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Downregulation of apolipoprotein A-IV in plasma & impaired reverse cholesterol transport in individuals with recent acts of deliberate self-harm

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***Background & objectives*: The major limiting factor in the prevention of suicide is the limited knowledge on molecular insights in individuals at risk. Identification of peripheral protein markers which can classify individuals at high-risk of suicide might aid in early diagnosis and effective medical intervention. The aim of the present study was, therefore, to analyze the differential regulation of plasma proteins in individuals with deliberate self-harm compared to controls.**

***Methods*: Using two-dimensional gel electrophoresis coupled with matrix-assisted laser desorption-ionization mass spectrometry, differentially expressed plasma proteins were identified in study participants with deliberate self-harm compared to age- and gender-matched controls. The finding was validated using mass spectrometry-based isotope-labelled relative quantification and Western blot analysis in a new set of individuals with deliberate self-harm and controls.**

***Results*: The plasma proteomic analysis showed that apolipoprotein A-IV (Apo A-IV) was downregulated by 2.63-fold (confidence interval: 1.52-4.54) in individuals with deliberate self-harm (n=10) compared to matched controls, which was consistent in mass spectrometry-based relative quantification and Western blot analysis performed in an independent set of individuals with deliberate self-harm (n=18). In addition, plasma levels of total cholesterol, esterified cholesterol and high-density lipoprotein (HDL) were observed to be significantly lower individuals with deliberate self-harm compared to controls.**

***Interpretation & conclusions*: Apo A-IV, which plays a crucial role in the esterification of free cholesterol, was found to be downregulated with concomitantly decreased levels of HDL, esterified cholesterol and total cholesterol in individuals with deliberate self-harm compared to matched controls. The present findings might provide a link between the differential regulation of plasma proteins and the previously reported results on altered cholesterol levels in individuals with deliberate self-harm.**

Key words Apolipoprotein A-IV - deliberate self-harm - esterified cholesterol - mass spectrometry - suicide - two-dimensional gel electrophoresis

Suicide is the third leading cause of death in the age group of 15-39 yr across the globe[1]. In India, suicide ranks as the first cause of death among the age groups 15-29 and 15-39 yr. The age standardized suicide death rates in 2016 was 17.9 per 100,000 population and reported a 40.1 per cent increase in 2016 compared to 1990[1].

Several demographic and psychological risk factors have been described in relation to acts of deliberate self-harm. Male gender, adolescents and young adults between 15 and 29 yr of age, past history and family history of self-harm, parental loss or separation, social isolation, presence of a psychiatric illness, chronic psychological distress and substance abuse are some of the factors that increase the risk of self-harm[2]. Concurrently, several investigators have also sought to understand the neurobiological underpinnings of suicidal behaviour[3].

Various neurobiological studies have shown that low levels of cerebrospinal fluid (CSF) 5-hydroxyindoleacetic acid, serotonin transporters and alterations in binding efficiency of serotonin to its receptors are associated with suicidal behaviour. However, the inherent practical difficulties and inconsistencies in results limit their use as a diagnostic molecular marker for identification of patients at high-risk for attempting suicide[4].

Development of a reliable biomarker linked to suicidal behaviour may help in early identification and prevention of suicide in individuals at high-risk. As suicidal behaviour involves interactions between genetic vulnerability, abnormalities in neurotransmitter pathways and environmental factors, it has been suggested that proteomic approach may be best suited for identification of biomarkers and molecular events related to the dynamic nature of this complex interaction[5]. In a CSF-based proteomic study, a protein with a mass of 33 kDa was reported to be unique in unmedicated suicidal attempters with major depression compared to non-attempters[6]. However, due to the low concentration of the protein in CSF, the protein was not identified. Schlicht *et al*[7] in a comparative proteomic analysis of autopsied tissue samples from the prefrontal cortex of suicide victims observed the differential expression of glial fibrillary acidic protein, manganese superoxide dismutase and α-crystallin chain B. However, variations in post-mortem interval, storage temperature of the body, tissue processing and duration of storage may affect alterations in the level of post-translational modifications and protein degradation[8].

In the present study, proteomic approach was adopted to analyze the differential expression of plasma proteins in individuals with recent deliberate self-harm. Two-dimensional gel electrophoresis (2D-GE) and matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS) platform were used to analyze the differential expression of plasma proteins in individuals with deliberate self-harm compared to age- and gender-matched controls. The observed differential regulation of plasma proteins was validated by relative quantification using stable isotope labelled peptides and Western blot analysis.

Material & Methods

The study was conducted at St. John's National Academy of Health Sciences (SJNAHS), Bengaluru, India, during 2011-2016. The study was approved by the Institutional Ethical Review Board. Individuals who presented to the Emergency department with an act of attempted suicide were included in the study based on the inclusion criteria. The inclusion criteria were as follows: (*i*) a recent deliberate self-harm, and (*ii*) clear sensorium and ability to provide a written informed consent. Those with the following exclusion criteria were excluded: (*i*) who had received medical intervention related to the present attempt before reaching the Emergency department, and (*ii*) those with significant medical complications including ventilator care and admission to intensive care unit. Age- and gender-matched healthy controls were chosen from the staff and students of SJNAHS. Individuals with deliberate self-harm and matched controls were recruited in two phases: discovery phase and validation phase. In the discovery phase, the differentially expressed plasma proteins in patients (n=10) compared to age- and gender-matched controls (n=10) were characterized by 2D-GE. In the validation phase of the study, the differentially expressed proteins were quantified using stable isotope label-based relative quantification technique and Western blot analysis in a separate set of individuals with deliberate self-harm (n=18) and controls (n=13).

Written informed consent was obtained from all participants after their medical condition was stabilized. Individuals with deliberate self-harm who presented with an altered level of sensorium were assessed for the level of consciousness using the Glasgow Coma Scale (GCS) and were interviewed only if there GCS score

was 15/15[9]. All the individuals underwent a detailed physical examination.

Clinical assessments: The demographic and clinical features were assessed using the following measures. The psychiatric diagnosis of individuals with deliberate self-harm was evaluated using the Mini-International Neuropsychiatric Interview (MINI) Plus[10]. The Beck's Suicidal Intent Scale (SIS) was used to assess the severity and intent of the suicidal attempt[11]. The Beck Hopelessness Scale (BHS), a 20-item self-reported inventory, was used to measure the extent of negative attitudes or pessimism about the future[12]. The Barratt Impulsiveness Scale-11 (BIS-11) was used to assess the behavioural construct of impulsivity associated with attempted suicide[13]. The mental health status of healthy controls was assessed using the Kessler Psychological Distress Scale (K10)[14]. Participants without any past psychiatric history or suicidal attempt and less than the recommended cut-off score of 20 on K10 were enrolled as healthy controls in the study.

Sample collection: Random venous blood (5 ml) was collected into Vacutainer tubes containing EDTA as an anticoagulant. Plasma was separated immediately and stored at −120°C until used for proteomic analysis. The mean delay time taken for collecting blood sample after the suicidal attempt was 6.9 h (range=30 min-48 h).

Sample processing and 2D gel electrophoresis (*2D-GE*): Fourteen major abundant plasma proteins were depleted from samples using affinity-based antibody depletion kit (Seppro IgY14, Sigma-Aldrich, USA). Twenty microlitres of crude plasma sample was loaded onto spin column, and protein depletion was done according to the manufacturer's instructions. Double-depletion strategy was used for effective depletion where depleted flow through was loaded onto the column and elution protocol was repeated. The depleted plasma sample was dialyzed and subsequently denatured and reduced by dissolving in resuspension buffer containing 7 M urea, 2 M thiourea and 65 mM dithiothreitol (DTT). The total protein concentration was estimated using the Bradford assay[15].

Isoelectric focussing was performed using 13 cm immobilized *p*H gradient (IPG) strips with *p*H gradient of 4-7 (GE Healthcare, UK). All samples from individuals with self-harm and matched controls (50 µg) were run in triplicates. Samples were made up to 250 µl using resuspension buffer, bromophenol blue (1%) and one per cent IPG buffer (*p*H 3-10). After passive rehydration, IPG strips were isoelectrofocussed at 25°C for a total of 55.1 kVh in the first dimension of separation. Focussed IPG strips were equilibrated with one per cent DTT w/v and alkylated using 2.5 per cent w/v iodoacetamide. The second-dimensional separation of proteins was done using 12 per cent sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by silver staining and imaged using Image Viewer (GE Healthcare, UK).

Differential expression analysis of protein spots: To identify the differentially expressed protein spots, the gel images were analyzed using ImageMaster 2D Platinum 7.0 software (GE Healthcare, UK). Detection of false spots was reduced using the following parameters: smoothness – 5, saliency – 12 and minimum area – 5. The spots in both individuals with deliberate self-harm and controls were matched, and percentage spot volume was estimated for each spot using ImageMaster 2D Platinum 7.0 software. In this software-based gel spot analysis, of the 313 matched protein spots, 35 were found to be differentially expressed between individuals with deliberate self-harm and controls. The average percentage spot volumes of each of these 35 spots were calculated across triplicate gels, and ratios (controls/those with deliberate self-harm) were estimated. Fourteen spots with ratios in the range of ≤ 0.7 and ≥ 1.3 were identified as either down- or upregulated. Spots were considered to be differentially regulated if the directional change was similar in at least 60 per cent or more of the individuals with deliberate self-harm-control matched pairs. Four of 14 spots were consistently differentially regulated. To estimate whether the identified spots were significantly different between the patient and the control groups, the mean and confidence interval (CI) of the ratios of the four protein spots were calculated. Proteins were considered as significantly down- or upregulated only if the CI excluded unity (null value).

Protein digestion and mass spectrometry analysis: The differentially expressed protein spots were in-gel digested using trypsin (Sigma-Aldrich, USA), and the proteolytic peptides were eluted following the methods described by Sumner *et al*[16]. The proteolytic peptides were analyzed in MALDI-MS (Synapt HDMS, Waters, UK) using α-cyano-4-hydroxycinnamic acid as matrix. Mass spectra were acquired in positive ion 'V' mode, in the range 900-3000 m/z using a 200 Hz solid-state laser (λ=355 nm). The mass spectra for each gel spot were searched against a Swiss-Prot human proteome database downloaded from UniProtKB

(*http://www.uniprot.org*) using ProteinLynx Global Server (PLGS) v2.5 and Mascot search algorithm (*http://www.matrixscience.com*). The parameters used in PLGS and Mascot search are as follows: peptide mass tolerance - 100 ppm, proteolytic enzyme - trypsin, fixed modifications - carbamidomethyl-cysteine, variable modification - methionine oxidation and one missed cleavage. The identity of the proteins was confirmed based on sequence assignments of tandem mass (MS/MS) spectra for at least two peptides.

Relative quantification of differentially expressed proteins using labelled peptide: Mass spectrometry-based quantification of a target protein is based on relative intensity of the isotopically labelled proteolytic peptide of the target protein that has been spiked in the experimental proteome sample. Since the ionization probabilities of the peptide of interest and its isotopically labelled analogue are the same, the target protein is quantified on the basis of relative intensities of the quantitative amount of labelled peptide spiked in the experimental proteome sample. The isotope labelling imparts a mass shift that makes it to appear with distinct m/z. In the present study, the relative concentrations of the differentially regulated plasma proteins were quantified by spiking stable isotope-labelled synthetic peptides of these proteins purchased from Cambridge Research Biochemicals, UK. Isotopically labelled peptides were selected based on the following criteria: tryptic peptides with high signal intensities in mass spectra, hydrophobic amino acid residue content <50 per cent, absence of methionine, cysteine and tyrosine residues and devoid of miscleavage sites[17]. In addition, the selected peptides were screened to ensure that these are unique to the differentially expressed proteins using blast analysis (*https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins*).

Relative levels of the differentially expressed plasma proteins were quantified in a different set of individuals with deliberate self-harm (n=18) and healthy controls (n=13), recruited in the validation phase of the study. The depleted plasma sample was reduced, alkylated, trypsin digested (1:10 enzyme:substrate ratio) and spiked with 100 fmoles of isotopically labelled peptides. Nano-liquid chromatography coupled to electrospray ionization mass spectrometry (nLC/ESI-MS) was used in the relative quantification experiment. The MS analysis of the digest was performed in triplicates using analytical C18 column (BEH-130, C18, 75 µm×250 mm, 1.7 µm) in a nanoAcquity UPLC (Waters, UK) connected to Synapt HDMS. The mobile phases were 100 per cent water with 0.1 per cent formic acid (FA) (solvent A) and 100 per cent acetonitrile with 0.1 per cent FA (solvent B). The peptides were eluted with 0.3 µl/min flow rate using a linear gradient of 0.1 per cent increase in solvent B. The data were acquired using MS^E mode of acquisition (50-1995 m/z) in positive ion 'V' mode with a source temperature of 70°C, capillary voltage 2.8 kV, cone voltage 30 V and an extraction cone voltage 4 V. Glu-fibrinopeptide was used as lockspray. The fragment ions were generated by linearly ramping the trap energy from 15-40 eV. Relative concentrations of the differentially expressed plasma proteins were calculated by comparing the total intensity of the two charge states (+1 and +2) of the labelled peptide to the total intensity of the unlabelled (endogenous) peptide in the experimental sample. The intensity of the isotopically labelled peptide was correlated with the spiked amount of the peptide. The concentrations of experimental peptide obtained from triplicate runs were averaged, and the ratio (individuals with deliberate self-harm/controls) was calculated.

Western blot analysis of differentially expressed proteins: The levels of the differentially expressed plasma protein identified in the proteomic analysis were estimated using Western blot analysis in a subset of individuals with deliberate self-harm (n=10) and controls (n=8) recruited during the validation phase. Proteins in the depleted plasma fractions (20 µg) were separated by SDS-PAGE in triplicates. The proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (0.2 µm, Novex, USA). The PVDF membrane was blocked with 3 per cent BSA in 1× phosphate-buffered saline, *p*H 7.4 and incubated with monoclonal Apo A-IV antibody (1:1000, Cell Signalling Technologies, USA). The blots were further incubated with horseradish peroxidase-conjugated goat anti-mouse antibody. The protein bands were developed by a chemiluminescent substrate (SuperSignal™ West Pico PLUS, Thermo Fisher Scientific, USA) and subsequently quantified using ImageJ software (*https://imagej.nih.gov/ij/index.html*). The densitometric intensity values for each patient-control pair were averaged, and the ratio was calculated and summarized as geometric mean and its CI (95%).

Estimation of plasma cholesteryl esters, high-density lipoprotein (*HDL*), *total cholesterol and lecithin-cholesterol acyltransferase* (*LCAT*) *activity*:

Plasma level of cholesteryl esters was estimated using Cholesterol/Cholesteryl Ester Quantitation Assay kit (Colorimetric/Fluorometric) (Abcam, USA). The esterified cholesterol was obtained as a fraction of total cholesterol, for both patients and controls. The total cholesterol and HDL were quantified using automated analyzer (Dimension Xpand Plus, Siemens, Germany). The activity of lecithin-cholesterol acyltransferase (LCAT) enzyme in plasma was measured using a commercially available fluorescence-based assay kit (Roar Biomedical, Inc., Sigma, USA).

Statistical analysis: The activity of LCAT, HDL and total cholesterol level of individuals with deliberate self-harm and matched controls were compared using the Wilcoxon signed-rank test (OriginPro v8.0, OriginLab Corporation, Northampton, MA, USA).

Results

The discovery phase of the study consisted of 10 individuals with deliberate self-harm and 10 age- and gender-matched healthy controls. Most of the individuals in the self-harm group did not have a current psychiatric diagnosis (n=9), and none of them had past or family history (first degree) of psychiatric disorders or past history of suicide attempt. The body mass index (BMI) was not significantly different between self-harm (18.81±2.01 kg/m^2) and matched controls (23.91±2.46 kg/m^2). The control group had more college-educated individuals (n=8) compared to those with deliberate self-harm (n=1). All those who attempted deliberate self-harm reported either chronic (n=4) or acute life stressors (n=6). The stressors were interpersonal in nature in eight, while two reported significant financial difficulties. The majority of the individuals with self-harm had minimal level of hopelessness and low-to-moderate level of suicidal intent. On BIS-11, the total score obtained for individuals with deliberate self-harm was 61±18.12, and in the non-planning domain, the score was 25.4±4.06. The clinical and demographical profiles of individuals recruited in the validation phase were similar to patients in the discovery phase. The demographic and clinical details of individuals and matched controls for both the discovery and validation phases are depicted in Table I.

Plasma proteomics: The differential expression analysis using ImageMaster 2D Platinum 7.0 showed 313 protein spots matched between patients and controls. Following the criteria described under methods, three spots were found to be downregulated and one was upregulated among 10 individuals with deliberate self-harm-control pairs. The mean ratio and 95 per cent CI for the three downregulated protein spots were 2.63 (CI: 1.52-4.54), 1.98 (CI: 1.76-2.23) and 1.49 (CI: 0.91-2.44), respectively. The mean ratio and 95 per cent CI for the upregulated protein spot were 0.44 (CI: 0.29-0.67). The protein spot with CI 0.91-2.44 was excluded in the subsequent analysis since it showed a CI with null value, whereas the rest of the three protein spots were significantly different between those with self-harm and the control groups. Fig. 1 shows the representative gel images of a matched healthy control (panel A) and an individual with deliberate self-harm (panel B). Insets Fig. 1A and 1B show the three-dimensional view of the intensities of the differentially expressed protein spot (spot 62) in matched participant and control pairs.

MALDI-MS analysis of the proteolytically digested protein spot 62 was identified as apolipoprotein-AIV (Apo A-IV) (accession number: P06727) with 61.4 (38 peptides) and 74 per cent (46 peptides) sequence coverage in PLGS and Mascot search algorithms, respectively. Fig. 2A shows the peptide mass fingerprint of the in-gel digest of protein spot 62. The Mascot score obtained for Apo A-IV was 132 ($P \leq 0.05$). Fig. 2B shows the MS/MS spectra of a tryptic fragment of Apo A-IV with 1104.5 m/z spanning residues from 135 to 143. The protein (spot 401) was identified as vitamin D-binding (VDB) protein (accession number: P02774), with 64.1 (30 peptides) and 68 per cent (34 peptides) sequence coverage in PLGS and Mascot search algorithms, respectively. The third protein spot with ratio 1.98 remained unidentified in MALDI-MS analysis most likely due to low protein concentration in the gel spot.

Relative quantification of plasma apolipoprotein A-IV and vitamin D binding: In the validation phase, the relative levels of Apo A-IV and VDB were quantified using isotopically labelled peptides in individuals with deliberate self-harm (n=18) and controls (n=13). We observed that Apo A-IV was significantly downregulated by 2.68-fold (95% CI: 1.83-3.937) in individuals with deliberate self-harm compared to controls. VDB did not show a significant fold difference (0.868-fold) between those with self-harm and the control groups, as CI (95% CI: 0.681-1.106) included null value, 1. Therefore, VDB was excluded from further analysis. Figure 3A depicts the overlaid

Table I. Demographic and clinical details of individuals with deliberate self-harm and age- and gender-matched healthy controls

Variables	Individuals with deliberate self-harm	Age- and gender-matched healthy controls
Discovery phase: 2D-GE-MALDI-MS-based proteomic analysis and esterified cholesterol measurement		
Sample size (n)	10	10
Age (yr)	26.7±5.51	26.9±5.23
Gender (male/female)	6/4	6/4
Method of suicidal attempt	Organophosphorus poisoning (n=6) (dichlorvos, quinalphos, cypermethrin, chlorpyrifos, pyrethroids and benzalkonium chloride) Drug overdose (n=2) (cetirizine and paracetamol) Wrist slashing (n=1) Self-inflicted burns (n=1)	
Location of residence	Rural (n=3) Urban (n=7)	Rural (n=2) Urban (n=8)
BIS-11 (total score), mean	61±18.12	
BIS-non-planning, mean	25.4±4.06	
BHS	40% (0-3 range), 30% (4-8 range), 10% (9-14 range), 0% (15-20 range)	
SIS	50% (15-19 range), 20% (20-28 range), 30% (above 29)	
Validation phase: Absolute quantification, Western blot analysis, LCAT activity, HDL and total cholesterol		
Sample size (n)	18	13
Age (yr)	26.6±3.29	25.1±3.41
Gender (male/female)	8/10	5/8
Method of suicidal attempt	Organophosphorus poisoning (n=7) (deltamethrin, pyrethroids, rodenticide, cleaning phenol, cypermethrin and prallethrin) Drug overdose (n=10) (thyroxine, paracetamol, vitamin tablets, zolpidem, glimepiride, carbamazepine and aluminium phosphide tablets) Paraphenylenediamine poisoning (n=1)	
BHS	17% (0-3 range), 33% (4-8 range), 28% (9-14 range), 22% (15-20 range)	
SIS	56% (15-19 range), 27% (20-28 range), 17% (above 29)	

2D-GE, two-dimensional gel electrophoresis; MALDI-MS, matrix-assisted laser desorption-ionization mass spectrometry; BIS-11, Barratt Impulsiveness Scale; SIS, Beck's Suicidal Intent Scale; BHS, Beck Hopelessness Scale; LCAT, lecithin-cholesterol acyltransferase; HDL, high-density lipoprotein

electrospray ionization (ESI) MS spectra of the doubly charged endogenous peptide of Apo A-IV 1104.6 m/z (552.8 m/z) and corresponding isotopically labelled peptide 1111.6 m/z (556.3 m/z) obtained from an individual with deliberate self-harm and the matched control.

Western blot analysis: The differential expression observed in proteomic analysis was validated using Western blot analysis. Figure 3B depicts the representative images of Western blot bands of Apo A-IV obtained for the two pairs of individuals with deliberate self-harm and matched controls. Western blot analysis showed that Apo A-IV was significantly downregulated by 2.015-fold (95% CI: 1.132-3.5858) in those with deliberate self-harm compared to matched controls.

Measurement of LCAT activity, level of HDL, total cholesterol and esterified cholesterol: To assess the functional correlation of the observed downregulation of plasma Apo A-IV, the plasma LCAT activity, levels of esterified and total cholesterol and HDL levels were estimated in individuals with deliberate self-harm and matched controls. The plasma LCAT activity obtained from the ratio of emission intensities (I_{470}/I_{390}) was not found to be significantly different between those with self-harm and controls (Table II). The fraction of esterified cholesterol with respect to total cholesterol was found to be lower in individuals with deliberate self-harm compared to controls. The total cholesterol level and HDL were observed to be lower in those with self-harm compared to matched controls (Table II).

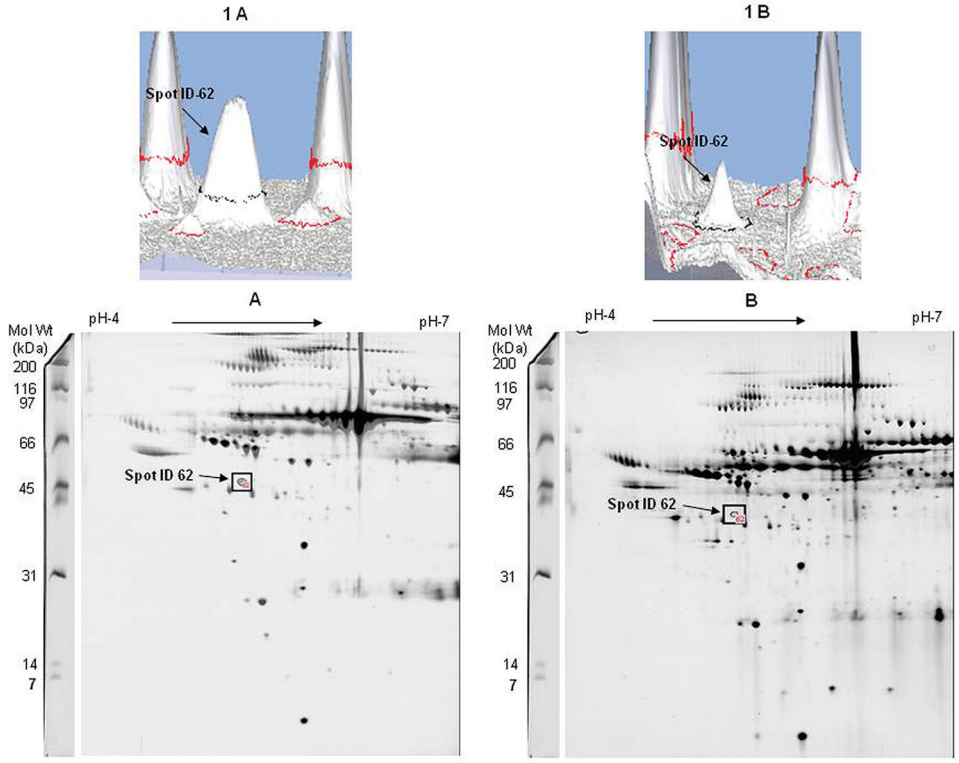

Fig. 1. Two-dimensional gel electrophoresis image of a participant with deliberate self-harm and matched control. Representative gel image of plasma proteins of an age- and gender-matched healthy control (panel A) and a participant with deliberate self-harm (panel B) with differentially expressed protein spot (spot ID - 62). Inset (1**A**) and (1**B**) depicts the three-dimensional view of the intensity of spot 62 present in a control and a participant with deliberate self-harm, respectively.

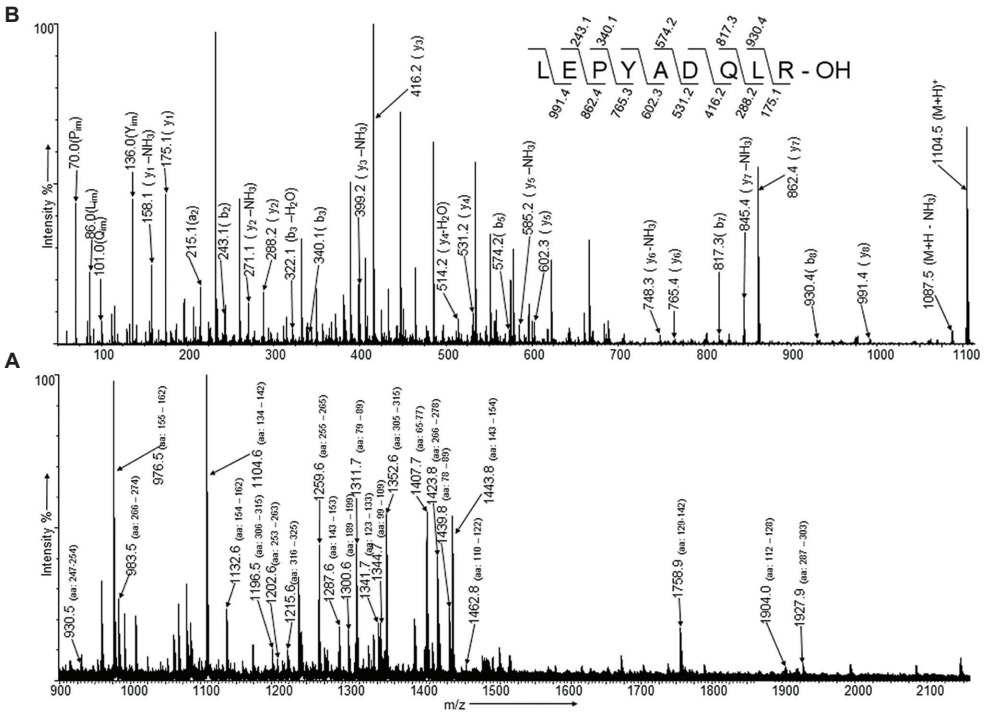

Fig. 2. MALDI mass spectra of tryptic digest of spot 62. (**A**) MALDI mass spectra of the tryptic peptides obtained from the in-gel digestion of spot 62. (**B**) Tandem mass spectra of the peptide with 1104.5 m/z with series of 'b', 'y' ions and neutral loses are labelled along with the sequence of the precursor peptide ion, obtained from tandem mass spectra is shown as inset.

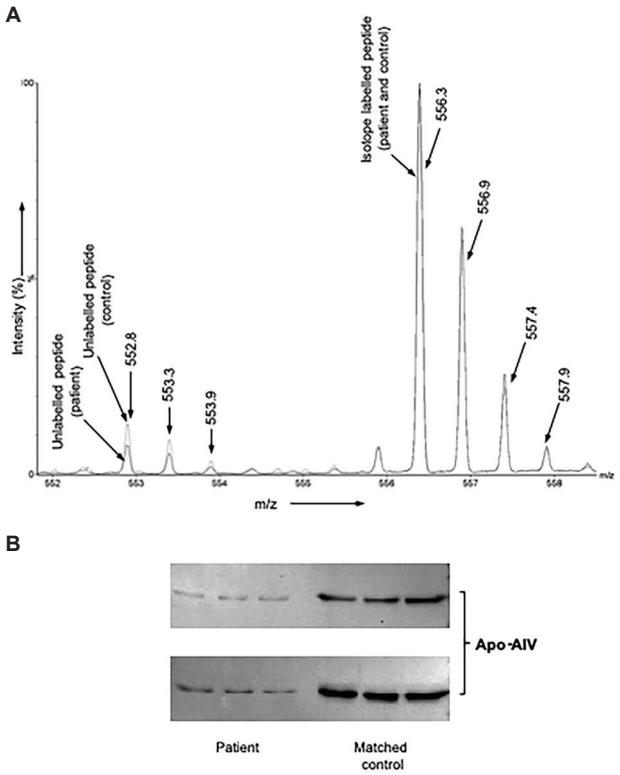

Fig. 3. Relative quantification and Western blot analysis. (**A**) Depicts the overlaid electrospray ionization mass spectra of the doubly charged endogenous peptide of apolipoprotein A-IV 1104.6 m/z (552.8 m/z) and corresponding labelled peptide 1111.6 m/z (556.3 m/z) obtained from an individual with deliberate self-harm and the matched control. (**B**) Western blot images of individuals with deliberate self-harm and matched controls.

Discussion

In the present plasma proteomic analysis, Apo A-IV protein was found to be downregulated in individuals with deliberate self-harm compared to matched controls. In addition, esterified cholesterol, total cholesterol and HDL were significantly lower in those with deliberate self-harm compared to controls. Although the activity of LCAT measured in plasma sample was slightly reduced in patients, it was not significantly different between patients and controls.

In this study, the majority of the acts of deliberate self-harm were impulsive in nature and in response to life stressors, and those individuals had minimal levels of hopelessness and low or moderate suicidal intent. The scores on BIS-11 and in the non-planning domain were similar to other studies in clinical population characterized by high degree of impulsiveness[18]. In addition, except for one person with self-harm, none of the others had a diagnosable psychiatric condition. This clinical profile was in agreement with several studies from India[19], wherein patients attempted suicide in response to psychosocial problems, and the proportion of diagnosable psychiatric condition in this population was fewer than that reported in the West[20]. Earlier studies have noted an association between downregulation of Apo A-IV and atherosclerosis[21]. The enhanced cholesterol efflux and protection against atherosclerotic lesions observed in transgenic mice overexpressed with human Apo A-IV underlines the role of Apo A-IV in reverse cholesterol transport and protection against atherosclerosis[22]. Studies have also suggested that Apo A-IV may possess antioxidant property and it is a potent inhibitor of oxidant-induced apoptosis in undifferentiated PC-12 cells[23]. The anti-atherogenic effect of Apo A-IV has been linked to its antioxidant property, where Apo A-IV was found to be an inhibitor of low-density lipoprotein oxidation[24]. In an earlier study on patients with attempted suicide, significantly elevated levels of oxidative stress markers such as nitric oxide metabolites and lipid hydroperoxides and decreased level of total antioxidants were reported[25]. Several studies have noted an association of elevated levels of pro-inflammatory cytokines and inflammatory proteins with suicidal behaviour suggesting a pro-inflammatory

Table II. Biochemical parameters of individuals with deliberate self-harm and healthy controls

Variables	Individuals with deliberate self-harm	Controls
Discovery phase (n)	10	10
Esterified cholesterol (%), median (Q1-Q3)	56.01 (49.80-61.63)**	65.19 (61.62-67.10)
Validation phase (n)	18	13
LCAT activity (I_{470}/I_{390}), median (Q1-Q3)	1.0812 (1.0494-1.1524)	1.0633 (1.0170-1.0755)
HDL cholesterol (mg/dl), median (Q1-Q3)	30 (26.25-38.25)*	37.5 (28.5-51)
Total cholesterol (mg/dl), median (Q1-Q3)	144.5 (132.5-158)*	160.5 (143.5-193)

*P^*<0.05, **<0.01 compared to controls. LCAT, lecithin-cholesterol acyltransferase; HDL, high-density lipoprotein

phenotype might be associated with individuals with deliberate self-harm[26]. The present study has shown an association between downregulation of plasma Apo A-IV and deliberate self-harm. Thus, the molecular insights underpinning the association between downregulation of Apo A-IV and oxidative stress in patients with attempted suicide need to be explored in future studies.

Apo A-1V is involved in reverse cholesterol transport through the activation of LCAT which converts free cholesterol to esterified cholesterol[27]. Our findings of low levels of esterified and total cholesterol and HDL in plasma of individuals with deliberate self-harm were in agreement with earlier studies that reported a significantly lower level of HDL cholesterol and esterified cholesterol in patients with a past history of serious suicidal attempt[28]. In another study on women aged between 17 and 39 yr, there was a significant association of low HDL cholesterol levels with suicidal attempt, independent of psychiatric diagnosis[29].

The lack of significant difference in LCAT activity between individuals with deliberate self-harm and the control group might be due to several reasons. First, apolipoproteins other than Apo A-IV such as Apo AI are involved in the activation of LCAT[30]. Second, our inability to find any significant difference in LCAT activity between patients and controls might be due to the relatively small sample size. Previous studies have shown that the ratio of unesterified cholesterol to esterified cholesterol is an index of LCAT activity[31]. Thus, the observed downregulation of Apo A-IV in the present study might result in a decreased activation of LCAT and consequently to lower level of esterified cholesterol in individuals with deliberate self-harm. Maes *et al*[28] proposed that the altered ratio of esterified cholesterol to free cholesterol in patients with attempted suicide may alter membrane viscosity, which might influence the functional activity of various neurotransmitter systems such as serotonin[32]. It has been shown that the presence of relatively high levels of cholesterol in cell membranes might impact its integrity.

While it has been well established that peripheral cholesterol metabolism and cholesterol metabolism in the CNS are independently regulated, impairment in blood-brain barrier permeability has been noted in patients with attempted suicide[33]. In a study on adolescents diagnosed with first-episode psychosis and mood disorder, mean serum S100B level which is indicative of increased permeability of blood-brain barrier[34], was significantly higher in adolescents with a high suicidal ideation[35]. Thus, it is possible that alterations in peripheral cholesterol metabolism may influence CNS mechanisms linked to suicidal behaviour.

A plasma-based proteomic study reported that various proteins involved in coagulation and inflammatory pathway were differentially regulated in patients diagnosed with major depressive disorder (MDD) who attempted suicide compared to MDD patients without suicide attempt[36]. In the present study, the majority of the suicidal attempts were impulsive in nature and in response to psychosocial stressors and did not have a diagnosable psychiatric condition.

Our study had a few limitations. The sample size was relatively small, partly due to exclusion of several individuals who developed medical complications as a result of deliberate self-harm and also due to inability to obtain a written informed consent in some with altered sensorium. In addition, the healthy controls, although were age and gender matched with patients, might not have been truly representative of general population as they were drawn from the institution. Furthermore, the majority of our patients did not have a diagnosable psychiatric illness.

In conclusion, downregulation of Apo A-IV, impaired reverse cholesterol transport, decreased level of esterified and total cholesterol and lower level of HDL were observed in individuals with deliberate self-harm compared to matched controls. The observed downregulation of Apo A-IV may be the link between the previously reported association of altered cholesterol levels with impulsivity and suicide. The findings need to be replicated in a larger sample including patients with a diagnosable psychiatric illness who have either attempted suicide and/or at risk of attempting suicide.

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