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This is the peer reviewed version of the following article:

Original:

Cortelazzo, A., De Felice, C., Guerranti, R., Signorini, C., Leoncini, S., Zollo, G., et al. (2016). Expression and oxidative modifications of plasma proteins in autism spectrum disorders: Interplay between inflammatory response and lipid peroxidation. *PROTEOMICS. CLINICAL APPLICATIONS*, 10(11), 1103-1112 [10.1002/prca.201500076].

Availability:

This version is available <http://hdl.handle.net/11365/1008483> since 2017-05-25T16:28:03Z

Published:

DOI:10.1002/prca.201500076

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Expression and oxidative modifications of plasma proteins in autism spectrum disorders: interplay between inflammatory response and lipid peroxidation

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Received: 07/14/2015; Revised: 05/04/2016; Accepted: 05/30/2016

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1002/prca.201500076](#).

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Abbreviations: **ASDs**, autism spectrum disorders; **APR**, acute-phase response; **OS**, oxidative stress; **4HNE**, 4-hydroxynonenal

Keywords: Acute-phase response proteins; 4-hydroxynonenal protein adducts; Autism spectrum disorders

Abstract

Purpose: A role for inflammation and oxidative stress is reported in autism spectrum disorders (ASDs). Here, we tested possible changes in expression and/or oxidative status for plasma proteins in subjects with ASDs.

Experimental design: To evaluate protein expression and protein adducts of lipid peroxidation-derived aldehyde, analysis of plasma proteins was performed in 30 subjects with ASDs and compared with 30 healthy controls with typical development, using a proteomic approach.

Results: Significant changes were evidenced for a total of 12 proteins. Of these, 10 were identified as proteins involved in the acute inflammatory response including alpha-2-macroglobulin, alpha-1-antitrypsin, haptoglobin, fibrinogen, serum transferrin, prealbumin, apolipoprotein A-I apolipoprotein A-IV, apolipoprotein J and serum albumin. In addition, significant changes occurred for 2 immunoglobulins alpha and gamma chains.

Conclusions and clinical relevance: Our present data indicate that an inflammatory response, coupled with increased lipid peroxidation, is present in subjects with ASDs. This information can provide new insight into the identification of potential plasma protein biomarkers in autism.

Statement of clinical relevance

Autism spectrum disorders (ASDs) are behaviourally defined neurodevelopmental disorders with clinical onset usually within the second year of life. A role for inflammation and oxidative stress processes is reported in ASDs. Identifying circulating markers related these two pathological processes may have high clinical significance to diagnose ASDs. Application of proteomics for identifying protein markers has an immense potential to gap this goal, given that no reliable biomarkers are, to date, detectable in ASDs. In our study, major expression changes in proteins identified as acute-phase response (APR) reactants, either positive or negative, were evidenced in the plasma samples from ASDs subjects. In addition, by using 4-hydroxynonenal as a lipid peroxidation marker, increased level of oxidative stress was evidenced for specific APR proteins. Therefore, the physiological function of the altered proteins could be compromised, with a series of predictable bioclinical consequences. Moreover, our findings revealed the potential of coupling clinical chemistry with classic bottom up and redox proteomics in order to underscore the coexistence of subclinical inflammatory and oxidative stress processes in ASDs, not detectable by traditional routine clinical chemistry.

1 Introduction

Autism spectrum disorders (ASDs) are behaviourally defined neurodevelopmental disorders with clinical onset usually within the second year of life [1], and mainly consist in social impairment, communication difficulties and restricted, repetitive, or stereotyped patterns of behaviour [2]. ASDs prevalence has raised from 1 in 5000 in the mid-1970s to 1 in 68 in 2010 in the USA, with a higher well known prevalence in boys (1 in 42) [3, 4]. To date, diagnosis of ASDs is based on the Diagnostic and Statistical Manual of Mental Disorders [5], while the cause remains unknown and is likely the result of a complex interaction between environmental factors and the genetic background [6]. A link between oxidative stress (OS) and the pathogenesis of ASDs has been reported by several research groups [7, 8], including our laboratory [9]. Whether redox imbalance is a cause or a consequence of autism it remains unclear [10]. In particular, decreased levels of two major plasma antioxidant proteins, i.e., ceruloplasmin and transferrin, have been associated in ASDs with increased lipid peroxidation [11]. Changes in plasma proteins by electrophilic lipoxidation products have the potential to lead to dysfunctional physiological consequences, including cell dysfunction, inflammatory response and apoptosis [12]. OS a key process in the pathogenesis and progression of several human diseases, including neuropsychiatric disorders, in which lipid peroxidation is a critical component [13-15].

Recent research suggests that inflammation may play a role in the pathophysiology of disorders marked by social cognitive deficits (i.e., autism and schizophrenia) [16, 17]. Increasing evidence indicates a possible role for inflammation in ASDs, as mediated by both central and peripheral mechanisms [18-23]. In particular, at the peripheral level, earlier suggestions indicate a possible involvement of proinflammatory cytokines and plasma protein changes [24-26].

Here, we applied proteomics to detect plasma protein changes in subjects with ASDs, in order to explore possible changes in expression and/or oxidative status of potential biomarkers of key physiopathological mechanisms.

2 Materials and methods

2.1 Clinical samples

A total of 30 subjects with ASDs (24 male, 6 female; mean age: 12.0 ± 3.0 years), as well as 30 healthy subjects of comparable age, and gender (20 male, 10 female; mean age: 11.7 ± 3.3 years) were enrolled. The autistic subjects were consecutively admitted to the Child Neuropsychiatry Unit of the University Hospital of Siena (Head: J.H.), and ASDs were diagnosed according with the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition [5], and evaluated using Autism Diagnostic Observation Schedule and Autism Behaviour Checklist. None of the autistic and healthy subjects showed clinical symptoms or signs of acute or chronic inflammation and/or infection. No specific comorbidities in the autistic cohort were present with the single exception of a coexisting celiac disease in one patient. None of the subjects were on anti-inflammatory or antioxidant drugs. On the other hand, in our case series only one third (10 out of 30) of the evaluated ASDs subjects were on psychotropic drugs: carbamazepine monotherapy (n = 2), risperidone monotherapy (n = 2), aripipazole monotherapy (n = 1), valproate monotherapy (n = 1), olanzepine plus valproate (n = 1), pericyazine plus valproate (n = 1), risperidone plus valproate (n = 1), topiramate plus valproate (n = 1). All the examined subjects were on a typical Mediterranean diet. The study was conducted after the approval by the Institutional Review Board and all written informed consents were obtained from either the parents or the legal tutors of the enrolled subjects in this study, in compliance with national legislation and the Code of Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association (Declaration of Helsinki). All samplings were carried out around 8 a.m. after overnight fasting. Blood was collected in heparinized tubes and all manipulations were carried out within 2 h after sample collection. Blood samples were centrifuged at 2400g for 15 min at 4°C, the platelet poor plasma was saved and the buffy coat was removed by aspiration. Plasma samples, contained phenylmethylsulfonyl fluoride (1 mM) as a protease inhibitor, were stored at -70°C until use.

2.2 Routine clinical biochemistry

All the hematology and clinical chemistry parameters were performed at the University Hospital Laboratory of Clinical Pathology (Siena, Italy). All clinical chemistry analytes were analyzed on Cobas 6000 system (Roche Diagnostic), using dedicated reagents and methods. Erythrocyte sedimentation rate (ESR) was performed by measuring the kinetics of aggregation of red blood cells by quantitative capillary photometry on TEST 1 analyzer

(Alifax). Complement component 3 (C3) and 4 (C4) were analyzed on BNII instrument (Siemens Healthcare) that uses the nephelometric technology. Prothrombin time and plasma fibrinogen concentration were analyzed on BCS-XP coagulometer (Siemens Healthcare). Albumin/globulin ratio was determined using the CAPILLARYS electrophoresis instrument (Sebia).

Lipid profile analytes including total cholesterol, high density lipoproteins (HDL) and triglycerides were determined by specific colorimetric enzymatic methods on Cobas 6000 system (Roche Diagnostic). Urine pH and urinary specific gravity values were determined by using Atlas instrument (Siemens Healthcare), and urine sediment was analyzed on Iris instrument (Beckman Coulter). The differential count of leukocytes was performed on the automated cell counters Sysmex-XE 2100 (Dasit) (Supplementary Table 1).

2.3 Protein expression analysis

Two-dimensional gel electrophoresis (2DE) was performed according to Görg et al. [27]. Samples containing 60 µg of proteins, determined with bicinchoninic acid protein assay reagent kit (Thermo Scientific), were combined with solubilizing buffer containing 8 M urea, 2 % 3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate, 0.3 % dithiothreitol (DTT), 2 % immobilized pH gradient (IPG) buffer. Samples were loaded into IPG strips 3-10 non linear on an Ettan IPGphor apparatus system (GE Healthcare, Uppsala, Sweden) and rehydrated for 7 h. Isoelectric focusing was carried out for a total of 32 kV h. After focusing, strips were combined with equilibration buffer containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 2 % w/v sodium dodecyl sulfate, 30 % v/v glycerol, and 1 % w/v DTT for 15 min. Then strips were equilibrated again with the same buffer except it contained 4 % w/v iodoacetamide instead of DTT and a trace of bromophenol blue. Strips were washed further for 10 min with Tris-glycine buffer. The second dimension was performed on an electrophoresis apparatus. IPG strips and a molecular weight standard were embedded at the top of a 1.5 mm thick vertical polyacrylamide gradient gel (8-16 % T) using 0.5 % w/v agarose and run at a constant current of 40 mA/gel at 20°C. Gels were stained by mass spectrometry (MS) compatible silver staining [28]. The exposure time for silver staining was also optimized in order to avoid overexposure of some gels with respect to others. Resulting electrophoretic gels were digitized and analyzed by ImageMaster 2D Platinum v7.0 software

(GE Healthcare). A reference gel image is shown in Figure 1, while other matched gels are reported as Supplementary Figure 1.

2.4 Oxidative post-translational modifications (PTMs) analysis

Samples containing 80 µg of proteins were subjected to 2DE, as previously described. Gels were electro-transferred (0.8 mA/cm²; 1 h, 40 min) onto a hybond enhanced chemiluminescence nitrocellulose membranes (GE Healthcare Europe GmbH, Milan, Italy) using a Pharmacia Biotech Nova Blot semi-dry transfer instrument. Resulting membranes were blocked for 1 h in Tris-buffered saline (TBS), pH 7.5, containing 0.5 % Tween 20 (TBST) and 3 % nonfat dry milk. Membranes were incubated overnight at 4°C with the primary antibody, goat anti 4-hydroxynonenal (4HNE) (1:1000) (Millipore Corporation, Billerica, MA, USA). Membranes were subsequently incubated for 1 h with the secondary antibody, mouse anti-goat IgG, horseradish peroxidase (HRP)-conjugated (1:2000) (Santa Cruz Biotechnology, Inc., CA). Bound antibodies, after extensive rinsing in TBST, were detected by using Immun-Star HRP Chemiluminescent Substrate (Bio-Rad, Milan, Italy). Western blot membranes were digitized and analyzed by Quantity One Imaging system (ChemiDoc XRS, Bio-Rad, Hercules, CA). A reference membrane image is showed in Figure 1, while other matched membranes are reported as Supplementary Figure.

In order to confirm that the most abundant identified proteins by MS/MS are also the most oxidized ones, we tested at least one protein by an orthogonal technique, i.e., immunoprecipitation of apolipoprotein A-I. Plasma samples (75 µl each) were incubated with 6 µl of mouse monoclonal anti apolipoprotein A-I antibody (cod. ab17278, Abcam, Cambridge, UK). Then, immune complex was incubated with 50 µl of Protein A-Sepharose (Sigma-Aldrich, Milan, Italy) and rotated at 4°C for 2 h. Samples were centrifuged at 10000g for 5 min and washed three times with 1 ml ice-cold phosphate-buffered saline. The pellet was mixed with 2X reducing sample buffer, boiled and loaded on electrophoretic gels. After western blot analysis membranes were incubated overnight at 4°C with the primary antibody, goat anti 4HNE (1:1000) (Millipore Corporation, Billerica, MA, USA) (results are shown as Supplementary Figure 2).

In order to validate 4HNE *in vivo* results, a model oxidizing condition was applied by using pro-oxidant molecule 2,2'-azobis(2-amidinopropane)hydrochloride (AAPH, specifically used

for lipid peroxidation). Newly prepared plasma samples from our ASDs case series (n = 10) as well as healthy controls (n = 10) were randomly selected (random number generation algorithm). The AAPH model of oxidizing condition was performed incubating plasma from healthy controls and ASDs subjects with AAPH for 1h at 37°C. Both ASDs and control plasma samples, untreated or treated with AAPH, were analyzed for their relative protein oxidation. Results were reported as Supplementary Figure 3.

2.5 Protein identification

Protein spots were carefully excised and subjected to in-gel trypsin digestion according to the method of Shevchenko et al. [28]. The supernatant containing tryptic peptides was dried by vacuum centrifugation. Prior to MS analysis, the peptide mixtures were redissolved in 10 µl of 5 % formic acid. Samples were analyzed using a split-free nano-flow LC system (EASY-nLC II, Proxeon, Odense, Denmark) coupled to a 3D-ion trap (model AmaZon ETD, Bruker Daltonik, Germany) equipped with an online nano-ESI sprayer (the spray capillary was a fused silica capillary, 0.090 mm o.d., 0.020 mm i.d.). For all experiments, a sample volume of 15 µl was loaded by the auto-sampler onto a homemade 2 cm fused silica precolumn (100 µm I.D.; 375 µm O.D.; Reprosil C18-AQ, 5 µm, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). Sequential elution of peptides was accomplished using a flow rate of 300 nl/min and a linear gradient from Solution A (2 % acetonitrile, ACN; 0.1 % formic acid) to 50 % of Solution B (98 % ACN; 0.1 % formic acid) in 40 min over the precolumn in-line with a homemade 15 cm resolving column (75 µm I.D.; 375 µm O.D.; Reprosil C18-AQ, 3 µm, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). The acquisition parameters for the mass spectrometer were as follows: dry gas temperature, 220°C; dry gas, 4.0 l/min; nebulizer gas, 10 psi; electrospray voltage, 4000 V; high-voltage end-plate offset, - 200 V; capillary exit, 140 V; trap drive: 63.2; funnel 1 in, 100 V out 35 V and funnel 2 in, 12 V out 10 V; ICC target, 200,000; maximum accumulation time, 50 ms. The sample was measured with the “Enhanced Resolution Mode” at 8100 m/z per second (which allows mono isotopic resolution up to four charge stages) polarity positive, scan range from m/z 300 to 1500, five spectra averaged, and rolling average of 1. The “Smart Decomposition” was set to “auto”. Acquired collision-induced dissociation spectra were processed in DataAnalysis 4.0, and deconvoluted spectra were further analyzed with BioTools 3.2 software and submitted to Mascot search

program (in-house version 2.2, Matrix Science, London, UK). A list identified plasma proteins from ASDs and healthy control plasma samples is shown in Table, whereas the inclusion criteria for protein identification and selection (i.e., peptide sequences and the relative score, peptide charge status) are reported in Supplementary Table 2.

2.6 Statistical analysis

Spot volume was expressed as a ratio of the percentage volume detected from the entire gel/membrane to minimize differences between samples (normalization). Only spots appearing in all gels/membranes of the same group were matched with those of the reference gel/membrane. The background was subtracted from all gels/membranes using the average-on-boundary method. All results were expressed as mean \pm SD. Statistical analysis of protein variations was performed using multiple *t*-test with a False Discovery Rate (*q*) of 0.05 (Graph-Pad Prism 6.01; GraphPad Software, Inc., CA, USA). A two-tailed *p*-value of less than 0.05 was considered statistically significant.

3 Results

3.1 Biometrics, blood pressure, routine clinical chemistry and blood cell counts

Mean body mass index, systolic blood pressure and diastolic blood pressure values for ASDs subjects were comparable with those of healthy controls. A total of 63.3% of patients were of normal weight, 26.7% overweight and 10.0 % underweight.

Interestingly, no significant changes in routine inflammatory markers such as ESR, high sensitivity C reactive protein, C3, C4 and fibrinogen were evidenced, as compared to healthy controls. Levels of markers for renal and liver function were within the reference range. Increased levels of triglycerides ($p = 0.0069$) and total cholesterol ($p = 0.032$) were observed. In addition, ASDs patients showed a relative neutrophilia ($p = 0.041$), lymphopenia ($p = 0.024$) with a decreased eosinophil counts ($p = 0.021$). Biometrics, blood pressure, routine clinical chemistry values, the lipid panel and blood cell counts are provided in Supplementary Table 1.

3.2 Positive acute-phase response proteins with increased expression

Pronounced differences in plasma proteins expression between ASDs and healthy subjects were observed, with an increase of several positive acute-phase response (APR) proteins. In particular, autistic subjects showed a significantly higher expression level of three protease inhibitors: alpha-2- macroglobulin (spot 1: 2.59 ± 0.79 % V vs. control 0.25 ± 0.10 % V, $p < 0.001$), alpha-1- antitrypsin (spot 2: 11.06 ± 0.71 % V vs. control 7.94 ± 0.69 % V, $p < 0.001$; spot 3: 3.14 ± 0.79 % V vs. control 0.21 ± 0.09 % V, $p < 0.001$) (Table 1, Fig. 1a and 1b, Fig. 2a). Another notable positive APR protein significantly increased in plasma of autistic subjects is haptoglobin (spot 4: 3.54 ± 0.56 % V vs. control 1.86 ± 0.65 % V, $p < 0.001$) (Table 1, Fig. 1a and 1b, Fig. 2a).

3.3 Negative acute-phase response proteins with decreased expression

In addition to highlight positive protein expression levels, we also observed decreased levels of several negative acute-phase proteins in autistic subjects. Relevant members of this protein group are serum transferrin (spot 7: 4.97 ± 0.83 % V vs. control 7.11 ± 0.69 % V, $p = 0.026$), prealbumin (spot 8: 2.03 ± 0.41 % V vs. control 6.60 ± 0.72 % V, $p < 0.001$), and apolipoprotein J (spot 12: 1.89 ± 0.55 % V vs. control 3.65 ± 0.46 % V, $p < 0.001$) (Table 1, Fig. 1a and 1b, Fig. 2a). In addition, albumin was found to be more fragmented in the plasma of ASDs subjects.

3.4 Evidence of aldehydes-derived adducts for several plasma proteins

To evaluate protein oxidation, we compared 4HNE protein adducts between plasma of healthy subjects and ASDs (Fig. 1c and 1d). The 4HNE protein adduct signal is significantly higher in autistic subjects (40.75 ± 2.23 % V vs. control 12.22 ± 1.19 % V, $p < 0.001$), indicating that lipid peroxidation increase. Conversely, a physiological degree of 4HNE was observed also in the healthy control group (Fig. 1c). Increased 4HNE adducts are observed in autistic subjects for positive APR proteins such as alpha-1-antitrypsin (spot 2: 6.39 ± 0.26 % V vs. control 4.96 ± 0.42 % V, $p = 0.033$), fibrinogen beta chain (spot 5: 3.33 ± 0.44 % V vs. control 1.83 ± 0.48 % V, $p < 0.001$), and for negative APR proteins, such as serum transferrin (spot 7: 6.33 ± 0.18 % V vs. control 3.40 ± 0.52 % V, $p < 0.001$) and serum albumin (spot 15: 6.25 ± 0.57 % V vs. control 2.01 ± 0.31 % V, $p < 0.001$). 4HNE adducts appear in the positive APR protein fibrinogen gamma chain (spot 6: 1.88 ± 0.23 % V, $p < 0.001$), and in negative APR proteins including prealbumin (spot 8: 1.86 ± 0.36 % V, $p < 0.001$; spot 9: 6.23 ± 0.18 % V, $p < 0.001$),

apolipoprotein A-I (spot 10: 1.81 ± 0.52 % V, $p < 0.001$), and in a serum albumin fragment (spot 14: 3.45 ± 0.44 % V, $p < 0.001$). In addition, 4HNE adducts appeared in Ig alpha-1 chain C region (spot 19: 1.86 ± 0.54 % V, $p < 0.001$) (Table 1, Fig. 1c and 1d, Fig. 2b).

Result of the immunoprecipitation of apolipoprotein A-I probed with anti 4HNE antibody showed that 4HNE is specifically linked to the protein revealed as most abundant by MS/MS analysis, and that the oxidation entity is more evident in ASDs patients, as reported in 2DE experiments. Results are reported as Supplementary Figure 2. Moreover, the data of the *in vitro* model of plasma with oxidizing condition (+ AAPH), confirm that the specific oxidized proteins observed in ASDs plasma belong to the whole set of plasma protein targets of the lipid peroxidation process. After AAPH test, significant variations were observed in ASDs subjects (AAPH +) for alpha-1-antitrypsin (spot 2), fibrinogen beta chain (spot 5), fibrinogen gamma chain (spot 6), apolipoprotein A-I (spot 10), albumin (spots 13 and 14) and for unidentified spots (from spot *a* to spot *e*). Unidentified protein/spots were evidenced after AAPH treatment of plasma from ASDs subjects. Spot *c* was evidenced also in the plasma from healthy controls. Of note, the ratio between ASDs and healthy controls in untreated plasma samples (AAPH -) is around 1:3 while in the AAPH treated plasma samples is around 1:1.5. Therefore, we can infer that AAPH treatment did not results in automatically homogeneous oxidation but it likely depends on specific protein identity (Supplementary Figure 3).

3.5 Increased immunoglobulins expression

We separately examined immunoglobulin variations. Figure 1a, Figure 1b and Table 1 show one spot of immunoglobulin alpha-1 chain (spot 21: 3.90 ± 0.91 % V vs. control 2.56 ± 0.64 % V, $p = 0.015$) and one spot corresponding to immunoglobulin gamma heavy chains (spot 24: 11.69 ± 0.59 % V vs. control 7.14 ± 0.78 % V, $p < 0.001$), significantly increased in the plasma of autistic subjects as compared to healthy controls (Fig. 2a).

4 Discussion

The present study demonstrates major changes in acute-phase proteins, either in expression and/or oxidative PTMs in subjects with ASDs, thus indicating the coexistence of a subclinical inflammatory status coupled with an enhanced lipid peroxidation process. Our approach

unveiled a significantly increase of four positive APR proteins in plasma of ASDs subjects. These proteins include alpha-2-macroglobulin, alpha-1-antichymotrypsin, haptoglobin and alpha-1-antitrypsin [29, 30]. Alpha-2-macroglobulin preferentially acts as inhibitor of coagulation proteinases, thus suggesting that it could play an important role in regulating hemostatic and inflammatory reactions [31]. Alpha-1-antichymotrypsin and alpha-1-antitrypsin belong to the serpin family and possess tissue protective properties mainly observed during the inflammatory response [29]. In particular, alpha-1-antichymotrypsin preferentially inhibits cathepsin G while alpha-1-antitrypsin mainly protects tissues from extracellular neutrophil elastase [32]. As haptoglobin is considered as a positive acute-phase protein, it exerts an anti-inflammatory activity. Therefore, its increased levels could attenuate the ongoing inflammatory process in ASDs subjects [30]. Likewise, we unveiled decreased levels for five negative APR proteins such as transferrin, prealbumin, retinol-binding protein 4, fetuin-A and apolipoprotein J. The decreased amount of these negative APR proteins, needed to divert available amino acids to the production of positive APR proteins required for host defence, could result from either reduced protein synthesis or increased degradation rate [33]. To this regard, we identified differentially expressed peptides corresponding to albumin fragments that increase (already evidenced in control plasma) and appear as new proteolytic products in plasma samples from subjects with ASDs.

Two major critical questions regarding acute phase reactants in ASDs include (a) the source for the inflammatory stimuli and (b) the relationship of APR proteins with the clinical phenotype of ASDs. A possible source for proinflammatory status could reside in exogenous toxicants. Specifically, exposure to phthalates and persistence of their metabolites in the environment have been previously associated with autism [34, 35]. Environmental triggers could be also associated with the increased immunoglobulins levels observed in our examined autistic cohort, and this evidence is in line with earlier reports on immune dysfunction in autism [36, 37]. Exposure to endogenous toxic sources has been also suggested, given prior reports on increased intestinal permeability (i.e., “leaky gut syndrome”) [38]. In addition, physical and/or psychological stress can lead to an inflammatory process, likely mediated by the release of key mediators of inflammation, including acute-phase proteins [39]. Therefore, multiple sources, either exogenous or endogenous, could potentially trigger an inflammatory response in ASDs. A possible

modulatory role by psychotropic drugs both on inflammation and OS has been reported, mainly in experimental settings with often contrasting results [40-46]. However, it should be emphasized that in our case series only one third (10 out of 30) of the evaluated ASDs subjects were on psychotropic drugs. Therefore, it appears to be unlikely that associated drugs could have a relevant effect of the observed plasma proteomics changes in terms of either APR or OS.

If changes of plasma APR proteins persist, an imbalance of protein activities could be related to phenotype severity in ASDs. To this regard, our study shows a significant decrease in serum transferrin levels that, as previously suggested, can lead to abnormal iron metabolism observed in autism [7]. A decrease in prealbumin, retinol-binding protein 4 and apolipoprotein J [47-49], could be related to the more general evidence of an altered lipid metabolism already reported in this condition [50].

Our study indicates that, although the pathogenesis of ASDs remains unclear, oxidative PTMs may play a role. We suggest that the physiological function of six oxidized plasma proteins could be compromised. These proteins include positive acute-phase proteins such as alpha-1-antitrypsin, fibrinogen beta and gamma chains and negative acute-phase proteins such as serum transferrin, prealbumin, apolipoprotein A-I and serum albumin. In particular, fibrinogen shows significant oxidative PTMs for its beta and gamma chains, instead to increase like other positive acute-phase reactants. We hypothesize that this modification could be interfering on the main fibrinogen function in the hemostatic system [51]. Our evidence of serum transferrin oxidation could be explained by a lack in the defence mechanism against reactive oxygen species, as previously reported in autism [11], knowing that it acts as an antioxidant by reducing the concentration of free ferrous ion. Oxidation of apolipoprotein A-I, a major component of HDL in plasma [24, 52] could lead to a cholesterol metabolism dysregulation, as previously reported in this condition [50]. Of interest, in our clinical series we observed increased total cholesterol and triglycerides levels. In addition, increased oxidative PTMs for prealbumin and serum albumin could be related to the dysregulation of the osmotic blood pressure, binding and transport of ions, hormones and fatty acids [53]. In general, our findings indicate that there is an enhancement of OS in subjects with ASDs, in line with prior reports [7, 21]. In particular, our data confirm that the specific oxidized proteins observed in ASDs plasma belong to the whole set of oxidizable

plasma protein targets during the lipid peroxidation process. To this regard, it can be speculated that the amino acid sequence of the human plasma proteins could have potential 4HNE binding sites and/or be differentially exposed to 4HNE.

Although the source/s of OS in ASDs remain/s unknown to date, possible endogenous sources could be related to dysregulation of critical factors and/or mitochondrial dysfunction [54]. On the other hand, OS from exposure to potential exogenous sources, such as phthalates, has been reported in ASDs [35].

The mechanisms leading to redox imbalance and its consequences in autism remain unknown, to date. Overall, the causes of increased OS and the sources of oxidants need to be explored in future studies. Whether the inflammatory response observed in autistic subjects could be responsible for the behavioural symptoms of the disorder remains to be ascertained. Nevertheless, our findings suggest the presence of a complex interplay between inflammation, OS and immune dysregulation in ASDs.

Acknowledgements

We thank Drs. Pierluigi Tosi, Silvia Briani and Roberta Croci (Azienda Ospedaliera Senese) for continued support; Dr. Carlo Scapellato (Head, Laboratory of Clinical Pathology, Azienda Ospedaliera Senese) for providing useful information regarding clinical chemistry data; Prof. Lucia Morbidelli and Prof. Sandra Donnini (Department of Life Science, University of Siena, Siena, Italy) for providing anti 4HNE antibodies; Prof. Cinzia Della Giovampola and Dr. Antonietta Capone (Department of Life Science, University of Siena, Siena, Italy) for providing the experimental material for apolipoprotein A-I immunoprecipitation; Prof. Emilia Maellaro and Dr. Barbara Del Bello (Department of Molecular and Developmental Medicine, University of Siena, Siena, Italy) for providing AAPH; professional singer Matteo Setti (www.matteosetti.com) for continued interest, support and charity concerts dedicated to Rett girls and children with autism spectrum disorders; Dr. Roberto Faleri (Central Medical Library) for online bibliographic assistance. This work was partially supported by the Regione Toscana (Bando Salute 2009; “Antioxidants (ω -3 polyunsaturated Fatty Acids, lipoic acid) supplementation in Rett syndrome: A novel approach to therapy,” RT no. 142).

Conflict of interest

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The authors have declared no conflict of interest.

5 References

- [1] Fombonne, E., Is there an epidemic of autism? *Pediatrics* 2001, *107*, 411-413.
- [2] Muhle, R., Trentacoste, S. V., Rapin, I., The genetics of autism. *Pediatrics* 2004, *113*, 472-486.
- [3] Weintraub, K., The prevalence puzzle: autism counts. *Nature* 2011, *479*, 22-24.
- [4] Center for Disease Control and Prevention, Prevalence of autism spectrum disorders—autism, developmental disabilities monitoring network, 11 sites, United States, 2010. *M. M. W. R. Surveill.* 2014, *63*, 1-21.
- [5] American Psychiatric Association, *Diagnostic and statistical manual of mental disorders*, American Psychiatric Press, Washington 2013.
- [6] Herbert, M. R., Contributions of the environment and environmentally vulnerable physiology to autism spectrum disorders. *Curr. Opin. Neurol.* 2010, *23*, 103-110.
- [7] Chauhan, A., Chauhan, V., Oxidative stress in autism. *Pathophysiology* 2006, *13*, 171-181.
- [8] Frustaci, A., Neri, M., Cesario, A., Adams, J. B. et al., Oxidative stress-related biomarkers in autism: systematic review and meta-analyses. *Free Radic. Biol. Med.* 2012, *52*, 2128-2141.
- [9] Ciccoli, L., De Felice, C., Paccagnini, E., Leoncini, S. et al., Erythrocyte shape abnormalities, membrane oxidative damage, and β -actin alterations: an unrecognized triad in classical autism. *Mediators Inflamm.* 2013, doi:10.1155/2013/432616.
- [10] Palmieri, L., Persico, A. M., Mitochondrial dysfunction in autism spectrum disorders: cause or effect? *Biochim. Biophys. Acta* 2010, *1797*, 1130-1137.
- [11] Chauhan, A., Chauhan, V., Brown, W. T., Cohen, I., Oxidative stress in autism: increased lipid peroxidation and reduced serum levels of ceruloplasmin and transferrin—the antioxidant proteins. *Life Sci.* 2004, *75*, 2539-2549.
- [12] Negre-Salvayre, A., Coatrieux, C., Ingueneau, C., Salvayre, R., Advanced lipid peroxidation end products in oxidative damage to proteins. Potential role in diseases and therapeutic prospects for the inhibitors. *Br. J. Pharmacol.* 2008, *153*, 6-20.

- [13] Halliwell, B., *Free Radicals in Biology and Medicine*, Oxford University Press, Oxford 2007.
- [14] Signorini, C., De Felice, C., Durand, T., Oger, C. et al., Isoprostanes and 4-hydroxy-2-nonenal: markers or mediators of disease? Focus on Rett syndrome as a model of autism spectrum disorder. *Oxid. Med. Cell. Longev.* 2013, doi:10.1155/2013/343824.
- [15] Csala, M., Kardon, T., Legeza, B., Lizák, B. et al., On the role of 4-hydroxynonenal in health and disease. *Biochim. Biophys. Acta* 2015, 1852, 826-838.
- [16] Onore, C., Careaga, M., Ashwood, P., The role of immune dysfunction in the pathophysiology of autism. *Brain Behav. Immun.* 2012, 26, 383-392.
- [17] Fineberg, A. M., Ellman, L. M., Inflammatory cytokines and neurological and neurocognitive alterations in the course of schizophrenia. *Biol. Psychiatry* 2013, 73, 951-966.
- [18] Chauhan, D. A., Chauhan, V., Brown, T., *Autism: Oxidative Stress, Inflammation, and Immune Abnormalities*, Taylor and Francis Group, CRC Press, Florida 2009.
- [19] Becker, K. G., Autism, asthma, inflammation, and the hygiene hypothesis. *Med. Hypotheses* 2007, 69, 731-740.
- [20] Depino, A. M., Peripheral and central inflammation in autism spectrum disorders. *Mol. Cell. Neurosci.* 2013, 53, 69-76.
- [21] Rossignol, D. A., Frye, R. E., A review of research trends in physiological abnormalities in autism spectrum disorders: immune dysregulation, inflammation, oxidative stress, mitochondrial dysfunction and environmental toxicant exposures. *Mol. Psychiatry* 2012, 17, 389-401.
- [22] Zimmerman, A. W., Jyonouchi, H., Comi, A. M., Connors, S. L. et al., Cerebrospinal fluid and serum markers of inflammation in autism. *Pediatr. Neurol.* 2005, 33, 195-201.
- [23] Vargas, D. L., Nascimbene, C., Krishnan, C., Zimmerman, A. W., Pardo, C. A., Neuroglial activation and neuroinflammation in the brain of patients with autism. *Ann. Neurol.* 2005, 57, 67-81.
- [24] Corbett, B. A., Kantor, A. B., Schulman, H., Walker, W. L. et al., A proteomic study of serum from children with autism showing differential expression of apolipoproteins and complement proteins. *Mol. Psychiatry* 2007, 12, 292-306.
- [25] Taurines, R., Dudley, E., Grassl, J., Warnke, A. et al., Proteomic research in psychiatry. *J. Psychopharmacol.* 2011, 25, 151-196.

- [26] West, P. R., Amaral, D. G., Bais, P., Smith, A. M. et al., Metabolomics as a tool for discovery of biomarkers of autism spectrum disorder in the blood plasma of children. *PLoS One* 2014, 9, e112445.
- [27] Görg, A., Obermaier, C., Boguth, G., Harder, A. et al., The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* 2000, 21, 1037-1053.
- [28] Shevchenko, A., Wilm, M., Vorm, O., Mann, M., Mass spectrometric sequencing of proteins silver stained polyacrylamide gels. *Anal. Chem.* 1996, 68, 850-858.
- [29] Law, R. H., Zhang, Q., McGowan, S., Buckle, A. M. et al., An overview of the serpin superfamily. *Genome Biol.* 2006, 7, 216.
- [30] Van Vlierberghe, H., Langlois, M., Delanghe, J., Haptoglobin polymorphisms and iron homeostasis in health and in disease. *Clin. Chim. Acta* 2004, 345, 35-42.
- [31] De Boer, J. P., Creasey, A. A., Chang, A., Abbink, J. J. et al. Alpha-2-macroglobulin functions as an inhibitor of fibrinolytic, clotting, and neutrophilic proteinases in sepsis: studies using a baboon model. *Infect. Immun.* 1993, 61, 5035-5043.
- [32] Gibson, T. L., Cohen, P., Inflammation-related neutrophil proteases, cathepsin G and elastase, function as insulin-like growth factor binding protein proteases. *Growth Horm. IGF Res.* 1999, 9, 241-253.
- [33] Gabay, C., Kushner, I., Acute-phase proteins and other systemic responses to inflammation. *N. Engl. J. Med.* 1999, 340, 448-454.
- [34] Kalkbrenner, A. E., Schmidt, R. J., Penlesky, A. C., Environmental chemical exposures and autism spectrum disorders: A Review of the Epidemiological Evidence. *Curr. Probl. Pediatr. Adolesc. Health Care* 2014, 44, 277-318.
- [35] Testa, C., Nuti, F., Hayek, J., De Felice, C. et al., Di-(2-ethylhexyl) phthalate and autism spectrum disorders. *ASN Neuro.* 2012, 4, 223-229.
- [36] Croonenberghs, J., Wauters, A., Devreese, K., Verkerk, R. et al., Increased serum albumin, gamma globulin, immunoglobulin IgG, and IgG2 and IgG4 in autism. *Psychol. Med.* 2002, 32, 1457-1463.
- [37] Enstrom, A., Krakowiak, P., Onore, C., Pessah, I. N. et al., Increased IgG4 levels in children with autism disorder. *Brain Behav. Immun.* 2009, 23, 389-395.

- [38] Coury, D. L., Ashwood, P., Fasano, A., Fuchs, G. et al., Gastrointestinal conditions in children with autism spectrum disorder: developing a research agenda. *Pediatrics* 2012, *130*, S160-S168.
- [39] Black, P. H., Stress and the inflammatory response: a review of neurogenic inflammation. *Brain Behav. Immun.* 2002, *16*, 622-653.
- [40] MacDowell, K. S., García-Bueno, B., Madrigal, J. L. M., Parellada, M., Arango, C. et al., Risperidone normalizes increased inflammatory parameters and restores anti-inflammatory pathways in a model of neuroinflammation. *Int. J. Neuropsychopharmacol.* 2013, *16*, 121-135.
- [41] Gómez, C. D., Buijs, R. M., Sitges, M., The anti-seizure drugs vinpocetine and carbamazepine, but not valproic acid, reduce inflammatory IL-1 β and TNF- α expression in rat hippocampus. *J. Neurochem.* 2014, *130*, 770-779.
- [42] Verrotti, A., Scardapane, A., Franzoni, E., Manco, R., Chiarelli, F., Increased oxidative stress in epileptic children treated with valproic acid. *Epilepsy Res.* 2008, *78*, 171-177.
- [43] Kato, T., Mizoguchi, Y., Monji, A., Horikawa, H. et al., Inhibitory effects of aripiprazole on interferon-gamma-induced microglial activation via intracellular Ca²⁺ regulation in vitro. *J. Neurochem.* 2008, *106*, 815-825.
- [44] Todorović, N., Tomanović, N., Gass, P., Filipović, D., Olanzapine modulation of hepatic oxidative stress and inflammation in socially isolated rats. *Eur. J. Pharm. Sci.* 2016, *81*, 94-102.
- [45] Motaghinejad, M., Motevalian, M., Shabab, B., Neuroprotective effects of various doses of topiramate against methylphenidate induced oxidative stress and inflammation in rat isolated hippocampus. *Clin. Exp. Pharmacol. Physiol.* 2016, *43*, 360-371.
- [46] Tutanc, M., Aras, M., Dokuyucu, R., Altas, M. et al., Oxidative Status in Epileptic Children Using Carbamazepine. *Iran J. Pediatr.* 2015, *25*, e3885.
- [47] Sousa, M. M., Berglund, L., Saraiva, M. J., Transthyretin in high density lipoproteins: association with apolipoprotein A-I. *J. Lipid Res.* 2000, *41*, 58-65.
- [48] Rocha, M., Bañuls, C., Bellod, L., Rovira-Llopis, S. et al., Association of serum retinol binding protein 4 with atherogenic dyslipidemia in morbid obese patients. *PLoS One* 2013, *8*, e78670.

- [49] Stuart, W. D., Krol, B., Jenkins, S. H., Harmony, J. A. Structure and stability of apolipoprotein Jcontaining high-density lipoproteins. *Biochemistry* 1992, *31*, 8552-8559.
- [50] Woods, A. G., Wormwood, K. L., Wetie, A. G. N., Ryan, J. P., Darie, C. C., Proteomics and Cholesterol in Autism. *Autism* 2013, *3*, 112.
- [51] Kamath, S., Lip, G. Y., Fibrinogen: biochemistry, epidemiology and determinants. *QJM* 2003, *96*, 711-729.
- [52] Nguyen, D., Nickel, M., Mizuguchi, C., Saito, H. et al., Interactions of apolipoprotein A-I with high-density lipoprotein particles. *Biochemistry* 2013, *52*, 1963-1672.
- [53] Nicholson, J. P., Wolmarans, M. R., Park, G. R., The role of albumin in critical illness. *Br. J. Anaesth.* 2000, *85*, 599-610.
- [54] De Felice, C., Signorini, C., Leoncini, S., Durand, T. et al., Oxidative stress: a hallmark of Rett syndrome *Future Neurol.* 2015, *10*, 179–182.

Figure captions

Figure 1. Comparison of plasma protein patterns from subjects with ASDs and healthy controls. Plasma proteome was evaluated in term of expression in healthy controls (Figure 1a) and autistic subjects (Figure 1b). Aldehydes-derived adducts for plasma proteins were assessed in healthy controls (Figure 1c) and autistic subjects (Figure 1d). Numbers that denote identified plasma proteins by MS are listed in Table 1 and reported in Figure 2. Molecular weight (MW, kDa) and pH markers are indicated.

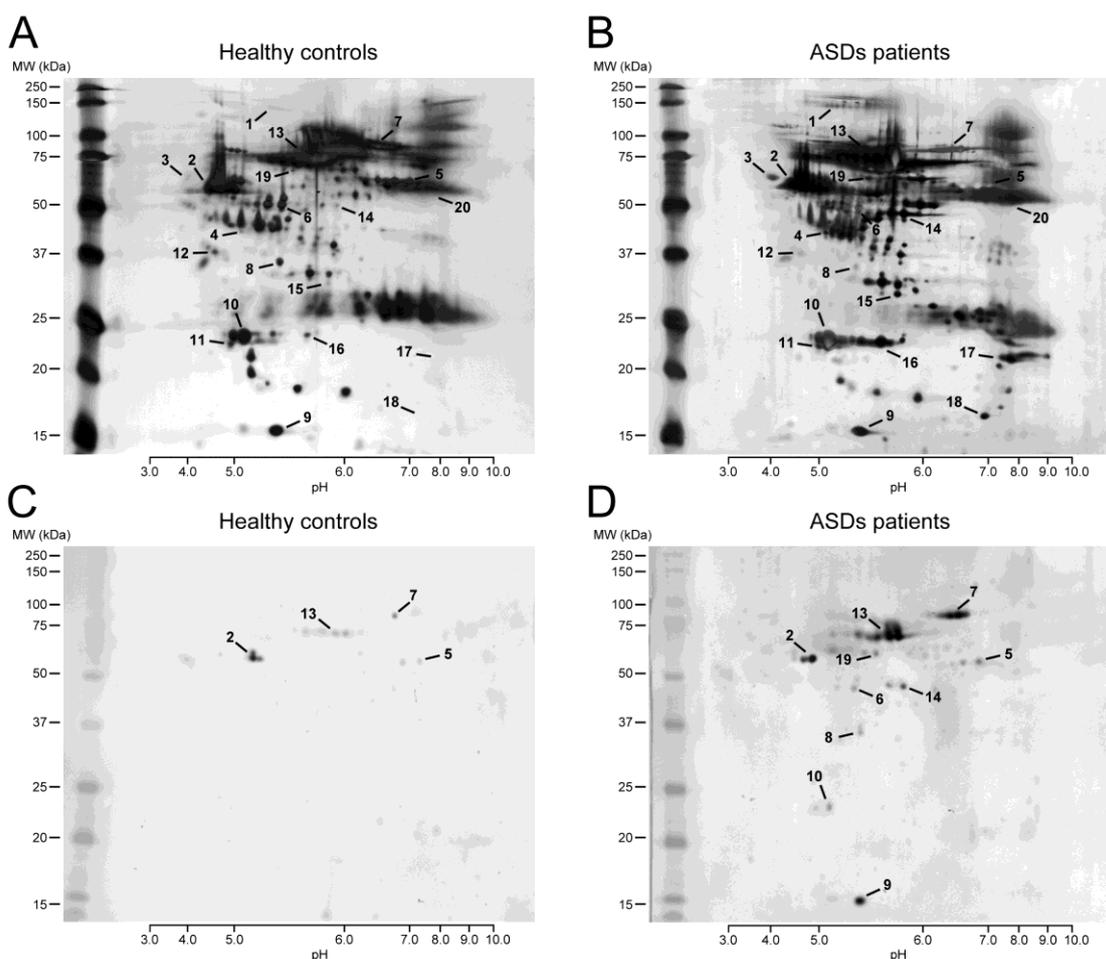


Figure 2. Protein expression levels and oxidative PTMs. Significant expression changes for 18 plasma proteins were evidenced in autistic subjects as compared to healthy controls (Figure 2a). The relative expression level of protein spots (% V) represents the relative amount of expressed proteins. Oxidative PTMs for plasma proteins were significantly

increased or appeared in autistic subjects as compared to healthy controls (Figure 2b). The relative oxidation of protein spots (% V) represents the relative degree of aldehydes-derived adducts of proteins. p -values of less than 0.05 were considered statistically significant ($*p<0.05$, $**p<0.01$). Spot results were reported as mean \pm SD of the percentage volume (% V). Spot number and short name, which denote the identified plasma proteins, are the same reported in Table 1.

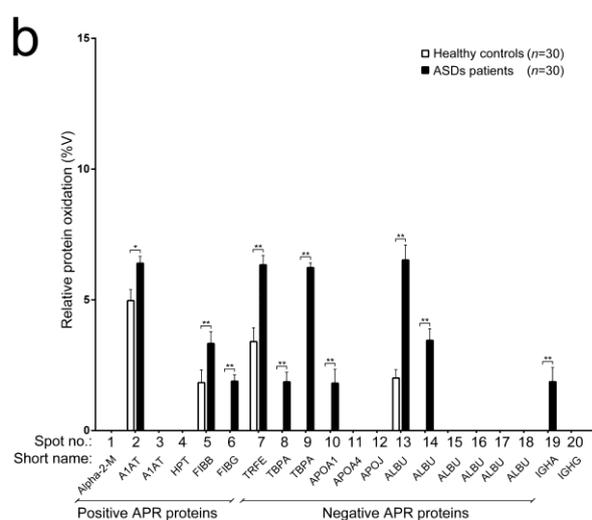
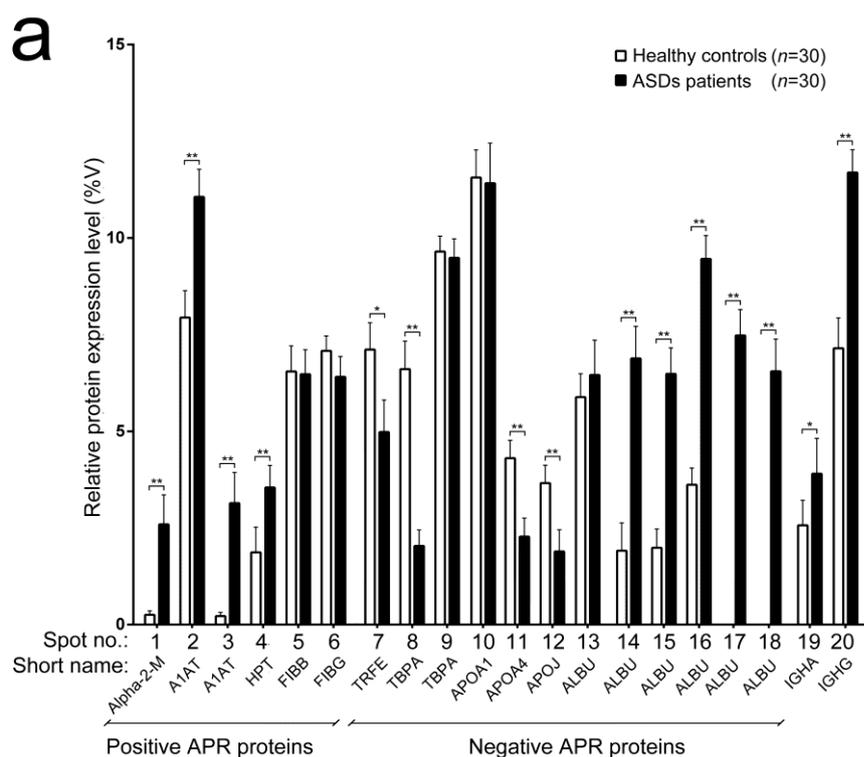


Table 1 legend**Table 1.** Identified plasma proteins by MS/MS

Spot no. ^{a)}	Accession no. ^{b)}	Protein and functional annotation	Short name	Peptides matches	Score
1	P01023	Alpha-2-macroglobulin ^{c)}	Alpha-2-M	33/51	703
2	P01009	Alpha-1-antitrypsin ^{c)}	A1AT	35/49	456
3	P01009	Alpha-1-antitrypsin ^{c)}	A1AT	85/105	316
4	P00738	Haptoglobin ^{c)}	HPT	25/34	187
5	P02675	Fibrinogen beta chain ^{c)}	FIBB	62/87	1033
6	P02679	Fibrinogen gamma chain ^{c)}	FIBG	13/19	132
7	P02787	Serum transferrin ^{d)}	TRFE	153/201	3150
8	P02766	Prealbumin ^{d)}	TBPA	49/59	548
9	P02766	Prealbumin ^{d)}	TBPA	3/3	104
10	P02647	Apolipoprotein A-I ^{d)}	APOA1	115/142	1634
11	P06727	Apolipoprotein A-IV ^{d)}	APOA4	53/72	312
12	P10909	Apolipoprotein J ^{d)}	APOJ	13/18	223
13	P02768	Serum albumin ^{d)}	ALBU	83/111	1159
14	P02768	Serum albumin (fragment) ^{d)}	ALBU	60/82	1147
15	P02768	Serum albumin (fragment) ^{d)}	ALBU	70/96	708
16	P02768	Serum albumin (fragment) ^{d)}	ALBU	49/66	669
17	P02768	Serum albumin (fragment) ^{d)}	ALBU	20/22	362
18	P02768	Serum albumin (fragment) ^{d)}	ALBU	47/72	881
19	P01876	Ig alpha-1 chain C region ^{e)}	IGHA1	10/16	73
20	P99006	Ig gamma heavy chain ^{e)}	IGHG	37/45	323

^{a)} Spot number as reported in Figure 1. ^{b)} Uniprot ID Accession number of ExPASy database. ^{c)} Positive acute phase response proteins. ^{d)} Negative acute phase response proteins. ^{e)} Immunoglobulins.