Original Article

# A distinct serum protein pattern in patients with paranoid schizophrenia

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## Abstract

**Introduction:** Schizophrenia is a chronic mental illness, whose aetiology is still unclear; therefore, information about differences in serum protein patterns may improve the understanding of the pathophysiology of schizophrenia. The goal of this study was to use the proteomic approach to identify altered protein levels in the serum samples from patients with schizophrenia.

**Methods:** Blood was collected from 10 patients with paranoid schizophrenia and 10 healthy volunteers matched by sex and age. Serum proteins were isolated by 2D gel electrophoresis. Proteins with altered levels were identified by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry.

**Results:** We uncovered major changes in the expression of such proteins as apolipoproteins of classes A4 and C3, transthyretin (TTR) and serum amyloid A1. An increase in expression was found only for apolipoprotein A4, whereas the expression of apolipoprotein C3, TTR and serum amyloid A1 was decreased. The observed differences in the expression of serum proteins (TTR and serum amyloid) are in good agreement with the results obtained by other research groups during analyses of cerebrospinal fluid or post-mortem brain tissues by other methods.

**Conclusion:** Such alterations of the expression of these proteins may indicate problems with regulation, for example, in the synthesis. On the other hand, the altered protein expression may simply reflect the pathophysiological status of patients, where these proteins could be candidates for biomarkers. Further research is needed to confirm the significance of the altered levels of these proteins in the pathogenesis of schizophrenia and to determine their suitability as biomarkers of schizophrenia.

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# Introduction

Although in recent years, great progress has been made in reducing mortality and in the treatment of common illnesses such as cancer and cardiovascular disease, the mortality caused by mental disorders remains unchanged (Kessler et al., 2005).

Schizophrenia (Sch) is a chronic mental illness, whose aetiology is still unclear. Schizophrenia is characterised by hallucinations, delusions (positive psychotic symptoms), affective problems (negative psychotic symptoms) and cognitive dysfunction (van Os and Kapur, 2009). A number of hypotheses have been proposed about the pathogenesis of Sch, for example, aberrations in the pathways of transmission of neurotransmitters dopamine and serotonin (Geyer and Vollenweider, 2008; Moore et al., 1999) or pathological changes in embryonic neurogenesis owing to variations in gene neuregulin-1 (Bramon et al., 2008), as well as oxidative-stress-mediated cell damage due to lowered levels of antioxidant defence enzymes in patients with schizophrenia (Ranjekar et al., 2003). Such pathogenesis may be caused by a dysfunction of some enzymes (proteins) as well as changes of their quantity in the blood of these patients.

At the same time, there is no full information about differences in serum protein patterns that can be used for typing of psychopathologies among individuals at risk of developing psychiatric disorders (Ivanova et al., 2013). Diagnosis and nosology rely on symptoms and accumulated clinical observations, and thus far, have been based mostly on interviews with patients and on patients' subjective complaints (Turck et al., 2005). Moreover, current medications still have substantial adverse effects and/or require weeks for therapeutic effects to manifest themselves; not all patients respond to current pharmacotherapy (Bystritsky, 2006). In sum, an insufficient understanding of psychiatric disorders at the molecular level and the lack of disease-specific changes in serum protein patterns prevent optimisation of diagnosis and treatment of such complex psychiatric disorders as Sch.

The proteomic approach, namely, the combination of 2D gel electrophoresis and matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS), allows researchers to reliably identify proteins isolated from human bodily fluids (Davalieva et al., 2016; Guest et al., 2016). Our exploratory study (Alekseeva et al., 2013) showed that 2D gel electrophoresis is suitable for isolation of proteins from blood of patients with mental disorders. Therefore, in the present work, clinical blood samples from patients with a diagnosis of Sch were tested to identify quantitative differences in the proteomic profile of serum.

# Materials and methods

### Chemicals
We used urea, SDS, glycerol, acetonitrile from Panreac (Spain), 3-[(3-chloramidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) from AppliChem (Germany), Bio-Lytes ampholytes from Bio-Rad (USA), dithiothreitol (DTT), HCOOH, $NH_4HCO_3$ from Sigma–Aldrich (USA), Tris-HCl, and bromophenol blue from Helicon (Russia), Coomassi Brilliant Blue G250 from Serva (Germany).

### Subjects
The study was conducted according to The Code of Ethics of the World Medical Association (Declaration of Helsinki revised in Fortaleza, Brazil, 2013) regarding experiments on human subjects. The patients in this study were recruited from the clinics of the Mental Health Research Institute (Tomsk, Russia). Written informed consent was obtained from each subject after we obtained approval of the study protocol from the Local Bioethics Committee of the Mental Health Research Institute. The inclusion criteria were a clinical diagnosis of Sch (according to International classification of diseases, 10th revision [ICD-10]: F20.0).

Groups of patients with paranoid schizophrenia and healthy volunteers were comparable by sex and age and did not differ statistically. Blood was collected from 10 subjects with Sch (six males and four females) and 10 control subjects (three males and seven females). All subjects were Caucasian inhabitants of the Tomsk region. The age of the patients was from 24 to 57 years (35 ± 13 years). The age of healthy group was from 26 to 56 years (39 ± 11 years). Blood sampling was carried out at the time of patient's admission to hospital, before prescribing psychotropic therapy. At the time of admission to the clinic, patients with Sch (7 from 10) were under the influence of maintenance therapy by Risperidone (4-6 mg/daily) or Haloperidol (10 mg/daily).

### Sample preparation
Venous blood (5 ml) was collected into a BD vacutainer anticoagulant (EDTA) tube between 07:00 and 08:00 am to avoid circadian fluctuation of the measured parameters. The blood was centrifuged

immediately after the sampling. After centrifugation (3000 rpm, 10 min, Eppendorf AG 22331), serum was separated and stored at −70 °C prior to proteomic analysis.

**Depletion of high-abundance plasma proteins**

Serum samples (500 µl) were subjected to procedures for removal of the proteins abundant in serum (i.e., transferrin, albumin, IgA, IgG, anti-trypsin and haptoglobin) by means of immunodepletion, i.e., an antibody-conjugated spin column: Multiple Affinity Removal Column Human 6 (Agilent Technologies, USA) (Pesic et al., 2011).

**Two-dimensional gel electrophoresis**

The 2DE technique was implemented in the same way as described in a study of Nasu-Hakola disease (Giuliano et al., 2014). Samples of each protein extract were applied to nonlinear (NL) gradient (pH 3–10), immobilised pH gradient (IPG) gel strips (18 cm long, Bio-Rad, USA). The pre-treatment procedure involved rehydration of the gel strips in a buffer composed of 9 M urea, 4% (w/v) CHAPS, 100 mM DTT, 0.2% (w/v) Bio-Lytes ampholytes and a small amount of bromphenol blue. Rehydration was carried out for 12 h at 20 °C at 50 V. The same voltage regimen was used for isoelectrofocusing of nonlinear gradient (pH 3–10) strips in accordance with a program in the Bio-Rad Ettan IPGphor system (15 min at 250 V, linear ramping from 150 to 10000 V in 5 h, and 5 h at 10000 V). The focused IPG strips were kept for 15 min at 25 °C in the following buffer: 6 M urea, 2% (w/v) SDS, 375 mM Tris-HCl (pH 8.8), 20% glycerol and 130 mM DTT. After the isoelectrofocusing stage, the strips were immobilized with 0.5% low-melting-point agarose and placed into a 20 × 18 cm slab (8–17% SDS-polyacrylamide gels). Electrophoresis was run at 40 mA (constant) in a PROTEAN II xi 2-D Cell device (Bio-Rad). The resulting 2DE gels were subjected to staining with 'Blue silver' (colloid Coomassie G-250), as described elsewhere (Candiano et al., 2004).

**Gel analysis**

Images of the stained gels were captured by means of a VersaDoc Imaging Model 3000 system (Bio-Rad, USA). Protein spots for subsequent analyses were selected manually using visual differences (in spot density in gels) between patients and healthy donors.

Spot densities were automatically quantified by an area-based approach (Brauner et al., 2014) using the Gel-Pro Analyzer (Media Cybernetics) software, version 4.0. To quantitate the spots, the so-called volume (% vol) was calculated as the sum of the pixel intensities in a 'spot area'. To calculate the volume represented by a protein spot on Coomassie-stained gels, the individual volume of each spot (SP) was divided by the sum of the volumes of three proteins (named *a*, *b*, and *c*) which served as a reference due to invariability of their expression levels (% vol = $SP_n/(SP_a + SP_b + SP_c)$), where n is the identification number corresponding to the selected protein spot on the 2D gel; a, b, and c are three invariable proteins. These three proteins are isoforms of apolipoprotein L (ApoL) (Lepedda et al., 2013). This procedure was performed on each of the three gels for each sample, i.e. 3 (gels) × 10 (samples) × 2 (groups) = 60 times. The fold change was calculated for each protein spot as ratio between mean values of Sch (% vol) and control (% vol). Only those spots with the fold change greater than 0.2 were selected for MALDI-TOF MS/MS analysis.

**In-gel digestion**

The digestion was carry out as described in (Giuliano et al., 2014). The selected spots were cut out of the 2D gel, sliced into smaller pieces and incubated on a thermo block at 25 °C with shaking for 15 min in a washing solution (a 1:1, v/v, mixture of 0.1 M $NH_4HCO_3$ and $CH_3CN$, pH 8.0). This protocol was repeated until destaining was complete. After the washing solution was removed (vacuum, 10 min, 60 °C), the pieces of the gel were resuspended in 30 µl of 50 mM $NH_4HCO_3$ pH 8.0 and digested with sequencing grade trypsin (Promega, USA; 0.5 µg, overnight, 37 °C). Next, the resultant peptides were sequentially extracted from the gel via incubation (15 min, 37 °C) with 30 µl of 50% $CH_3CN$, then with 0.1% HCOOH, and lastly with 50 µl of $CH_3CN$ (100%). Every extraction step included 10-min stirring with subsequent centrifugation and discarding of the supernatant. The first supernatant and the solutions obtained by means of the above extraction steps were mixed and dried. For subsequent MS analyses, the peptide mixture was solubilised in 10 µl of 0.1% HCOOH.

## Protein identification by MALDI-TOF MS/MS and peptide mass fingerprinting

Mass-spectrometry analysis was carried out in the Core Facility of Mass Spectrometric Analysis (ICBFM SB RAS). The HCCA (α-Cyano-4-hydroxycinnamic acid) matrix was prepared by the dried-droplet method. One microliter of each peptide extract was manually spotted onto a MTP 384 ground steel target (Bruker Daltonics) and dried at ambient temperature. The proteins were identified using peptide mass fingerprinting followed by database searches. In case of ambiguous identification, the proteins were identified by fragment ion analysis. MS and MS/MS spectra were obtained on an Autoflex Speed mass spectrometer (Bruker Daltonics, Germany). The peptide calibration standard, Peptide Mix II (Bruker Daltonics), was used for external calibration. MS and MS/MS spectra were recorded in positive reflective mode in the range 600–4000 Da by means of the FlexControl software (Bruker Daltonics) and were analysed in the FlexAnalysis software (Bruker Daltonics). The NCBI database and MASCOT software (Matrix Science, London, UK) were used for the searches of peptide masses to identify proteins (Perkins et al., 1999). The database searches were done at the following settings: trypsin cleavage, one missed cleavage allowed; mass accuracy of the precursor and fragment ions, 150 ppm and 0.5 Da, respectively. For further consideration, only the proteins that were detected in all three gels for each sample were assumed to be identified definitively.

## Statistical analysis

To verify reproducibility of the data, 2DE maps were obtained in triplicate for each of 10 samples in two groups. Statistical analysis of % vol spots was performed using Statistica 13.0 software (TIBCO Software Inc., CA, USA). Resulting data are presented as mean ± SD. Statistically significant differences in the level of protein expression between patients with schizophrenia and healthy donors were evaluated using the Student's t-test analysis. The criterion of statistical significance was $P<0.05$.

# Results

In this study, we examined blood serum of healthy people and patients with paranoid schizophrenia to search for quantitative and/or qualitative differences in proteins associated with this mental disorder. We
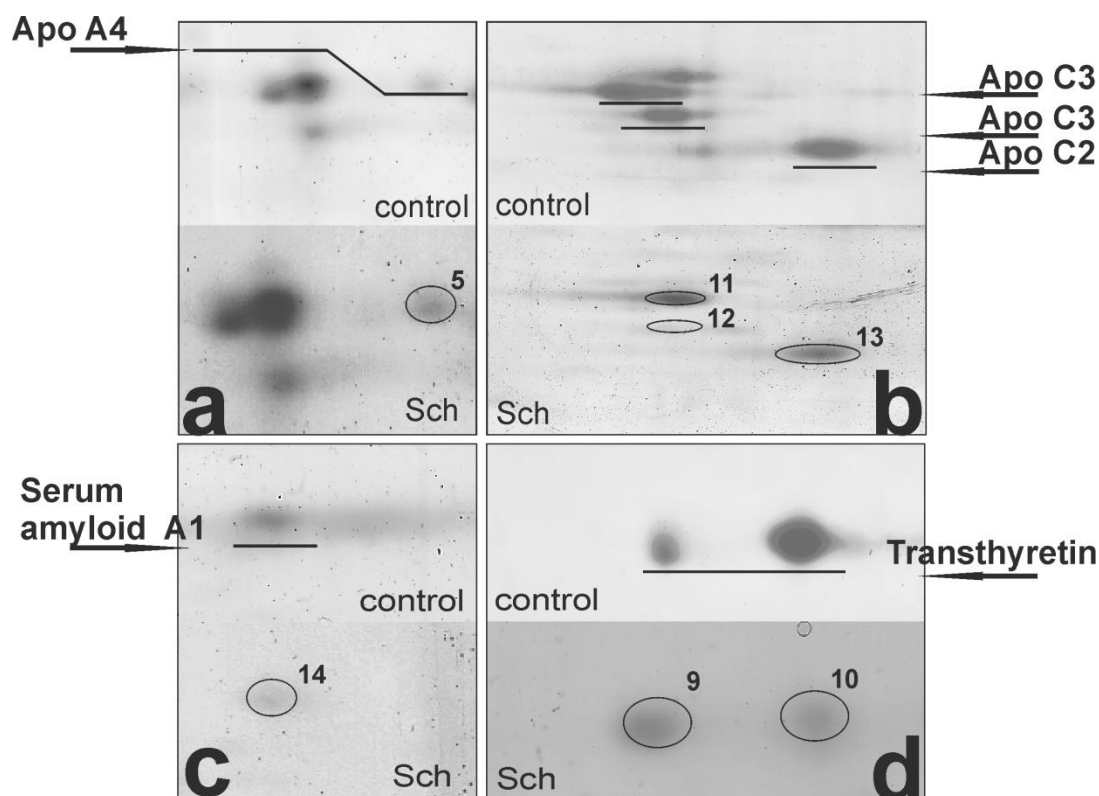


**Fig.1.** Examples of proteins with differential expression in human serum are presented in the enlarged sections of the 2DE profile. a) Apolipoprotein A4 (Apo A4); b) Apolipoprotein C3 and C2 (Apo C3 and C2); c) serum amyloid A1; d) transthyretin (the protein is present in two spots because of post-translational modifications). Sch: schizophrenia.

**Table 1:** The list of proteins from human serum analysed by 2DE and identified by MALDI-MS/MS after in-gel digestion with trypsin. Values are shown as mean ± SD.

| Protein | NCBI database no. | Protein ID | pI | MW (kDa) | Score (individuals/ significant) | Sch (% vol) | Control (% vol) | Fold Change (+/-)* Sch vs control |
|---|---|---|---|---|---|---|---|---|
| 1 | gi157831596 | α2-antitrypsin | 5.37 | 44.3 | 48/45 | 3.39 ± 0.42 | 4.48 ± 0.32 | -1.32 |
| 2 | gi338305 | SP 40 | 5.74 | 36.7 | 17/10 | 1.8 ± 0.21 | 2.35 ± 0.17 | -1.31 |
| 3 | gi3337390 | Haptoglobin | 6.14 | 38.2 | 35/29 | 2.14 ± 0.13 | 1.75 ± 0.21 | +1.22 |
| 4 | gi114318993 | Transthyretin (dimer) | 5.16 | 20.2 | 120/84 | 2.26 ± 0.35 | 3.8 ± 0.25 | -1.68 ** |
| 5 | gi11957960 | Apolipoprotein A4 | 5.28 | 28.9 | 43/32 | 3.77 ± 0.66 | 2.05 ± 0.31 | +1.84 ** |
| 6 | gi223976 | Haptoglobin hp2α | 6.23 | 41.7 | 39/20 | 1.67 ± 0.23 | 2.3 ± 0.21 | -1.38 |
| 7 | gi296653 | Haptoglobin hp2α | 6.23 | 41.5 | 18/10 | 1.24 ± 0.18 | 1.76 ± 0.13 | -1.42 |
| 8 | gi296653 | Haptoglobin hp2α | 6.25 | 41.5 | 39/15 | 1.48 ± 0.23 | 2.32 ± 0.4 | -1.56 |
| 9 | gi4507725 | Transthyretin | 5.52 | 15.9 | 75/52 | 1.86 ± 0.08 | 1.98 ± 0.15 | -1.06 |
| 10 | gi4507725 | Transthyretin | 5.52 | 15.9 | 65/60 | 1.61 ± 0.14 | 2.34 ± 0.31 | -1.45 ** |
| 11 | gi4557323 | Apolipoprotein C3(1) | 5.23 | 10.8 | 112/70 | 1.33 ± 0.35 | 2.46 ± 0.39 | -1.85 ** |
| 12 | gi4557323 | Apolipoprotein C3(2) | 5.23 | 10.8 | 115/68 | 1.62 ± 0.48 | 5.31 ± 1.77 | -3.28 ** |
| 13 | gi4557323 | Apolipoprotein C2 | 5.42 | 11.2 | 104/78 | 1.82 ± 0.32 | 2.28 ± 0.48 | -1.25 |
| 14 | gi40316910 | Serum amyloid A1 | 6.28 | 13.5 | 134/90 | 1.98 ± 0.61 | 4.63 ± 1.05 | -2.34 ** |
| 15 | gi19626079 | Albumin fragment | 6.20 | 22.4 | 56/40 | 1.35 ± 0.28 | 2.78 ± 0.63 | -2.06 ** |

* The fold change is ratio between Sch (% vol) and control (% vol). Symbols "-" and "+" mean a 'decrease' and 'increase' of protein levels of patients with schizophrenia, respectively.

** Differences between Sch and control were significant at the $P<0.05$
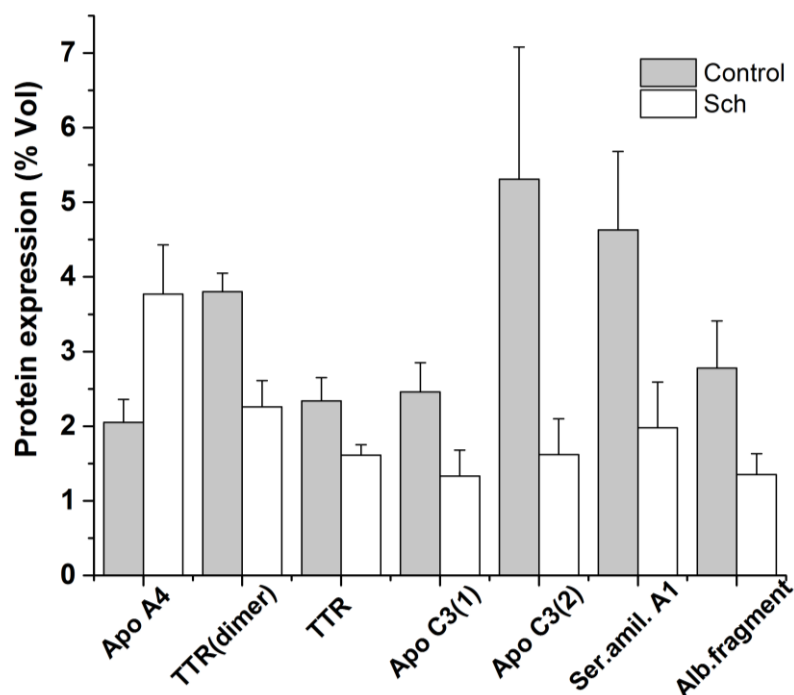


**Fig.2.** Relative plasma proteins expressions are presented of all found proteins with statistical significance difference ($P<0.05$) in protein levels between healthy people (control) and patients with schizophrenia. Apo A4 – apolipoprotein A4; TTR – transthyretin; Apo C3 - apolipoprotein A3; Ser. amil. A1 – serum amyloid A1; Alb. fragment - albumin fragment. Significant differences are determinate by unpaired Student's t-test. Data are expressed as mean ± SD.

analysed differences in serum protein patterns by comparing the gels between the patients and healthy controls. The analysis of protein patterns in serum was focused on those protein spots that differed in 2D gels between the patients and healthy controls. As a result, 15 protein spots were isolated and identified by peptide mass fingerprinting and MS/MS analysis. The list of proteins identified in the NCBI database is shown in Table 1. Some proteins – haptoglobin, transthyretin (TTR), and apolipoprotein C3 – shown in Table 1 are present in more than one spot on a gel and have different pI values. Perhaps this phenomenon is due to various post-translational modifications or partial processing. It was found that only the serum level of ApoA4 was increased (1.8-fold) as compared to the control group (Figs. 1a and 2).

The decrease in the serum concentration relative to the control group was observed for ApoC3 and for ApoC2 in patients with schizophrenia (Figs. 1b and 2). This downregulation was on average from 1.8- to 3-fold for ApoC3, and smaller for ApoC2: only 1.25-fold. Other proteins with a decreased concentration are serum amyloid A1 (2.3-fold), albumin fragment (2-fold) and TTR (Figs 1c, 1d and 2). In the case of TTR, we observed a decrease in the serum concentration of its dimer and one of monomeric forms (protein 10 in Table 1) among the patients with schizophrenia (~1.7-fold and ~1.5-fold, respectively), whereas the serum level of another TTR monomeric form was found to be unchanged relative to the control group (protein 9 in Table 1).

# Discussion

This study shows the use of proteomic analysis of human plasma for determination of altered protein levels of patients with schizophrenia. Our findings support other reports on altered protein levels in serum and cerebrospinal fluid in schizophrenia (Chow and Loh, 2011; Yang et al., 2006). For example, it was observed a 1.23-fold increase of ApoA4 level in patients with schizophrenia (Yang et al., 2006).

Albumin fragment is a part of major protein – albumin. In the procedure of sample preparation we purified the serum from whole major proteins such as albumin by means of immunodepletion. Unspecific albumin fragment could not be removed at this stage. In case if ratio of this albumin fragment to whole albumin is the same both for patients with schizophrenia and control group, concentration decreasing of albumin fragment could represents change in level of expression the albumin. Such decrease in serum albumin levels in patients with schizophrenia were found previously (Huang, 2002; Pae et al., 2004). Nevertheless, we do not consider changes in albumin concentration to be reliable, due to depletion stage of high-abundance plasma proteins in the procedure of sample preparation.

Apolipoproteins ApoC2 and ApoC3 are synthesised in the liver and are components of very low-density lipoproteins (VLDLs). Apolipoprotein C2 activates extrahepatic lipoprotein lipase, whereas apolipoprotein C3 can inhibit lipoprotein lipase and activate LCAT (Eckel, 1989; Wang and Eckel, 2009). Previously, it was found that the expression of apolipoproteins is altered in schizophrenia, bipolar disorder and other psychiatric disorders (Zheng et al., 2012). The authors found that low-density lipoproteins (LDLs) and VLDLs are the most prominent factors differentiating depressed patients from healthy controls, and that plasma unsaturated lipid concentrations are elevated in the depressed group. Thus, there is growing evidence that deregulated lipid homeostasis may play a common role in the pathophysiology of psychiatric disorders such as schizophrenia.

Inflammatory amyloid A1 is among the so-called acute phase proteins, which have both direct and indirect bactericidal and/or bacteriostatic properties. According to the classical theory of inflammation, in the acute phase of inflammation, the serum concentration of amyloid A1 increases 100- to 1000-fold (Baumann and Gauldie, 1994), whereas in our study, we observed a 2.3-fold decrease in the serum concentration of this protein in patients with schizophrenia. Perhaps this result is due to decreased immunity in patients with psychiatric disorders or to the presence of comorbidities. In addition, it is possible that a decrease in serum amyloid A1 concentration is related to downregulation of antioxidant-defence enzymes in patients with schizophrenia (Ranjekar et al., 2003) because high-density lipoproteins (HDLs) inhibit oxidative modification of LDLs via the activity of their associated enzymes and apolipoproteins (Vohnout et al., 2011). If HDLs become so-called "dysfunctional HDLs" because of accumulation of oxidants derived

from an inflammatory reaction, such HDLs inhibit the HDL-associated antioxidant enzymes and reduce the ability of apolipoproteins A1 to promote ABCA-1-mediated cholesterol efflux (Vohnout et al., 2011). In the literature, there are data on a strong positive relation between cholesterol levels and pathophysiological features of mood disorders. The link between mental health (brain) and cholesterol is believed to be based on hypothetical neuron-associated mechanisms. Cholesterol is an integral component of the plasma membrane of neurons and is present in myelin. Furthermore, cholesterol performs crucial functions in the development, stability, and workings of the synapse (Chattopadhyay and Paila, 2007). Overall, aberrations in cholesterol in a psychiatric illness may substantially affect the mood via synaptic stability and lowered serotonergic activity.

TTR is a liver-derived secretory protein and is the major serum carrier of thyroid hormones: thyroxine and tri-iodothyronine. TTR is also involved in the transport of retinol via an interaction with retinol-binding proteins. Several studies were conducted in an attempt to identify disease biomarkers that could advance the understanding of the pathogenesis of schizophrenia. In some of these studies, a link between TTR and schizophrenia was found (Huang et al., 2006; Wan et al., 2006). In ref. (Huang et al., 2006), it was estimated that 3% of TTR in ventricular cerebrospinal fluid and 10% of TTR in lumbar cerebrospinal fluid are derived from blood. To assess the involvement of blood TTR in the changes observed in the cerebrospinal fluid of patients with schizophrenia, those authors also studied serum TTR levels in the same people (simultaneously with cerebrospinal fluid collection) by an ELISA. They observed a significant moderate decrease in TTR concentration in serum samples of patients with schizophrenia compared to controls. Nevertheless, there was no association between cerebrospinal-fluid and serum TTR levels in the same individuals, indicating that the protein levels of TTR are regulated by different systems in serum and in cerebrospinal fluid.

# Conclusion

In the present study, we identified differentially expressed proteins in the serum from patients with schizophrenia by proteomic analysis. We showed differential expression of such proteins as TTR, serum amyloid A1 and apolipoproteins of classes A4 and C3. Furthermore the increase in the expression was found only for apolipoprotein A4, whereas the expression of apolipoprotein C3, TTR and serum amyloid A1 was decreased.

Such alterations of the expression of these proteins may indicate problems with regulation, for example, in the synthesis. On the other hand, the altered protein expression may simply reflect the pathophysiological status of patients with schizophrenia, where these proteins could be candidates for biomarkers. Nevertheless, to confirm the significance of the altered levels of these proteins in the pathogenesis of schizophrenia, and to determine their suitability as biomarkers of schizophrenia, further research is needed.

# Conflict of interest

The authors declare that they have no conflicts of interest related to the contents of this article.

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