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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.



## 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.