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Serum protein profiling of early and advanced stage Crohn's disease

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ABSTRACT

Crohn's disease (CD) represents a highly debilitating disease of difficult diagnosis and increasing incidence. Serum protein profiling of early stage Crohn's disease (ES) CD was investigated in order to improve the comprehension of the very early pathologic mechanisms and to support the difficult diagnostic procedures currently available. Inflammatory proteins and complement 3 chain C (C3c) were over-represented during ES CD, clusterin, retinol binding protein, α1-microglobulin and transthyretin were under-represented. A C3c isoform was found to be present only during ES CD. By now, lack of specific antibodies to detect isoforms made it impossible to perform alternative validation.

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1. Introduction

Crohn's disease (CD), together with ulcerative colitis (UC), is the most common form of inflammatory bowel disease (IBD) [1]. CD is a chronic inflammatory condition of unknown aetiology that can affect any portion of the digestive tract, but most frequently the terminal ileum and/or the colon [2]. Genetic factors, an abnormal immune response to microbial infections and unbalance in the gut-microbiota are thought to be involved in disease pathogenesis [3,4].

The diagnosis and management of IBD still presents a number of challenges for treating physicians. The presence of intestinal inflammation is a primary criterion for diagnosis and differentiation from other diseases, no definitive diagnostic test exists as a gold standard for CD diagnosis, which is made on the basis of history and physical examination, supplemented with objective findings from laboratory, radiological, endoscopic and histological studies [5]. The latter involve invasive procedures, which are often a burden for the patient. Consequently, in an attempt to overcome these

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problems, a number of laboratory markers for the disease have been evaluated [6,7].

The presence of active gut inflammation in patients with IBD is associated with an acute phase reaction and the migration of leukocytes in the gut [8,9]. This promotes the production of a large number of proteins, detectable in serum and stools. In serum, elevated acute phase markers (e.g. erythrocyte sedimentation rate, orosomucoid (alpha-1-acid glycoprotein) and C-reactive protein, CRP) can be found. However, while these markers, and especially CRP, have been shown to correlate with disease activity and prognosis of IBD, their specificity is insufficient, and they may reflect general inflammation [10–12]. Faecal calprotectin and lactoferrin are the products of activated neutrophil cells and they are released into the faeces. They are more specific biomarkers of IBD, and offer a promising way to confirm intestinal inflammation, but they cannot differentiate between IBD and other intestinal inflammations such as intestinal infections or non-steroidal anti-inflammatory drug enteropathy [8–12]. Thus, the utility of testing these biomarkers in routine clinical practice needs to be explored further, including its cost. Serological studies evaluating antibodies against *Saccharomyces cerevisiae* (ASCA) and perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA) may provide adjunctive support for the diagnosis of CD and its differential diagnosis with UC, but they also suffer from a lack of sensitivity and specificity [13–16].

The investigation of molecular mechanisms related to CD is crucial to the development of new diagnostic and therapeutic strategies. Although higher levels of acute phase proteins have already been well described in CD [17], a qualitative analysis of isoforms of these proteins during the disease state, and in particular during the various stages of pathology, has never been reported. Meuwis et al. [12] suggested the haptoglobin subunit (Hpα 2) as a potential biomarker for the clinical phase of CD, but this study did not consider changes related to treatment and in the ES CD. Moreover, the role of protease inhibitors as α1-antitrypsin (A1AT) and α1-antichymotrypsin (AACT) has been reported to be important, in the pathophysiology of this disease [18]. Crohn's disease-related epithelial injury and ulceration are thought to be due to cytokine-mediated alterations in the balance between matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs), resulting in degradation of the lining of the gut [19]. We therefore aimed to investigate the proteomic profile of the ES vs. AS CD in comparison to HCs in order to explore the differential expression of acute phase proteins or protein isoforms characteristic of the pathological status, according to disease duration and treatment.

2. Materials and methods

2.1. Patients

The institutional ethics committee of Sacco Hospital of Milan approved the experimental protocol (Protocol n° 239/07/83/06/AP, 13/04/2007). All subjects provided written informed consent before enrollment. Serum samples were collected from 13 healthy controls (HC), 8 early stage (ES) CD patients and 36 advanced stage (AS) CD patients for comparative proteomic analysis. Of the 36 AS CD samples, 16 were excluded as they were receiving more than one anti-inflammatory drug at the time of serum collection, 20 were chosen to be included in this study. It has been decided to avoid multiple drugs treatments to do not excessively interfere with the serum proteome.

The diagnosis of CD was based on published international criteria [5]. ES and AS CD were defined according with the duration of the disease, as previously described [20–22], following these criteria: *Early* = first attack of CD in a patient with no previous history of any gastrointestinal symptoms or surgery; *Advanced* = CD in a patient with at least five years history from the time of initial diagnosis and with persistent clinical activity requiring immunosuppressors, immunomodulators, steroids or surgery. ES CD patients had never received corticosteroids, antimetabolites or biological therapy and serum was collected within 3 months from the diagnoses (median 1 month). We excluded any patient who had an immediate need for surgery, severe comorbidity, documented chronic infection, a positive stool culture for pathogens, or a malignancy. AS CD patients were treated or with systemic steroids, including oral budesonide or prednisone, or with monoclonal antibodies against TNFα (i.e. infliximab and certolizumab pegol), or with oral immunosuppressors (i.e. azathioprine or methotrexate).

Table S1 has been added as supporting information and summarizes the baseline demographic and clinical characteristics of the enrolled patients.

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.euprot.2014.02.010.

2.2. Samples

A single blood sample was taken from each HC/CD subject after overnight fasting, and after 10–15 min of rest. Blood clotting was achieved by standing tubes vertically at room temperature (22 °C) for 60 min. After blood clotting, samples were centrifuged at 1500 × *g* for 10 min at 4 °C and the supernatants (serum) were stored in 1 mL aliquots at -80 °C until use.

2.3. 2D electrophoresis

Three experimental replicates were performed for each sample to minimize technical gel-to-gel variation. The gels were stained with coomassie G250, for evaluation of linear protein expression and for mass spectrometric analysis. Immobiline Dry strips (pH 3–10, length 18 cm, GE-Healthcare) were rehydrated with 350 μl buffer containing 8 M urea, 4% CHAPS, 65 mM DTT, 1% ampholine and 0.002% bromophenol blue [23,24] for 14 h at room temperature. Serum samples were defrosted and diluted in a buffer containing 8 M urea, 4% CHAPS, 65 mM DTT, 1% ampholine pH 3.5–10 and 0.002% bromophenol blue. 100 μg of protein were loaded by cathodic cup loading. Isoelectric focusing was performed using the Ettan IPGphor III IEF System (GE Healthcare) with a total of 140 kVh. Strips were equilibrated in a solution containing 6 M urea, 30% glycerol, 2% SDS and 50 mM Tris–HCl (pH 8.8), with the addition of 1% w/v DTT in the first step, and 2.5% w/v iodoacetamide in the second step.

For the second dimension, proteins were separated by SDS-PAGE on 10% polyacrylamide gels using Ettan Daltsix (GE-Healthcare) according to the following procedure: 30 min at a constant current of 12 mA followed by 24 mA per gel until the bromophenol blue front reached the bottom of the gel. Gels were then stained with Coomassie G250 or silver staining compatible with mass spectrometry [25]. Ultra narrow customized IPG strips (pH 4–5.5, length 13 cm [26,27]) were rehydrated with 350 μl rehydration buffer and 1 mg of sample protein was loaded by cathodic cup loading. For the second dimension, proteins were separated by SDS-PAGE on 10% polyacrylamide gels using EttanDalt six (GE-Healthcare) according to the following procedure: 30 min at a constant current of 12 mA followed by 24 mA per gel until the bromophenol blue front reached the bottom of the gel. Gels were then stained with Coomassie G250 or MS modified silver staining [25]. Ultra narrow customized IPG strips (pH 4–5.5, length 13 cm [26,27]) were rehydrated with 350 μl rehydration buffer and 1 mg of sample protein was loaded by cathodic cup loading.

For the second dimension, proteins were separated by 8–16% gradient–sodium dodecyl sulphate–polyacrylamide gel electrophoresis using an Ettan Daltsix (GE-Healthcare). Gels were stained using Coomassie G250.

2.4. Image analysis

All images were acquired using a PHAROS FX laser scanner (Bio-Rad) at 100 μm resolution. Gel images were imported into Progenesis SameSpots (v4.5; Nonlinear Dynamics, Newcastle, UK) for analysis [28].

All imported images were processed with Progenesis SameSpots to check image quality (saturation, dimension). The aligned images were then automatically analyzed using the 2D analysis module for spot detection, background subtraction, normalization, and spot matching, and all spots were manually reviewed and validated to ensure proper detection and matching.

2.5. Statistical analysis

Statistical analysis was performed using the Progenesis Stats module on the log-normalized volumes for all spots. Mann–Whitney test and one-way ANOVA were used to confirm the p value between different groups, p-values under 0.05 were considered statistically significant. FDR (false discovery rate) and power analysis were also calculated, q values <0.05 and power values >0.8 respectively were considered to be significant. Multivariate statistical analysis was carried out using the Progenesis Stats module to perform principal components analysis (PCA) [29].

2.6. Protein identification by mass spectrometry

2.6.1. In situ digestion

Protein spots of interest were excised from the Colloidal Coomassie-stained preparative gel. The excised spots were first washed with acetonitrile (ACN) and then with 0.1 M ammonium bicarbonate. Protein samples were reduced by incubation in 10 mM dithiothreitol for 45 min at 56 °C. The cysteine residues were alkylated by incubation in 55 mM iodoacetamide for 30 min at room temperature in the dark. The gel particles were then washed with ammonium bicarbonate and ACN. Enzymatic digestion was carried out with trypsin (12.5 ng/μl) in 50 mM ammonium bicarbonate pH 8.5 at 4 °C for 1 h. The buffered solution was then removed and a fresh aliquot of the enzyme/buffer solution was added for 16 h at 37 °C. A minimum reaction volume, sufficient to achieve the complete rehydration of the gel was used. Peptides were then extracted, washing the gel particles with 50 mM ammonium bicarbonate and 0.1% trifluoroacetic acid (TFA) in 50% ACN at room temperature, then lyophilized and resuspended in 30 μl of 0.1% formic acid/3% ACN for MS analysis.

2.6.2. MALDI-TOF Mass spectrometry

Positive Reflectron MALDI-TOF mass spectra were recorded on a Voyager DE STR instrument (Applied Biosystems, Framingham, MA). The MALDI matrix was prepared by dissolving 10 mg of α-cyano-4-hydroxycinnamic acid in 1 ml of ACN/water (90:10 v/v). Typically, 1 μl of matrix was applied to the metallic sample plate and 1 μl of each sample was then added. Acceleration and reflector voltages were set up as follows: target voltage at 20 kV, first grid at 95% of target voltage, delayed extraction at 600 ns to obtain the best signal-to-noise ratios and the best possible isotopic resolution with multipoint external calibration using peptide mixture purchased from Applied Biosystems. Each spectrum represents the sum of 1500 laser pulses from randomly chosen spots per sample position. Raw data were analyzed using the computer software provided by the manufacturers and are reported as monoisotopic masses.

Database searches with the measured monoisotopic peptide masses were performed against the Swiss Prot database using the peptide search MASCOT (http://www.matrixscience.com). Search parameters were typically set to 70 ppm maximal tolerance, one missed cleavage site for tryptic peptides allowed, and the modifications accepted were carboamidomethylation with iodoacetamide of cysteines and possible artefactual oxidation of methionines.

2.6.3. NanoLC mass spectrometry

Tryptic peptides derived from each sample were analyzed by nLC–MS/MS analysis using a 4000 Q-Trap (Applied Biosystems) coupled to an 1100 nano HPLC system (Agilent Technologies). The samples were loaded on an Agilent reversed-phase pre-column cartridge (Zorbax 300 SB-C18, 5 × 0.3 mm, 5 μm) at 10 μl/min (A solvent 0.1% formic acid, loading time 5 min). Peptides were separated on an Agilent reversed-phase column (Zorbax 300 SB-C18, 150 mm × 75 μm, 3.5 μm), at a flow rate of 0.3 μl/min with a 0–65% linear gradient in 60 min (A solvent 0.1% formic acid, 2% ACN in MQ water; B solvent 0.1% formic acid, 2% MQ water in ACN). Nanospray source was used at 2.5 kV with liquid coupling, with a declustering potential of 20 V, using an uncoated silica tip from NewObjectives (O.D. 150 μm, I.D. 20 μm, T.D. 10 μm). Data were acquired in information-dependent acquisition (IDA) mode, in which a full scan mass spectrum was followed by MS/MS of the 5 most abundant ions (2 s each). In particular, spectra acquisition of MS/MS analysis was based on a survey Enhanced MS Scan (EMS) from 400 m/z to 1400 m/z at 4000 amu/s. This scan mode was followed by an Enhanced Resolution experiment

(ER) for the five most intense ions and then MS2 spectra (EPI) were acquired using the best collision energy calculated on the bases of m/z values and charge state (rolling collision energy) from 100 m/z to 1400 m/z at 4000 amu/s. Data were acquired and processed using Analyst software (Applied Biosystems).

LC MS Data were analyzed using Analyst software (version 1.4.1) and MS/MS centroid peak lists were generated using the MASCOT.dll script (version 1.6b9). The threshold of MS/MS centroid peaks was taken to be 0.1% of the base peak. MS/MS spectra having less than 10 peaks were rejected. MS/MS spectra were searched against the Swiss Prot database using the licensed version of Mascot 2.1 (Matrix Science), after converting the acquired MS/MS spectra into the mascot generic file format. The query was performed with the maximal tolerance for parent masses of 200 ppm and a maximal tolerance for fragments of 0.2 Da, searching peptides on 2+ and 3+ charge state. At the most two miss-cleavage for tryptic peptides were allowed, and the modifications accepted were carboamidomethylation of cysteines as fixed modifications and oxidation of methionines and pyro-glu N-term Q as variable post-translational modifications. The taxonomy was restricted to Homo sapiens Spectra with a MASCOT score <25 were rejected. However, spectral data were manually validated and contained sufficient information to assign peptide sequence.

2.6.4. *Western blot analysis*

Protein extract from sera samples, 7 μg and 5 μg/lane were loaded on a SDS-PAGE system 10%, transfer blotting was achieved by a semi dry apparatus (NOVABLOT) on low fluorescence PVDF. Membranes were incubated for 2 h using a commercial monoclonal antibody (NB100-64339, Novus Biologicals) specific against C3c complement and successively for 1 h with a secondary antibody (A3562, SIGMA).

3. Results

All samples were analyzed using 3–10 pH IPG strips and obtained data have been considered as main data. Furthermore ultra narrow 4–5.5 [30] pH 2D electrophoresis have been employed to confirm obtained data and to better annotate the supposed specific protein isoforms.

3.1. *HC vs. ES CD*

When comparing ES CD and HC, image analysis highlighted 10 proteins whose expression was significantly altered in the disease state, these proteins were successfully identified by MS (Fig. 1a and b, Table 1). In particular, α1-antitrypsin (A1AT, 36.7% higher in ES CD), α1-antichymotrypsin (AACT, 50.2% higher in ES CD), and complement 3 fragment C (C3c, 270% higher in ES CD) were up-represented, haptoglobin (HPT, 32% higher in ES CD) expression showed a tendency to up-regulation ($p = 0.08$) (Fig. 1a).

On the other hand, RETBP, CLUS, AMBP, and TTHY were significantly under-represented in ES CD compared with HC, with levels 49.8%, 51%, 74% and 56.6% lower ($p \leq 0.05$), respectively (Fig. 1b).

Table 1 – Differentially represented proteins during ES CD identified by MS. ↑ = up-regulation in ES Crohn's disease patients; ↓ = down-regulation in ES Crohn's disease patients.

Spot number	Uniprot accession number	Protein name	ES CD vs. HC		AS CD vs. ES CD		AS CD vs. HC		Mascot score	Sequence coverage (%)	Theoretical Mr/pI
			Ratio of means	p-Value	Ratio of means	p-Value	Ratio of means	p-Value			
1	P01009	α1-Antitrypsin	1.367686	0.00054	0.706468	4.00812E−05	0.966226	0.61547942	255	73	60.59/4.70
2	P01011	α1-Antichymotrypsin	1.497752	0.003195	0.81714	0.174199	1.223873	0.139132	373	87	54.93/5.00
3	P00738	Haptoglobin	1.314226	0.121067	1.255693	0.176734	1.650265	0.000394	203	63	40.42/5.36
4	P01024	Complement 3 fragment C	3.760332	9.46E−09	0.442775	2.47E−07	1.664982	0.00608	263	58	40.91/4.84
5	P02753	Retinol binding protein 4	0.5027	0.000762	2.195367	0.000813	1.10361	0.447741	131	71	20.10/5.23
6	P10909	Clusterin	0.489887	7.9E−08	2.229412	0.002048	1.092159	0.513289	163	87	37.21/4.85
7	P02760	Alpha-1-microglobulin	0.260326	1.9E−06	4.221332	0.005435	1.098921	0.658607	218	65	38.9/5.95
8	P02766	Transthytretin	0.438517	1.87E−06	1.866248	5.25E−05	0.818381	0.021019	263	91	35.39/5.52
9	P02766	Transthytretin isoform pI 4.7	HC = 0	–	AS = 0	–	HC = 0	–	170	80	35.39/4.72
10	P01024	Complement 3 fragment C isoform pI 4.9	HC = 0	–	0.404369	0.027986	HC = 0	–	255	57	38.80/4.95

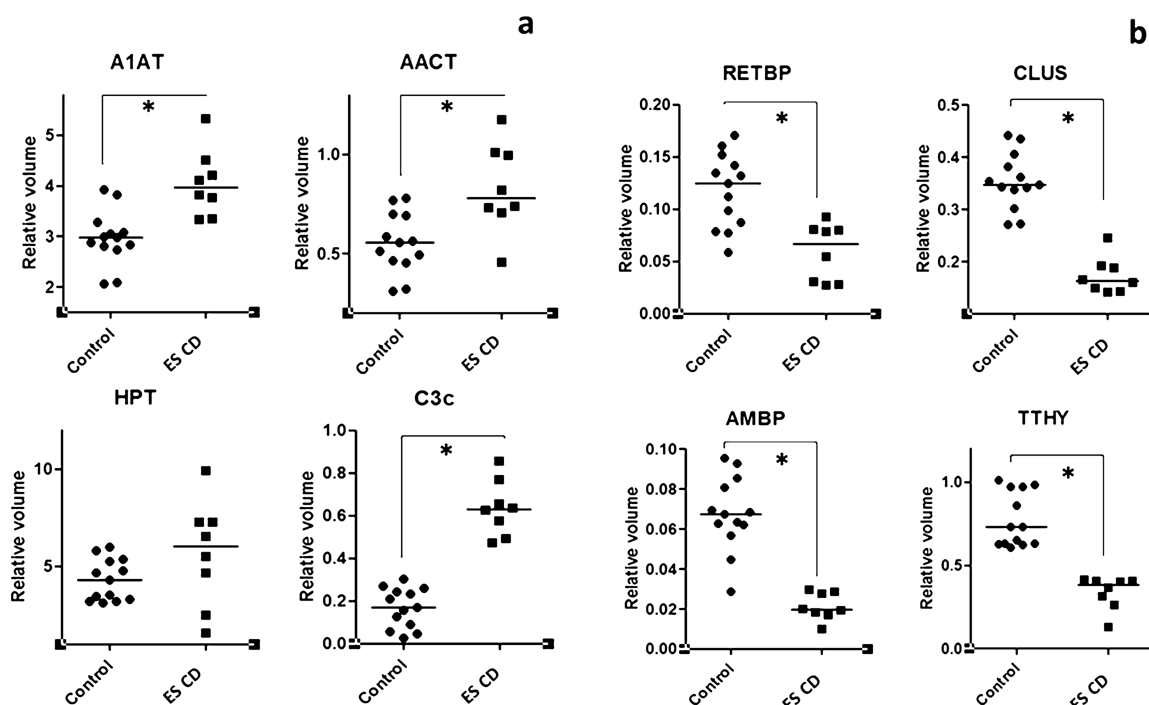

Fig. 1 – (a) Up-represented serum proteins in ES CD ($n = 8$) and HCs ($n = 13$). A1AT (α1-antitrypsin): HCs (2.96 ± 0.54) vs. CD (4.05 ± 0.65); AACT (α1-antichymotrypsin): HCs (0.55 ± 0.15) vs. CD (0.83 ± 0.22); HPT (haptoglobin): HCs (4.30 ± 1.03) vs. CD (5.67 ± 0.86); C3c (complement 3 fragment c): HCs (0.17 ± 0.09) vs. CD (0.63 ± 0.13) (*$p \leq 0.05$). (b) Down-represented serum proteins in ES CD ($n = 8$) and HCs ($n = 13$): RETBP (retinol binding protein): HCs (0.12 ± 0.03) vs. CD (0.06 ± 0.03); CLUS (clusterin): HCs (0.35 ± 0.06) vs. CD (0.17 ± 0.03); AMBP (α1-microglobulin): HCs (0.067 ± 0.016) vs. CD (0.017 ± 0.012); TTHY (transthytretin): HCs (0.78 ± 0.17) vs. CD (0.34 ± 0.1). *$p < 0.05$ for Mann Whitney test carried out for each sampling data point between HC and CD proteins.

Furthermore, two proteins apparently absent in HC samples were identified in ES CD samples: one isoform of transthyretin at pI 4.7 and one isoform fragment of complement C3c at pI 4.9 (Fig. 2). Both isoforms were found in 3–10 and 4–5.5 pH range experiments, as well as they were successfully identified by MALDI TOF and nLC–MS/MS analysis.

3.2. *ES CD vs. AS CD*

As shown in Fig. 3a, the proteins over-represented during the ES of CD did not maintain higher relative levels during the AS of the disease, and their expression was similar to HC. Notable exceptions to this, however, were C3c complement fragment and, to a greater extent, haptoglobin (C3c $p = 0.024$; HPT $p = 0.01$). Proteins which were under-represented during the ES of CD, were also observed to return to normal (HC) levels in AS CD (Fig. 3b). Notably, no differences were observed within the AS CD patient group according to the type of anti-inflammatory treatment (*data not shown*).

3.3. *PCA analysis of all groups*

To provide evidence for the differences between serum samples, multivariate unsupervised PCA analysis was performed. Fig. 4 represents the biplot of the first two principal components (PC).

The biplot showed an effective separation of samples in two classes: ES CD samples (pink, negative scores of PC1), and HCs (cyan, positive scores of PC1). Spots that contribute to differences between the two groups are present as loadings in the PCA plot (spot numbers, Fig. 4) and confirm the results obtained by non-parametric univariate analysis.

Fig. 5 shows the biplot which also includes samples from CD AS. The graph highlights the similarity between the AS CD (violet) and HCs (cyan) that would be included in the same group using this analysis. This confirms that in AS CD protein expression returns similar to control values.

A validation of the C3 fragment profile has been attempted using commercial monoclonal antibody NB100-64339, Novus Biologicals. Such a validation has been performed in the early stage Crohn's disease samples as considered more significant (Fig. 6). Unfortunately the specific fragment isoform of C3 complement highlighted in the 2DE was not detected by this antibody which is recognizing a protein band at 115 kDa supposedly associated with the intact C3 protein. This band shows an overall trend similar to of C3c complement isoform, detected by the 2DE, however these results, do not show a statistically significant differential expression but only a suggestive profile.

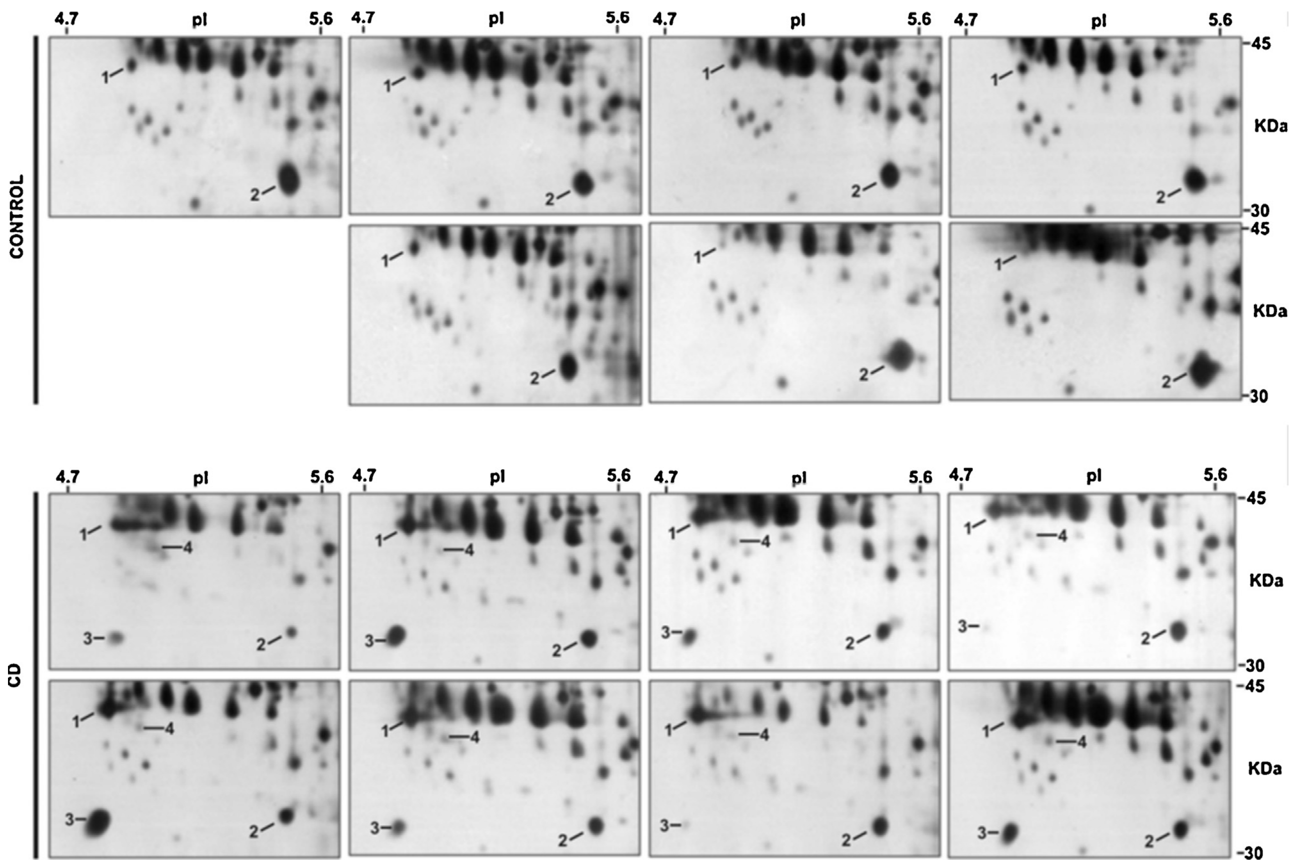

Fig. 2 – Details of 2-D map (pI 4.7–5.6; kDa 45–30). 1 = complement 3 fragment c; 2 = transthyretin; 3 = transthyretin isoform pI 4.7; 4 = complement 3 fragment c isoform pI 4.9.

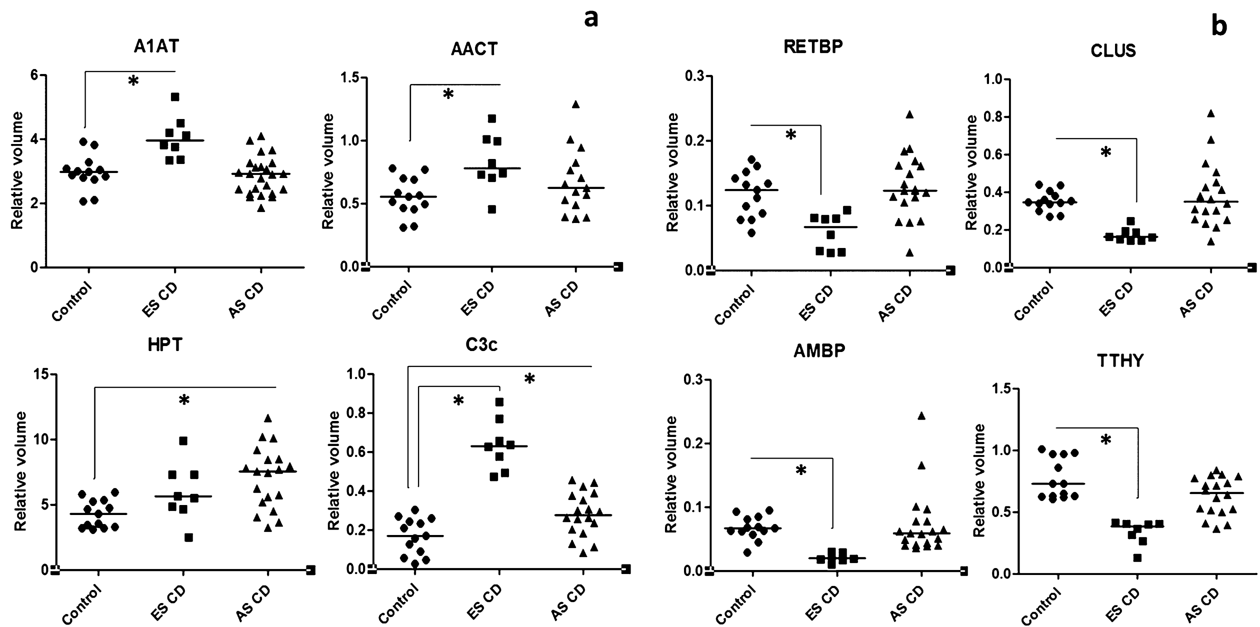

Fig. 3 – Expression profiling of up-represented proteins during AS of Crohn's disease: only HPT and C3c complement were up-represented compared to HCs (HPT $p = 0.01$; C3c $p = 0.024$) ($^{*}p \leq 0.05$).

4. Discussion

The differential protein display was evaluated in ES CD patients vs. HC and in AS CD patients vs. ES CD.

4.1. HC vs. ES CD

Image and multivariate (PCA) analysis of 2D maps showed an overexpression of α1-antitrypsin, α1-antichimotrypsin and

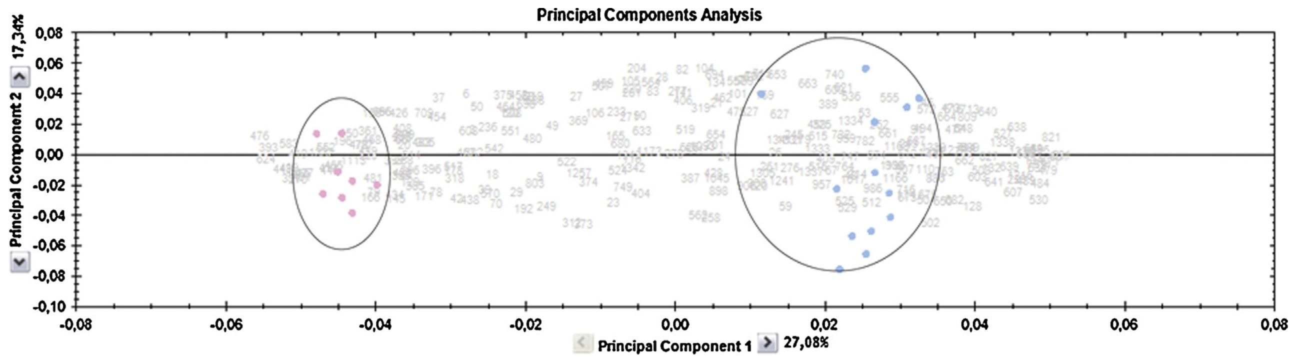

Fig. 4 – Biplot of the first two components of the principal component analysis of HCs and ES CD. Ovals indicate the areas where the data points of the two groups are distributed pink spots: ES CD, cyan spots: HCs.

complement factor 3 chain c during ES CD (Fig. 1a) in comparison to HC, haptoglobin showed a over-represented trend. On the other hand, clusterin, retinol binding protein, α1-microglobulin and transthyretin (Fig. 1b) were under-represented. These are mostly inflammation-related proteins that have already been described during Crohn's disease. However, this is an innovative study because of the presence of a rare group of Crohn's disease patients that have been investigated during the ES of this disease. The analysis of the serum proteome of this particular pool of samples revealed, as described below, the presence of differential expression of the described proteins and their isoforms.

4.2. *ES CD vs AS CD*

Obtained results highlighted the presence of important differences between the proteomic profiles of HC and ES CD, however, the relative levels of these proteins came back to levels similar to HC in AS CD patients (Fig. 3). Different immune activation patterns have been previously described for T-cell responses in a study in children, where variations in mucosal T-cell immune regulation during the course of human IBD were observed [20]. In particular, at the onset of CD, mucosal T cells appear to mount a typical Th1 response that resembles an acute infectious process, but this response is lost with progression to AS CD, suggesting that patients with the initial manifestations of IBD may represent an ideal population in which immunomodulation has optimal therapeutic efficacy.

Moreover, the overexpression of some proteins in the ES CD may reflect their potential role in disease pathogenesis according to the particularities of their functions in the inflammatory cascade, or simply be an aspecific marker of inflammatory activation.

Differentially represented proteins and isoforms during ES and AS CD in comparison to HC: α1-antitrypsin and α1-antichymotrypsin are protease inhibitors and positive acute-phase proteins whose plasma concentrations are increased as a result of inflammation. During acute phase response, overexpression of these two proteins is crucial in the irreversible inhibition of protease activity. Both proteins are synthesized primarily in the liver, but isoforms of A1AT have been previously shown to be present also in gut Paneth cells [31], and variable associations have been found between A1AT levels or polymorphisms and CD, with conflicting results [32–34]. Notably, an excess of tissutal protease inhibitors like A1AT has been described [31] and this was associated with a disturbance in the normal processing of antimicrobial peptides, like defensins, which then allows increased exposure of the intestinal mucosa to luminal micro-organisms [35], triggering an excessive immune activation in IBD. On the other hand, the downregulation of these antiproteases, that was observed in AS CD, may suggest new mechanisms of disease control by the various anti-inflammatory treatments used or changes in the mechanisms of immune activation during the

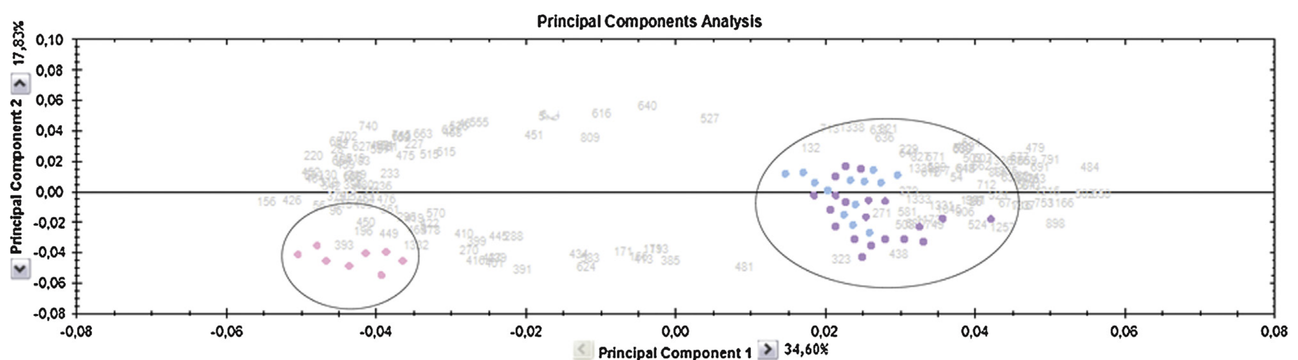

Fig. 5 – Biplot of the first two components of the principal component analysis of HCs and ES/AS CD. Ovals indicate the areas where the data points of the two groups are distributed pink spots: ES CD samples. Violet spots: AS CD, cyan spots: HCs.

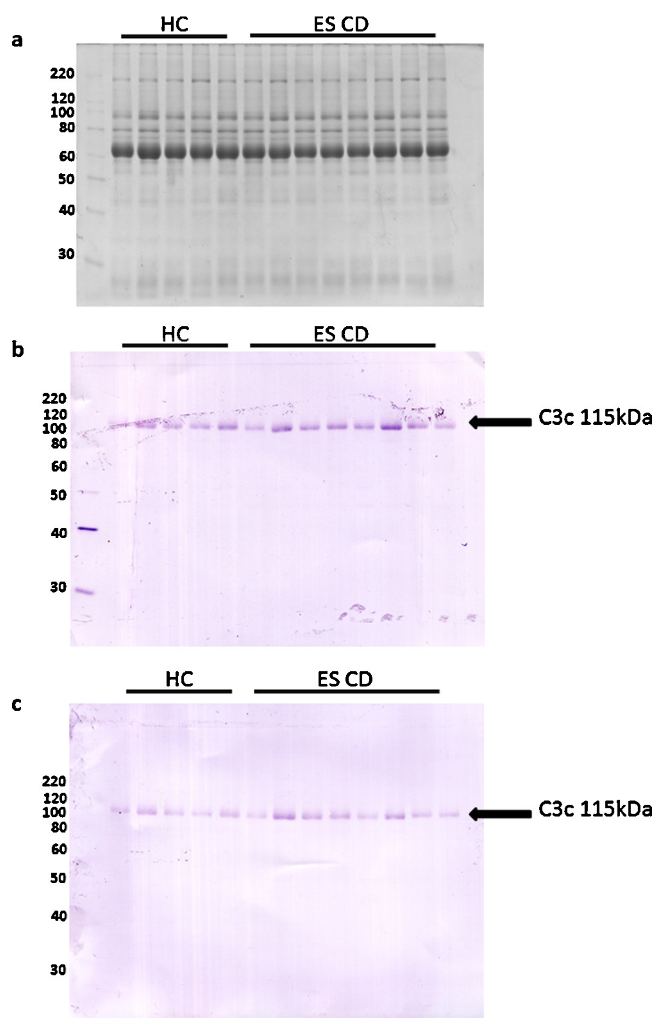

Fig. 6 – Immunoblotting of the C3c. (a) One dimensional electrophoresis (coomassie staining). (b) One dimensional immunoblotting with 7 μg per lane protein load. (c) One dimensional immunoblotting with 5 μg per lane protein load.

advanced phases of the disease. As described before, recent findings highlighted that an impaired metabolism of proteases and proteases inhibitors could be linked to the etiopathogenesis of this disease [19].

4.3. *Complement C3c*

Like other acute phase proteins, complement C3c is over-represented. This data confirms results from previous studies [36]. Complement-derived proteolytic products mediate functions which contribute to pathogen disruption and elimination [37]. However, the inappropriate activation of the complement system has been involved in pathogenetic mechanisms of immunological and inflammatory diseases, including IBD, as shown in several studies [17,38–49]. The central role of complement component 3 (C3) is due to the complexity of the functional regulation of its fragments, including the major fragment, C3b, which anchors the convertase assembly effecting activation of fragments C3 and C5. C3c is a fragment derived from the proteolysis of C3b; C3c over-representation was found not only during the ES CD but also during AS disease, irrespective of various treatments, suggests a dysregulated complement metabolism that could be related to disease pathogenesis and to the maintenance of gut inflammation. Recent metaproteomic studies suggest the possibility to evaluate the differences between the normal and the pathological microbiota [50]. Furthermore, image analysis revealed a C3c isoform at pI 4 [50]. Furthermore, image analysis revealed a C3c isoform at pI 4.9 apparently missing in HC samples, further highlighting the specific role of the complement system in CD immune activation as well as the need to better explore the role of this isoform. A putative explanation of the role of the complement system in other autoimmune pathologies have already been proposed [30] but the provided explanation does not include the description of the role or the provenience of C3c fragment. However the differential representation of such a C3 isoform may be interesting, we could not independently validate such an evidence by the use of an immunochemical method with commercial antibodies. Only a trend profile of the intact Complement C3 protein could be detected. Such a partial result may be due to a number of factors affecting the superimposition of 2DE data with classical Western blot detection. The problem of validation of specific isoforms of proteins that are significantly different in 2D electrophoresis is a question regarding the availability of epitope qualified and validated reagents. Also in this case, there are no commercial antibodies available against a specific isoforms and the commercial products are directed to a specific undisclosed sequence of the entire proteins.

4.4. *Clusterin*

The present study shows also that clusterin, retinol binding protein, α1-microglobulin and transthyretin are underrepresented during ES CD. An over-expression of clusterin is reported in a wide variety of models of stress and disease, including withdrawal of growth factors and exposure to toxic agents [51]. Moreover clusterin expression [51]. Moreover clusterin expression increases in humans usually during intense oxidative stress, although this phenomenon is not observed in rheumatoid arthritis [52]. These findings suggest a possible role for clusterin in protecting cells from oxidative stress during pathological status and inflammatory response. Accordingly, strongly enhanced levels of clusterin were previously described in the ileum of CD patients, correlating with disease activity [53]. Increased level of clusterin during ES CD is therefore expected to protect tissues against oxidative stress damage caused by inflammation [54,55]. The under-representation of clusterin in ES CD observed in our study, is therefore an unexpected result, and warrants further investigation to explore the etiopathogenic implications; for example, to study if impaired protection from oxidative stress, could be responsible for tissue injury and impaired mucosal barrier. The result may also suggest potential analogies between CD and rheumathoid arthritis pathogenesis [56].

4.5. α1-Microglobulin

α1-Microglobulin is an immunosuppressive plasma protein synthetized by the liver. The down-regulation observed in this study has never been reported before and deserves further investigation as it may reflect a loss of immunoregulatory function which could influence the balance between pro- and anti-inflammatory pathways of immune response. However it has been interestingly documented a considerably increased excretion of α1-microglobulin in patients affected by Crohn's disease. Its increased excretion is so high to induce scientists to speculate α1-microglobulin in urine as putative biomarker for the evaluation of IBD activity index [57].

Retinol binding protein (RBP) is synthesized primarily in the liver, and requires retinol binding to trigger its secretion [58,59]. In vivo, RBP binds the larger protein, transthyretin, while in vitro one tetramer of TTR binds two molecules of retinol binding protein. However, the concentration of RBP in plasma is limiting and the complex isolated from serum is composed of both TTR and RBP (in a one to one stoichiometry) with a resultant molecular mass of about 80 kDa. The binding of RBP to TTR prevents extensive loss of the low molecular weight RBP through glomerular filtration and may also restrict free partitioning of RBP into the intercellular space outside the vascular system [58,60,61]. Retinol binding protein is a vitamin A carrier; its decrease in CD has been previously described [62] as a sign of intestinal malabsorption. The most interesting characteristic of RBP is due to its common positive correlation with systemic inflammation that is the phenomenon that characterizes CD [63]. But surprisingly, obtained results, confirmed its down-regulation during ES CD in spite of what was expected. However a recent study by Keicho and colleagues found RBP decreased in patients with tuberculosis [64]. This experimental evidence suggests again a putative microbial implication in the pathogenesis of CD particularly if considering that *Mycobacterium avium* subsp. *paratuberculosis* has been often described as the putative cause of CD [65] and could be found in cow milk for human consumption [66]. Moreover particular attention needs to be paid to microbial pathogens due to the recent evidences in the field of multidrug resistance [67].

4.6. Transthyretin

TTR is mainly synthesized by the liver and then secreted into the circulation. It is normally considered a negative acute-phase protein as well as a nutritional index [68]; our results match favourably with previous studies showing decreased levels of TTR in active CD [18], as well as its increase after effective treatment with infliximab [69]. Its concentration was found to be decreased also in rheumatoid arthritis confirming the already described analogies between this two pathologies [70]. In ES CD it has been found an isoform at pI 4.7. The presence of this isoform only in ES CD could be linked to a putative PTM of this protein linked to the pathological status. The putative role of TTR as biomarker has been already described for other pathologies [71].

5. Conclusion

Our collected evidences highlighted a differential protein display of several serum proteins in ES CD patients. In addition it was found a transthyretin isoform (pI 4.7) and C3c fragment isoform (pI 4.9) which has not been described before in type of clinical samples. These isoforms could help to explain the molecular changes observed during the clinical phase of CD and in particular the role of C3c complement in the complement cascade activation and in the disease progression. The C3c isoform is of particular interest as it was still represented in 78% of AS CD patients after anti-inflammatory therapies. This observation may indicate that other factors (for example, microbial components) could still play an important role in the advanced phases of the disease. In particular it is important to quote that many other proteomic studies have investigate the acute phase proteins in other diseases similar to CD as rheumatoid arthritis [72]. None of this studies performed on serum described an augmented expression of C3c complement as it was found in this study [70]. Another study conducted by Cylwik and colleagues describes the analysis of all serum acute-phase proteins during the course of rheumatoid arthritis highlighting that C3 complement concentration was not influenced by the disease activity [73]. Moreover even if C3 complement has been described as autoantigen during IBD [74], the over-representation of C3c has never been described during this kind of pathologies. The relevance of C3 complement and isoforms as putative biomarker for other pathologies has also been described highlighting the relevance of further studies on this topic [75–77]. All this experimental evidences could suggest that C3c proteolytic isoforms expression in Crohn's disease could be disease specific and encourages further investigation in wider populations [78] in particular in the light of the difficulties actually encountered in the diagnoses of this pathology. Although the detection of the described isoforms could represent a difficult task since the currently available commercial antibodies are not recognizing this isoforms. The development of new generation affinity binders isoform specific will represent a key milestone for the construction of diagnostic tests for the early phases of this pathology.

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