD *vs* controls

Spot no.	*Protein name*	*Swiss Prot Acc no.*	*pI*	*MW kDa*	*Protein score*	*Expectation value*	*Protein levels (fold change)*	*Biological functions*
3349	Complement C3 precursor	P01024 CO3_HUMAN	6.02	188.6	245	3.97 E-21	(– 3.07)	Activation of the complement system and mediator of local inflammatory process
3369	Complement C3 precursor	P01024 CO3_HUMAN	6.02	188.6	222	7.93 E-19	(– 3.72)	Activation of the complement system and mediator of local inflammatory process
3519	Complement C3 precursor	P01024 CO3_HUMAN	6.02	188.6	349	1.58 E-31	(– 3.16)	Activation of the complement system and mediator of local inflammatory process
3521	Complement C3 precursor	P01024 CO3_HUMAN	6.02	188.6	303	6.30 E-27	(– 2.33)	Activation of the complement system and mediator of local inflammatory process
3522	Complement C3 precursor	P01024 CO3_HUMAN	6.02	188.6	505	3.97 E-47	(– 2.99)	Activation of the complement system and mediator of local inflammatory process
2339	Complement Component C6 precursor	P013671 CO6_HUMAN	6.31	108.4	244	5E-21	(– 2.24)	Formation of the lytic C5b_9m complex
1663	Ig gamma-3 chain C region (heavy chain disease proteins HDC)	P01860 IGHG3_HUMAN	7.89	33.2	154	5E-12	(– 2.54)	—
3577	Serum amyloid A-4 protein precursor	P35542 SAA4_HUMAN	9.27	14.8	118	1.99 E.08	(– 2.92)	Major acute phase reactant apolipoprotein of HDL complex

pI: Isoelectric point; MW: Molecular weight, n = 3

As expected, significantly high BAC and levels of FAEEs were found only in the ALD group. Total lipids were higher in both groups *vs* controls, but the mean value was statistically significant only in the NAFLD group. Our findings from proteomic studies suggest that innate and acquired immunity could be significantly compromised in ALD *vs* NAFLD. Detailed results are described below:

Differentially Altered Plasma Proteins

We found multiple spots of differentially altered proteins in all the groups (Tables 2 to 4). Out of a total of 39 proteins detected in all the groups, only a few proteins were significantly altered in the plasma of patients in group 1 (ALD *vs* control, Figs 1A to C; Table 2), group 2 (NAFLD

Table 3: Differentially altered proteins in the plasma of patients diagnosed with NAFLD *vs* controls

Spot no.	*Protein name*	*Swiss Prot Acc no.*	*pI*	*MW (kDa)*	*Protein score*	*Expectation value*	*Protein levels (fold change)*	*Biological functions*
3365	Haptoglobin precursor	P00738 HPT_HUMAN	6.13	45.9	137	2.51 E-10	(2.28)	Protecting kidneys from damage by hemoglobin
3625	Haptoglobin precursor	P00738 HPT_HUMAN	6.13	45.9	184	5.0 E+15	(2.12)	Protecting kidneys from damage by hemoglobin
3728	Haptoglobin precursor	P00738 HPT_HUMAN	6.13	45.9	130	1.26 E-09	(2.15)	Protecting kidneys from damage by hemoglobin
3730	Haptoglobin precursor	P00738 HPT_HUMAN	6.13	45.9	96	3.16 E-06	(2.48)	Protecting kidneys from damage by hemoglobin

pI: Isoelectric point; MW: Molecular weight, n = 3

Table 4: Differentially altered proteins in the plasma of patients diagnosed with NAFLD *vs* ALD

Spot no. NAFLD	*Protein name*	*Swiss Prot Acc no.*	*pI*	*MW (kDa)*	*Protein score*	*Expectation value*	*Protein levels (fold change)*	*Biological functions*
3734	Complement C3 precursor	P01024 CO3_HUMAN	6.02	18.86	76	3.16 E-04	(2.30)	C3 plays central role in activation of complement system and local inflammatory process
2779	Complement C4-B precursor	P0C0L5 C04B_HUMAN	6.73	19.42	77	7.92 E-09	(2.17)	Activation of classical pathway of complementary system, mediator of local inflammatory process
3197	Complement C4-B precursor	P0C0L5 C04B_HUMAN	6.73	19.42	133	6.30 E-10	(2.30)	Activation of classical pathway of complementary system, mediator of local inflammatory process
3200	Complement C4-B precursor	P0C0L5 C04B_HUMAN	6.73	19.42	110	1.26 E-07	(2.23)	Activation of classical pathway of complementary system, mediator of local inflammatory process
3029	Complement C4-B precursor	P0C0L5 C04B_HUMAN	6.73	19.42	116	3.16 E-08	(2.21)	Activation of classical pathway of complementary system, mediator of local inflammatory process
2341	Complement component C6 precursor	P13671 C06_HUMUN	6.31	10.84	494	5.0 E-46	(2.07)	Involved in formation of lytic C5b_9m complex
2049	Immunoglobulin J chain	P01591 IGJ_HUMAN	4.62	16.04	141	9.98 E-11	(–2.04)	Serves to link to monomer units of either IgM or IgA
2723	Leucine-rich alpha-2 glycoprotein precursor	P02750 A2GL_HUMAN	6.45	38.38	412	7.93 E-38	(–2.04)	—

pI: Isoelectric point; MW: Molecular weight, n = 3

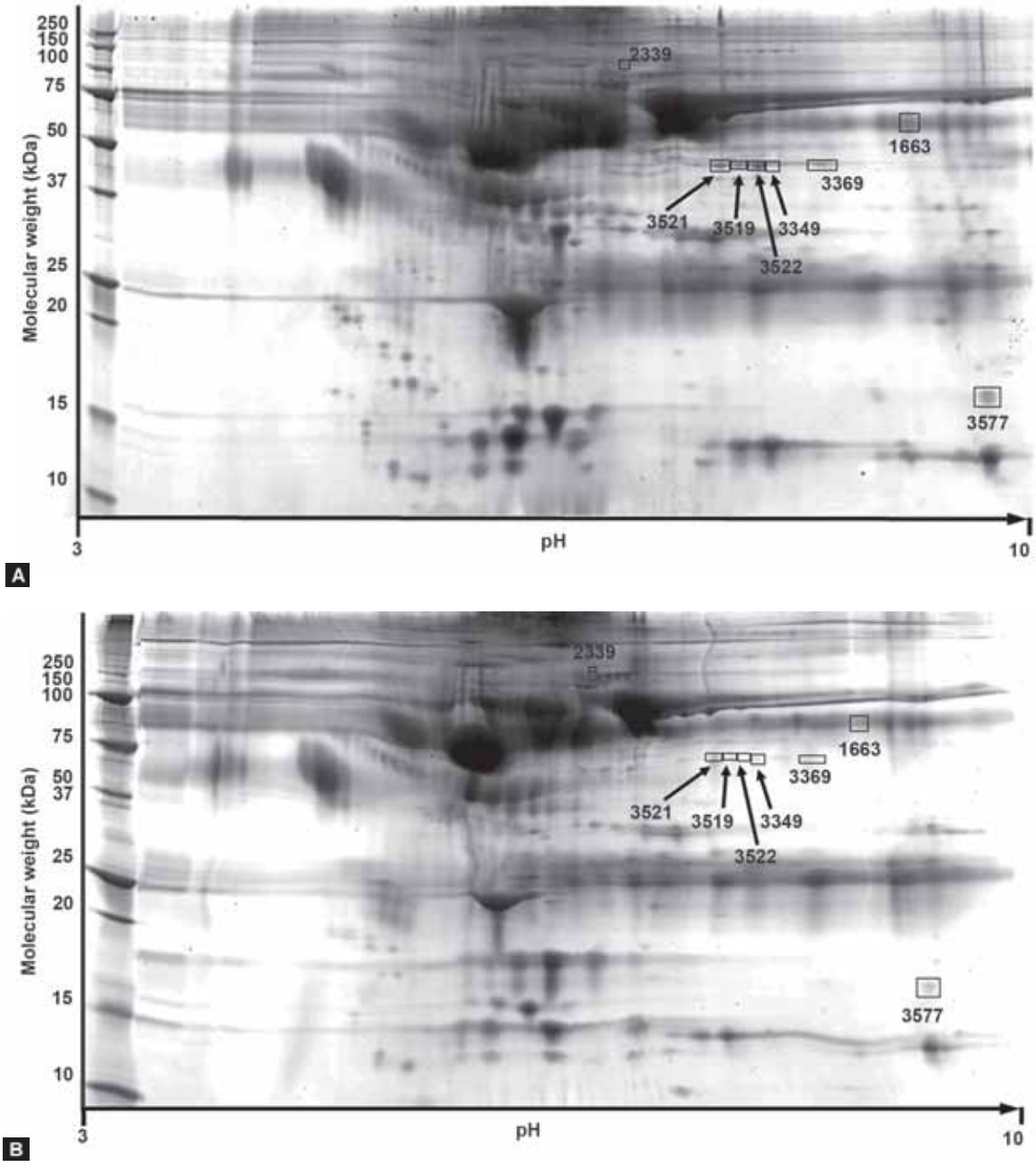
vs control, Figs 2A to C; Table 3) and group 3 (NAFLD *vs* ALD, Figs 3A to C; Table 4). In group 1, precursors of complement C3 and complement C6, Ig gamma-3 chain C region and serum amyloid A-4 protein precursor were all significantly decreased (Table 2). In contrast, only haptoglobin precursor showed significantly higher levels in patients with NAFLD (Table 3). NAFLD in group 3 showed elevated levels of precursors of complements C3, C4-B, C6 and lower levels of immunoglobulin J chain and leucine-rich alpha-2 glycoprotein precursor (Table 4). However, changes in mature form of complements could not be detected by our proteomic studies. Despite of similarities between ALD and NAFLD regarding the pathogenesis and disease progressions, our proteomic results indicate significant differences between ALD and NAFLD.

BAC

BAC averaged 253 mg% with a standard error of the mean of 51 in patients diagnosed with ALD as compared with < 15 mg% in all the patients of NAFLD group (Table 1). Surprisingly, one patient with ALD showed relatively low BAC (44 mg%). High BAC in ALD group could be a signature of acute alcohol abuse/ingestion.

Plasma Lipids and FAEEs

Total plasma lipids averaged 3.86, 5.81 and 8.35 mg/ml in control, ALD and NAFLD groups, respectively. The mean values of the total lipids increased to ~150 and 215% in ALD and NAFLD groups, respectively, than controls (Fig. 4). However, the increases in total lipids were



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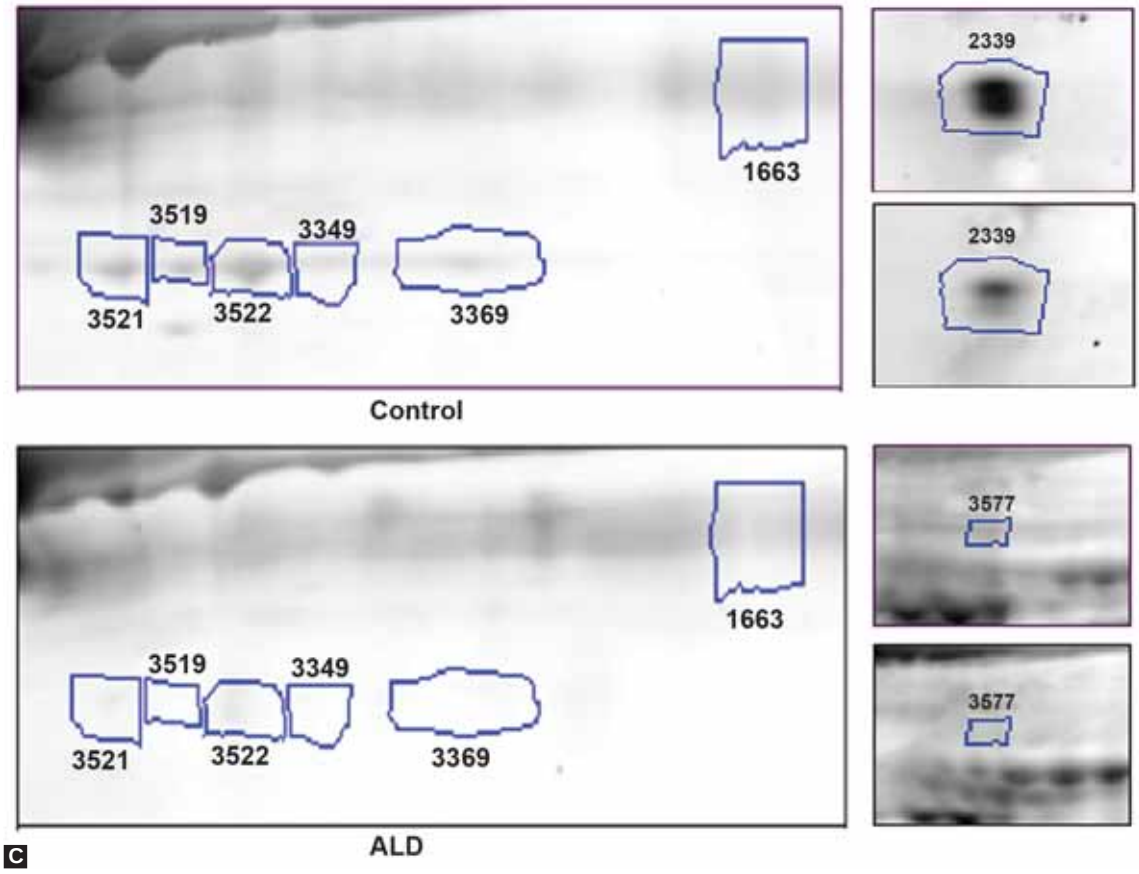


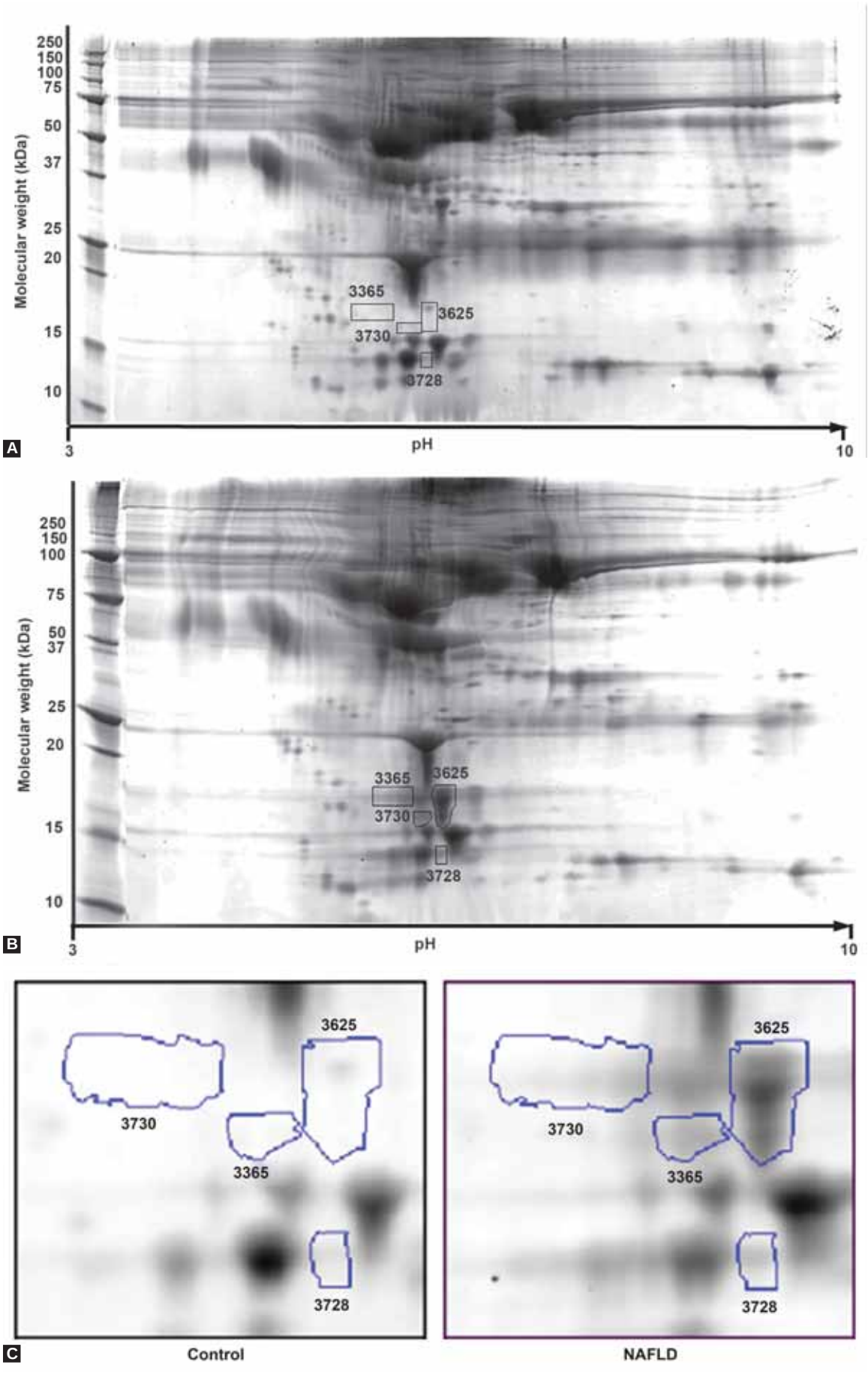
Figs 1A to C: Representative 2-DGE gel image of plasma from control group (A) and ALD group (B). The protein spots differentially altered are marked by the spot numbers and shown in Montage window (C) for the relative intensity. More than 2-fold change in protein concentration with p-value of ≤ 0.05 were considered significant

statistically significant in NAFLD group only. For the analysis of FAEEs, the recovery of internal standard was found to be > 70%. Significantly higher levels of FAEEs were found in the plasma samples of ALD group as compared with no detectable amounts in NAFLD group and controls (Fig. 5). Among three major FAEEs found in the ALD group, palmitic acid (16:0) ethyl ester was found to be ~3-fold greater than each stearic acid (18:0) and oleic acid (18:1) ethyl esters. Significant amount of plasma FAEEs can clearly distinguish ALD from NAFLD.

DISCUSSION

ALD as well as NAFLD initiate from early reversible fatty liver and both progress to similar terminal disease conditions. It is well-known that liver is a primary site for the biosynthesis of lipids, proteins and secretion of circulating complements. However, the key lipidomic and proteomic changes between the ALD and NAFLD are not well-defined. Elevated levels of total lipids in the plasma of both ALD and NAFLD groups appear to reflect fatty liver. However, it is well-known that high BAC and presence of plasma FAEEs are associated with acute and chronic alcohol abuse, one of the major causes of liver disease.[7] Although it has been reported that complement pathway contributes to inflammatory response, its activation can also enhance the process of fibrosis.[14] Our data on plasma proteome suggest a compromised complement system in patients with ALD. Increased triacylglycerol and cholesterol esters and decreased phosphatidylethanolamine have been reported by us in the livers of rats after long-term ethanol exposure.[11] Plasma lipidome and proteome appear to reflect a chemical insult or onset of pathological changes in the liver and also provides strong possibility to identify potential biomarker(s) for liver diseases of different etiologies. Although ALD and NAFLD originate from a similar fatty liver state, proteomic and lipidomic findings of this study strongly suggest remarkable etiological and metabolic differences between the ALD and NAFLD.

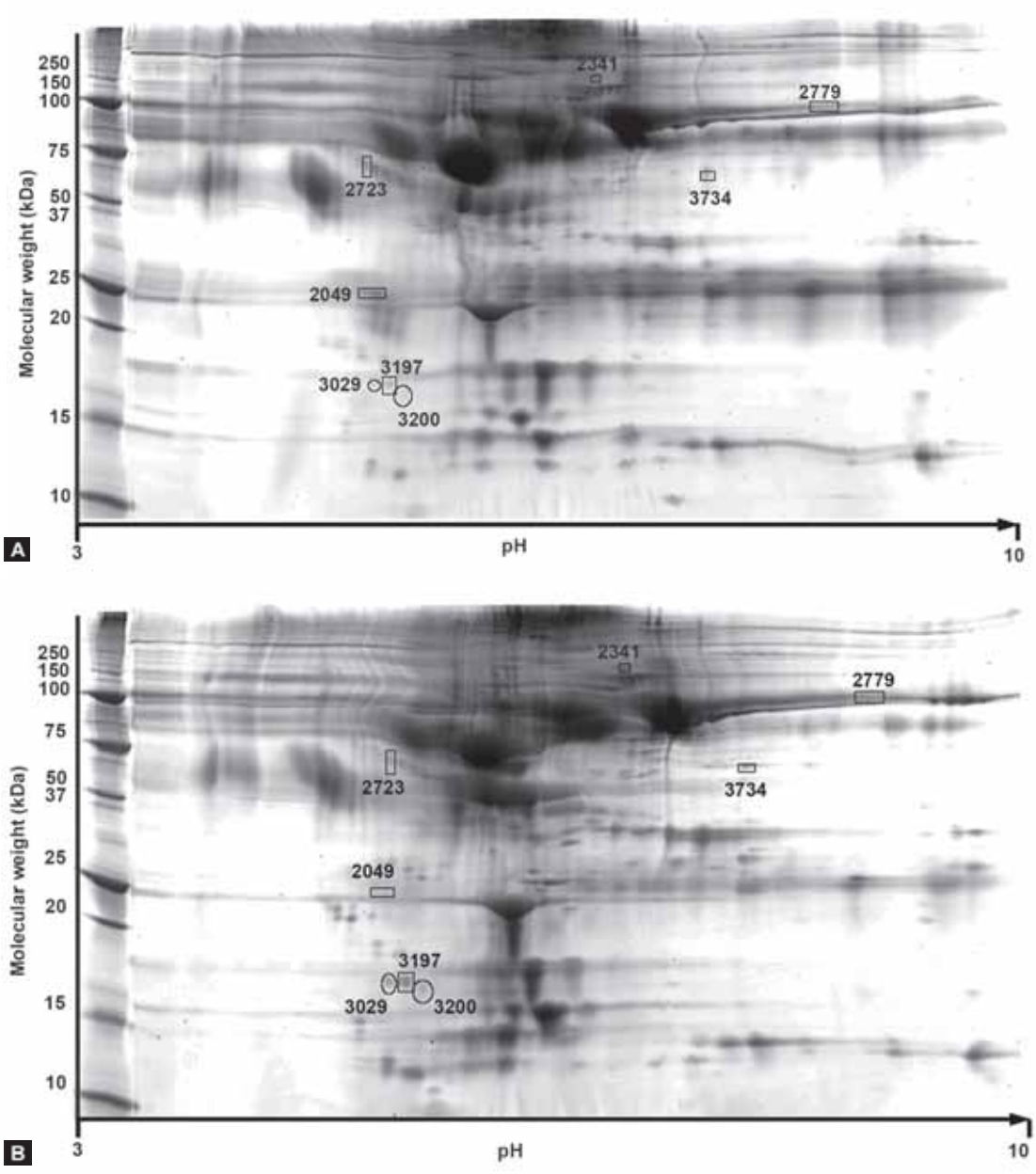
Complements are the plasma and membrane proteins and participate in innate and adoptive immunity, cell killing and host defense against infection by various mechanisms.[15] About 80 to 90% of plasma complement components are synthesized in the hepatocytes. Activation of complement pathway can occur via classic, lectin or alternative pathways ultimately resulting in activation of C3. Although complement activation may not contribute to the clinical and histological features of ALD but can promote



Figs 2A to C: Representative 2-DGE gel image of plasma from control group (A) and NAFLD group (B). The protein spots differentially altered are marked by the spot numbers and shown in Montage window (C) for the relative intensity (see Fig. 1)

inflammatory responses by librating small peptide fragments involved in cellular activation.[16,17] Most of the previous findings reported in the literature suggest that ethanol-induced liver injury is mediated, at least in part, by an inflammatory response involving innate immune system.[14,18] A high prevalence of infection has also been reported in patients diagnosed with ALD.[19-22] Therefore, it is possible that decreased levels of complement precursors be related to impaired hepatic synthesis of various components of complements.[23,24]

Complement C3 plays a central role in activation of the complement system and C3a anaphylatoxin as mediator of local inflammatory process and induces contraction of smooth muscle, increases vascular permeability and causes histamine relapse from mast cells and basophilic leukocytes (SwissProt primary accession number-P01024). However, the functions of IgG-3 chain C region (heavy chain disease protein) and biological significance of decreased levels of serum amyloid A4 protein (major acute phase reactant) precursor in ALD are not known. Mechanism(s) by which levels of immunoglobulin J (P01591, helps to bind IgM and IgA immunoglobulin) and leucine-rich alpha-2 glycoprotein (P02750, no recorded function available yet) are decreased in plasma of patients with NAFLD *vs* those with ALD need to be investigated. Several spots for precursors of complement C3 and C6, and haptoglobin found in respective gels of ALD or NAFLD groups could be related to their posttranslational modifications. Such structural modifications of the proteins could be key factors for initiation and progression of ALD or NAFLD. Therefore, haptoglobin



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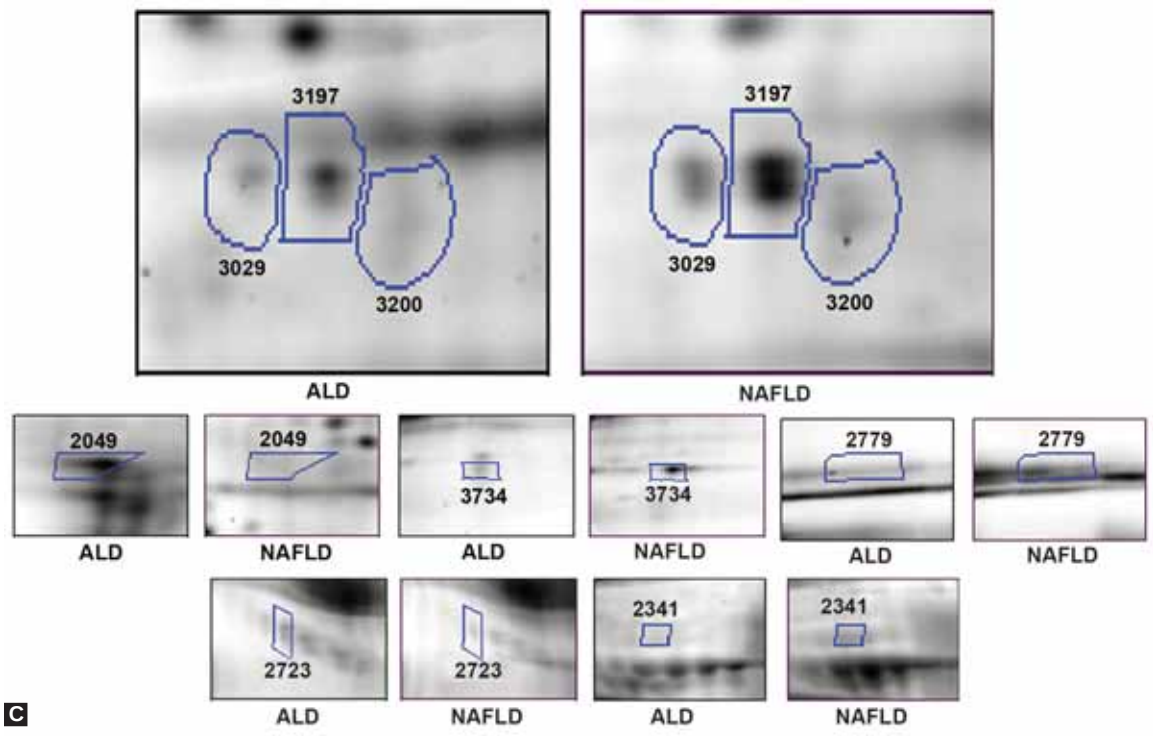

Figs 3A to C: Representative 2-DGE gel image of plasma from ALD group (A) and NAFLD group (B). The protein spots differentially altered are marked by the spot numbers and shown in Montage window (C) for the relative intensity (for details regarding the gels and spots, see Fig. 1)

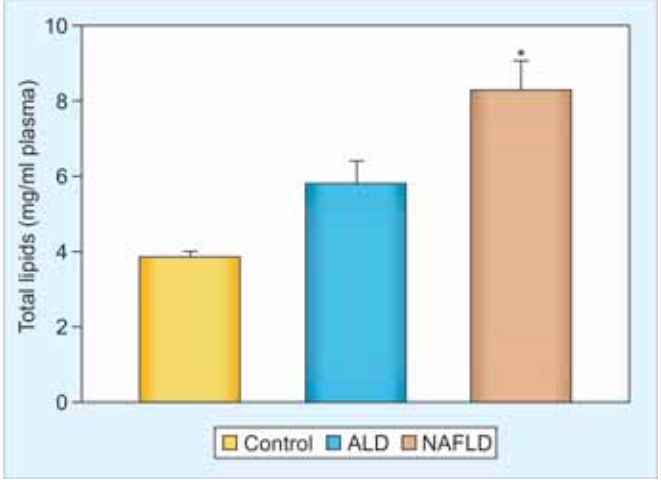

Fig. 4: Total lipids in the plasma of patients diagnosed with ALD or NAFLD (mg/ml plasma). *p-value < 0.05

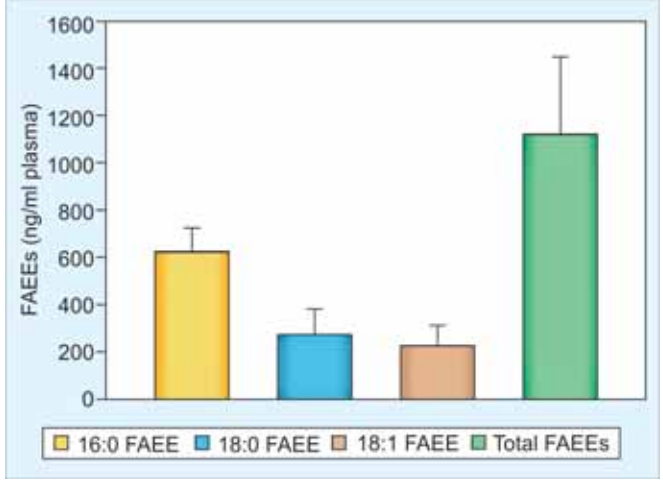

Fig. 5. Individual and total FAEEs in the plasma of patients diagnosed with ALD. FAEEs were not detectable in the plasma of controls and NAFLD group (data not shown)

acts as an antioxidant and plays an important role in modulating host-defense responses to inflammation and infection.[25] It can also bind to free plasma hemoglobin and allows degradative enzymes to gain access to the hemoglobin and at the same time preventing loss of iron through the kidneys and protecting the kidneys from damage by hemoglobin. Increased levels of haptoglobin precursor, as observed in NAFLD group, are also found in patients with ovarian and pancreatic cancers.[26-28] Therefore, etiopathogenesis could be vastly different for ALD and NAFLD based upon our proteomic and lipidomic data.

BAC is a most commonly used metric of intoxication for medical purposes. However, BAC as well as metabolism of ethanol could be a determining factor for target organ toxicity.[8,9] Among several oxidative and nonoxidative ethanol metabolites, plasma FAEE levels correlate better with extent of chronic alcohol abuse.[7] Several fold greater BAC and the plasma FAEEs found only in the ALD group are parallel to our previous findings.[7] One of the potential sources of FAEEs in the plasma could be the FAEE synthase (FAEE synthesizing enzyme)-catalyzed biosynthesis or their circulation after their formation in the livers or pancreas.[7,9,29]

Several isoforms of hepatic FAEE synthase, purified and identified to be various isoforms of carboxylesterases, can be released in circulation during liver injury.[30,31] Significantly high FAEE levels could be due to high BAC and/or presence of FAEE synthase in the plasma of the ALD group.[9,32] It has been reported that FAEEs cause hepatocellular injury and have greater half-life than ethanol itself.[8,33-35] Therefore, endogenously formed FAEEs could play a critical role in etiopathogenesis of ALD.

CONCLUSION

Complements, haptoglobin, BAC and plasma FAEEs can be considered as key factors for understanding the mechanisms of ALD and NAFLD. It is possible that innate and adaptive immunity are important targets of toxicity by alcohol and its metabolites. The role of complements C3, C6, haptoglobin precursors, immunoglobulin J chain and leucine-rich alpha-2 glycoprotein in etiopathogenesis of ALD or NAFLD and/or their potential as biomarker candidate(s) need to be investigated in larger cohorts. Therefore, a comprehensive study to identify altered plasma proteins (proteome) and lipids (lipidome) in larger cohorts could be important in understanding the mechanisms and for developing biomarkers of ALD or NAFLD.

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