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Biomarker discovery for inflammatory bowel disease, using proteomic serum profiling

Marie-Alice Meuwis [a,1], *Marianne Fillet* [a,1], *Pierre Geurts* [b], *Dominique de Seny* [c], *Laurence Lutteri* [a], *Jean-Paul Chapelle* [a], *Vincent Bours* [d], *Louis Wehenkel* [b], *Jacques Belaiche* [e], *Michel Malaise* [c], *Edouard Louis* [e], *Marie-Paule Merville* [a,*]

[a] *Laboratory of Clinical Chemistry, GIGA Research, CHU, University of Liège, B34, 4000 Liège, Belgium*
[b] *Department of Electrical Engineering & Computer Sciences, B28, University of Liège, Belgium*
[c] *Laboratory of Rheumatology, CHU, University of Liège, Belgium*
[d] *Laboratory of Human Genetics, CHU, University of Liège, Belgium*
[e] *Department of Hepato-Gastroenterology, CHU, University of Liège, GIGA Research, University of Liège, B34, Sart Tilman, 4000 Liège, Belgium*

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ABSTRACT

Crohn's disease and ulcerative colitis known as inflammatory bowel diseases (IBD) are chronic immuno-inflammatory pathologies of the gastrointestinal tract. These diseases are multifactorial, polygenic and of unknown etiology. Clinical presentation is non-specific and diagnosis is based on clinical, endoscopic, radiological and histological criteria. Novel markers are needed to improve early diagnosis and classification of these pathologies. We performed a study with 120 serum samples collected from patients classified in 4 groups (30 Crohn, 30 ulcerative colitis, 30 inflammatory controls and 30 healthy controls) according to accredited criteria. We compared protein sera profiles obtained with a Surface Enhanced Laser Desorption Ionization-Time of Flight-Mass Spectrometer (SELDI-TOF-MS). Data analysis with univariate process and a multivariate statistical method based on multiple decision trees algorithms allowed us to select some potential biomarkers. Four of them were identified by mass spectrometry and antibody based methods. Multivariate analysis generated models that could classify samples with good sensitivity and specificity (minimum 80%) discriminating groups of patients. This analysis was used as a tool to classify peaks according to differences in level on spectra through the four categories of patients. Four biomarkers showing important diagnostic value were purified, identified (PF4, MRP8, FIBA and Hpα2) and two of these: PF4 and Hpα2 were detected in sera by classical methods. SELDI-TOF-MS technology and use of the multiple decision trees method led to protein biomarker patterns analysis and allowed the selection of potential individual biomarkers. Their downstream identification may reveal to be helpful for IBD classification and etiology understanding.

* *Corresponding author*. Tel.: +32 4 366 24 75; fax: +32 4 366 45 34.
E-mail address: mpmerville@ulg.ac.be (M.-P. Merville).

[1] These authors equally contributed to this work.

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1. Introduction

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders affecting the gastrointestinal tract. The two main forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC). Their etiopathogenesis has not been fully elucidated but involves a complex interplay among genetic and environmental factors [1–3]. Recent studies on experimental animal models of IBD as well as the discovery of gene variants or loci, selectively associated with specific forms of IBD have highlighted the heterogeneity of mechanisms leading to IBD [4,5]. Despite this heterogeneity, clinical manifestations of these diseases appear quite stereotyped including diarrhea, abdominal pain, fever and degradation of the general physical condition.

There is currently no easy diagnostic tool for these pathologies. Biological markers potentially useful in IBD include proteins of inflammation such as C-reactive protein (CRP), fecal calprotectin and several antibodies [6,7]. However, these biomarkers have many limitations. Acute inflammatory markers, such as CRP or fecal calprotectin cannot differentiate between infectious colitis and flare of IBD [8–12]. Anti-*saccharomyces* antibodies (ASCA) and perinuclear anti-neutrophil cytoplasmic antibody (pANCA) are the only available commercial tests that can be helpful for CD and UC discrimination. Although, they show a quite good specificity, their sensitivity is rather low and they are therefore not recommended for broad clinical practice [13–16]. Finally, the recent identification of genetic factors predisposing to IBD did not lead to novel and relevant diagnostic tools since such factors are also present in a significant proportion of the general population [17,18]. Therefore, initial diagnosis still relies on the combination of several biological and morphological tests, including gastrointestinal endoscopies and histology, and is based on standardized validated diagnostic criteria [19,20]. However, even using these invasive methods, differential diagnosis between IBD and self-limited colitis as well as between the two main forms of IBD is still difficult. This is a relevant clinical question since optimal management and treatments may differ among these entities [21,22]. Beyond this initial diagnosis, there are various degrees of severity among IBD and responses to standard therapies vary between patients due to disease heterogeneity. Again, powerful predictive factors do not exist and clinicians often manage patients empirically and secondarily adapt the therapeutic strategy according to clinical evolution [23,24].

Analysis of the proteome signature of each patient, although technically difficult, could be a more pragmatic and realistic approach to these questions, in particular for inflammatory processes [25–27]. Indeed the proteome represents the net result of interactions between genetic background and environmental factors and may be considered as the signature of a disease, involving small circulating proteins or peptides from degraded molecules as often encountered in those types of inflammatory and dysimmune pathologies. Surface Enhanced Laser Desorption Ionization-Time of Flight-Mass Spectrometer (SELDI-TOF-MS) technology is a rapid and sensitive technique, which offers the possibility to analyze many samples in a very short time period [28,29]. The sensitivity of mass spectrometer allows detection of peptides at the femto molar range. This means that low abundant serum proteins can be detectable in appropriate conditions [30–33]. Many teams including ours have already undertaken studies of protein profiling with SELDI-TOF-MS, in order to discover new specific biomarkers for various pathologies at different stages and on different sample origins [34,35].

In this paper, we present a pilot study based on serum profiling and robust statistical approach which attempts to answer questions regarding the potential interest of proteomics in IBD study and classification. We compared profiles from IBD versus non-IBD inflammatory pathologies and healthy controls. The same samples were tested with non-invasive markers for IBD, namely ASCA and ANCA, for comparison. We further selected and identified several potential biomarkers among the highly discriminating ones, which might be interesting to better understand IBD pathophysiology.

2. Materials and methods

2.1. Patients

Experimental protocol was approved by the ethic committee of our academic hospital and patients enrolled gave their informed consent for the study. A total of 120 serum samples from patients affected by various pathologies and healthy controls were prospectively collected in 10 cm^3 serum separator vacutainer tube. Clotting was allowed to occur within a minimum of 30 min and a maximum of 4 h before centrifugation at 3000 rpm for 10 min. All sera were aliquoted and immediately frozen at −80 °C, until thawed for SELDI-TOF-MS analysis (Ciphergen Biosystems Fremont, CA, USA).

Samples were classified in four categories according to the considered pathologies: Crohn's disease (CD), ulcerative colitis (UC), healthy controls (HC) and inflammatory controls (IC). IC grouped patients presenting inflammatory pathologies affecting the bowel other than IBD, such as diverticulitis or pathogen caused enterocolitis, as well as two other chronic inflammatory diseases: asthma and rheumatoid arthritis. Diagnoses of IBD patients were realized by gastroenterologists specialized in IBD, according to widely accepted criteria [36]. CD was considered as clinically active or inactive according to Harvey–Bradshow index (HBI) [37]. UC was considered active when clinical symptoms were confirmed by the presence of significant lesions at rectosigmoidoscopy including erosions and spontaneous or contact bleeding. Vienna classification was used to describe the localization and behavior of CD population at the time of sampling [38,39]. The HC group was composed of 30 healthy controls showing CRP level <6 mg/l (CRPXL Tina-quant® ROCHE Diagnostics, GmbH). Diverticulitis clinical diagnoses were confirmed by abdomen CT scanner. Pathogen caused enterocolitis were defined by acute onset of intestinal symptoms associated with stool culture positive for a specific pathogen. Rheumatoid arthritis patients were fulfilling the 1987 ACR criteria [40] and asthmatics the 1987 ATS criteria [41]. ASCA tests (Euroimmun, Germany) (Inova diagnostis, USA) and ANCA (The Binding Site, UK) test were realized on every sample according to manufacturers' recommendations, in order to correlate our results to existing

tests. In CD patients, the three main causative mutations in the *Card*15 gene were genotyped from genomic DNA either with Taqman MGB probes (Custom Taqman SNP genotyping assays—ABI, USA) for SNP12-G808R and SNP13-1007fs or by direct sequencing (Terminator Ready Reaction kits using a 3100 automated DNA analyzer, ABI, USA) for SNP8-R702W. Population was caucasian and other details on patient characteristics are summarized in Table 1.

2.2. Protein chip arrays preparation and analysis

Optimization of chips preparation and engine reading's parameters were achieved to obtain optimal profiles in terms of peaks number and resolution. A quality control serum sample was used as described previously [34]. The standardized sample preparation was used to minimize sources of variability. Two types of chip arrays were selected: Q10 and CM10 (anions and cations exchangers, respectively), both at pH 4. Chips were read on a Protein Biological System II (ProteinChip reader, Ciphergen Biosystems, USA) in the mass on charge (m/z) range: 0–25,000. The complete protocol was described previously by de Seny et al. [34]. Each sample was analyzed in quadruplicate.

2.3. Peak detection and clustering

Calibration, base line substraction, matrix saturated signal removing and normalization steps were realized as previously described [34]. Peak detection and peak cluster formation were performed with the ProteinChip Biomarker Wizard software Version 3.0 (Ciphergen Biosystems, Inc.-USA-CA-Fremont).

2.4. Data analysis

2.4.1. Decision tree boosting

Data were analyzed by a machine learning algorithm called decision tree boosting, as described by Geurts et al. [42], using the software PEPITo™ (PEPITe, Belgium).

2.4.2. Estimation of sensitivity and specificity

To obtain an unbiased estimation of the sensitivity and specificity of a classification provided by boosting for a given disease, leave-one-out cross-validation was used [34,42]. Sensitivity was estimated by the proportion of patients from the disease group well classified (true positives) and the specificity by the proportion of patients from the other groups that were well classified (true negatives).

2.4.3. Selection of potential biomarkers

Biomarkers were evaluated individually by univariate and multivariate analysis following the approach adopted in [43]. The discriminative power of peaks was assessed according to the associated P value calculated using the non-parametric Mann–Whitney test. In the multivariate analysis, we computed an ensemble of 100 decision trees, by Boosting methods from which it was possible to determine the relative relevance or contribution of each peak to the classification: importance (imp%). The measure of variable importance we used for a tree, was the Shannon information measure [44]. To further stabilize ranking with respect to data randomness, the

Table 1 – Characteristics of the patients and subjects of the study

Crohn disease patients: CD	
Treatment	
Immunosup.	53.3% (16/30)
Corticoids	26.7% (8/30)
Anti-TNF	30.0% (9/30)
Antibiotics	10.0% (3/30)
Mesalazine	46.7% (14/30)
Pathology localization	
Ileal	20.0% (6/30)
Ileo-colonic	60.0% (18/30)
Colonic	20.0% (6/30)
Pathology behavior	
Stricturing	6.7% (2/30)
Fistulizing	50.0% (15/30)
Non-stricturing non-fistulizing	43.3% (13/30)
Perianal disease	36.6% (11/30)
Miscellaneous	
CARD 15 variant carriers	56.6% (17/30)
Active (HBI > 6)	50.0% (15/30)
ASCA+[1]	73.3% (22/30)
ASCA+[2]	80.0% (24/30)
ANCA+	10.0% (3/30)
CRP mg/l (median-range)	4.9 (0.3–119)
Female	66.7% (20/30)
Male	33.3% (10/30)
Smokers	56.7% (17/30)
Extra. dig. manifestations	30.0% (9/30)
Age, years (median-range)	34 (20–53)
Disease duration, years (median-range)	13 (<1–29)
Ulcerative colitis patients: UC	
Treatment	
Immunosup.	16.7% (5/30)
Corticoids	13.3% (4/30)
Anti-TNF	None
Antibiotics	13.3% (4/30)
Mesalazine	90.0% (27/30)
Pathology localization	
Pancolitis	30.0% (9/30)
Left sided colitis	56.7% (17/30)
Proctitis	13.3% (4/30)
Miscellaneous	
Active	50.0% (15/30)
ASCA+[1]	0.0% (0/30)
ASCA+[2]	0.0% (0/30)
ANCA+	63.3% (19/30)
CRP mg/l (median-range)	7 (0–190)
Female	26.7% (8/30)
Male	73.3% (22/30)
Smokers	26.7% (8/30)
Extra. dig. manifestations	16.7% (5/30)
Age, years (median-range)	51 (31–73)
Disease duration, years (median-range)	5 (<1–15)
Inflammatory control patients: IC	
Treatment	
Immunosup.	10.0% (3/30)
Corticoids	10.0% (3/30)
Anti-TNF	6.7% (2/30)
Antibiotics	20.0% (6/30)
Sulfasalazine	6.7% (2/30)
Pathology	
Rheumatoid arthritis	33.3% (10/30)
Asthma	16.7% (5/30)

Table 1 (Continued)

Infectious enterocolitis	23.3% (7/30)
Diverticulitis	26.7% (8/30)
Miscellaneous	
ASCA+[1]	16.7% (5/30)
ASCA+[2]	6.7% (2/30)
ANCA+	3.3% (1/30)
CRP mg/l (median-range)	14.4 (1.2–315.8)
Female	56.7% (17/30)
Male	43.3% (13/30)
Age, years (median-range)	54 (23–76)
Healthy control: HC	
ASCA+[1]	3.3% (1/30)
ASCA+[2]	10.0% (3/30)
ANCA+	0.0% (0/30)
Female	53.3% (16/30)
Male	46.7% (14/30)
Age, years (median-range)	47(21–77)

variable importances presented in the result section (imp%) were averages of the relative importance obtained by randomly sampling 50 times 70% of the patients without replacement from the whole set of patients.

2.4.4. *Correlation between specific biomarkers and patients characteristics*

Correlation coefficient (r) and associated P value were determined with the non-parametric Spearman test and by Mann–Whitney, respectively. P values were considered statistically relevant when inferior to 0.05.

2.5. *Purification and identification of biomarkers*

2.5.1. *Antibodies, immunodepletion and Western blotting*

Specific antibody for PF4 used in WB and in immunodepletion experiments was a polyclonal rabbit (Gentaure, Belgium). For immunodepletion it was coupled with protein G+ agarose beads (Santa Cruz Biotechnology, USA) as recommended by manufacturer. Polyclonal rabbit anti-FLAG antibody (Santa Cruz Biotechnology) or protein G+ beads alone were used as negative controls. Polyclonal chicken antibody for Haptoglobin (Ab14248, Abcam, UK) recognizes different allotypes: Hp1-1, Hp1-2 and Hp2-2. Western blotting detection on serum were realized on a single sample, typical of each active disease or healthy control.

2.5.2. *ELISA assay for PF4*

Specific ELISA for PF4 (American Diagnostica Inc., USA) was carried out as recommended by manufacturer except for the optimal sera dilution, which was 1000. The data are expressed in ng of PF4 per ml of sera (1 IU is equivalent to 1 ng of PF4).

2.5.3. *Poly-acrylamide gel electrophoresis and in gel trypsin digestion*

Twelve percent SDS-MES Nu PAGE were used for protein separation and Western blotting, as well as silver staining coloration kit (Invitrogen-Life Technologies, USA). Band excision and in gel trypsin digestion were realized as described in [45] using trypsin (Promega, USA). Solubilization of peptides for MS–MS analysis was obtained in 0.1% formic acid.

2.5.4. *Purification of peptide FIBA*

A pool of several digestive IC sera was first depleted with protein A-agarose as recommended by the manufacturer (Sigma, USA). The supernatant was diluted in 100 mM sodium acetate buffer, 30 mM NaCl, 1.4 M Urea, 0.4 M Thiourea and 0.4% CHAPS at pH 4 and loaded on CM-Sephacryl column (Biosepra, France) equilibrated in pH 4 sodium acetate buffer, 30 mM NaCl, 1.4 M Urea, 0.4 M Thiourea and 0.4% CHAPS. The non-retained fraction and washes were pooled to be concentrated by precipitation with icy cold acetone. Solubilization of pellets was done in 0.5% TFA. Last desalting and concentration steps were realized with C8 column (Amersham, UK) as recommended by manufacturer and the eluted fraction, in 70% ACN was stored for further MALDI MS–MS analysis.

2.5.5. *MS–MS analysis*

MALDI MS–MS peptides analysis were performed with a MALDI-TOF–TOF (Bruker, France), with saturated CHCA (Ciphergen, USA) prepared as recommended by the manufacturer. The monoisotopic peptide showing a mass at 1465 Da was selected and submitted to further fragmentation. Analysis of fragmented ions produced was made on MASCOT (http://www.matrixscience.com).

The peptides sequences spectra of Hpα2 were obtained using nanochromatography (Agilent, USA) on-line coupled with an ion-trap mass spectrometer (MSD Trap XCT, Agilent, USA). Eight microliter of sample were injected, concentrated and desalted on a C18 nanoprecolumn (Agilent). Peptides were then eluted and separated on a C18 column at 200 nl/min. The separation was performed using a gradient of solvent A (100% H_2O, 0.1% HCOOH) and solvent B (97% ACN, 3% H_2O, 0.1% HCOOH). Detection was carried out in positive mode and the spectra were acquired on a m/z 300–2000 range. The protein identifications were performed using MASCOT (http://www.matrixscience.com).

3. Results

Several important factors influence the reproducibility of the results obtained with SELDI-TOF-MS. We processed samples according to a standardized procedure as described in our previous reports [42].

3.1. *Classification models, specificity, sensitivity and accuracy*

We first studied the ability of the boosting algorithm to accurately discriminate the different diseases groups (CD versus UC, IBD versus controls), either considering the entire cohort (n = 120) or only patients with clinically active disease (n = 60). Considering the entire database, 120 samples were profiled in quadruplicate and gave a total of 480 mass spectra. Peak detection with the Ciphergen software resolved a total of 137 peaks on CM10 and 37 peaks on Q10 arrays in the m/z ratio between 1000 and 25,000. The quadruplicates repeatability was ranging from 12 to 14% depending on the patient groups. These percentages were in agreement with those announced by Ciphergen (20–30%).

Table 2 – Sensitivities, specificities and accuracies obtained by decision-tree boosting analysis

	Peaks detected by the Ciphergen software		
	Sensitivity	Specificity	Accuracy
Q10 arrays			
IBD vs. all controls	88.3% (53/60)	96.7% (58/60)	92.5% (111/120)
CD vs. UC	76.7% (23/30)	86.7% (26/30)	81.7% (49/60)
Active IBD vs. IC	96.7% (29/30)	100.0% (30/30)	98.3% (59/60)
Active CD vs. active UC	60.0% (9/15)	86.7% (13/15)	73.3% (22/30)
CM10 arrays			
IBD vs. all controls	85.0% (51/60)	95.0% (57/60)	90.0% (108/120)
CD vs. UC	85.0% (51/60)	95.0% (57/60)	90.0% (108/120)
Active IBD vs. IC	93.3% (28/30)	96.7% (29/30)	95.0% (57/60)
Active CD vs. active UC	93.3% (14/15)	86.7% (13/15)	90.0% (27/30)

Percentage of sensitivity, specificity and accuracy obtained on CM10 and Q10 chip arrays, for each comparison, with peaks detected by the Ciphergen software. Four spectra per patient were considered; a sample being classified in a given category when three out of its four spectra gave the same results in the boosting analysis. The specificity and sensitivity, as the accuracy were calculated by leave-one-out.

Table 2 shows the sensitivity, the specificity and the accuracy obtained by decision tree boosting on Q10 and CM10 arrays. Sensitivity, specificity and accuracy of multivariate models were calculated taking into account that a sample is considered as classified in a given patient category when tree of its four spectra give the same result. The different group comparisons considering all the patients (IBD versus all controls and CD versus UC) gave sensitivities ranging from 76.7 to 90%, on Q10 arrays and from 85 to 90%, on CM10 arrays. When we considered only patients with active disease, sensitivities were even higher for the comparisons ''active IBD versus IC'' and particularly (93.3%), ''active CD versus active UC'' (93.3%), on CM10. In every cases, specificities (83.3–100%), as well as accuracies (73.3–98.3%) obtained were rather

Table 3 – ASCA and ANCA tests

	All			Active		
	ASCA+		pANCA+	ASCA+		pANCA+
	1	2		1	2	
(A)						
CD	73.3% (22/30)	80.0% (24/30)	6.6% (2/30)	73.3% (11/15)	86.7% (13/15)	4.5% (1/15)
UC	0.0% (0/30)	0.0% (0/30)	60.0% (18/30)	0.0% (0/15)	0.0% (0/15)	66.7% (10/15)
IC	16.7% (5/30)	6.7% (2/30)	3.3% (1/30)	16.7% (5/30)	6.7% (2/30)	3.3% (1/30)
HC	3.3% (1/30)	10.0% (3/30)	0.0% (0/30)			

	Sensitivity	Specificity	Accuracy
(B)			
IBD vs. all controls			
1	66.7% (40/60)	90.0% (54/60)	78.3% (94/120)
2	70.0% (42/60)	62.2% (56/60)	81.7% (98/120)
CD vs. UC			
1	66.7% (20/30)	60.0% (18/30)	63.3% (38/60)
2	76.7% (23/30)	56.7% (17/30)	66.7% (40/60)
Active IBD vs. IC			
1	70.0% (21/30)	33.3% (9/30)	50.0% (30/60)
2	76.7% (23/30)	23.3% (7/30)	50.0% (30/60)
Active CD vs. active UC			
1	73.3% (11/15)	66.7% (10/15)	70.0% (21/30)
2	86.7% (13/15)	66.7% (10/15)	76.7% (23/30)

(A) Percentage of ASCA+ and ANCA+ determined on the same patients and subjects cohort. (B) Percentage of sensitivity, specificity and accuracy calculated for CD and UC patients using the combination of ASCA and ANCA tests. Only ASCA+/ANCA− patients were considered as true positives when considering CD patients and true negatives for UC patients. ASCA−/ANCA+ were the only true positives status accepted for UC and true negatives for CD patients. Other combinations, ASCA+/ANCA+ or ASCA−/ANCA− were both considered as non-CD and non-UC and were only involved in accuracy calculation, as false positives or false negatives. IBD patients were ASCA+ and/or ANCA+. Subjects showing ASCA−/ANCA-were the only true negatives considered in the ''IBD vs. controls'' or either ''active IBD vs. IC'' comparisons. The combination of ANCA and ASCA status obtained by two different kits (Euroirnrnun = 1 and Inova = 2) were evaluated for every groups of subjects.

high. Sensitivities, specificities and accuracies reached with this proteomic approach were similar or higher than those obtained with ASCA and ANCA tests performed on the same serum sample set, as detailed for the entire group and for the active patients (Table 3A). Table 3B shows the sensitivity, specificity and accuracy obtained with combined ASCA and ANCA results, generally leading to better sensitivity and specificity than ASCA or ANCA alone. Finally, the proteomic method and downstream multivariate analysis provided similar or higher accuracy than these antibody biomarkers kits, particularly for active diseases.

3.2. Potential biomarkers selection

Boosting decision-tree method also provided information about peaks which presented high potential of discrimination. Table 4 illustrates the first 10 most discriminating peaks obtained with the boosting method, on Q10 and CM10 arrays. The quantity of information that a peak brings in the ensemble decision trees was referred as the percentage of importance (imp%) and was used to rank the potential biomarkers. We also provided in Table 4, the *P* values calculated by univariate analysis with the Mann–Whitney test. We then made the assumption that highly informative peaks were good biomarker candidates. Informative peaks were different considering either the entire group of patients or only patients with active disease: active IBD versus IC and IBD versus controls. In many cases, the most discriminating peaks were often associated with a low *P* value.

Among the most discriminating biomarkers relevant through all the statistical questions addressed (Table 4), several mass ranges or peaks were found to be present in more than one comparison. For example, a peak at 2666 was found discriminating for active IBD versus IC and was also able to discriminate UC from CD and active UC from active CD, on CM10 array. Many biomarkers appeared differentially represented on spectra of CD or UC patient groups and seemed to be specifically correlated to these pathologies. We found that both types of biomarkers were combined in the model of classification designed by boosting which was aiming at discriminating IBD from controls.

3.3. Identification of biomarkers

Some statistically relevant biomarkers have been successfully purified and identified (see Table 4, peaks labelled with a asterisk (*)). Purification strategy was different for each biomarker identified and briefly described below.

Purification on CM-Sephacryl and further MS–MS sequencing using MALDI-TOF–TOF were successful for the identification of a singly charged peptide at *m/z* 1465 Da observed on Q10 (Table 4). Fig. 1A shows the sequence of FIBA peptide obtained by MS–MS. The imp% and *P* value of FIBA in two diagnosis questions are given in Fig. 1B. Fig. 1C shows the distribution of

Table 4 – The 10 most discriminating biomarkers, on Q10 and CM10 arrays ranked according to imp(%) determined by boosting and associated *P* value calculated by Mann–Whitney test (if $<10^{-12}$ to ~0)

Peaks detected on Q10						Peaks detected on CM10					
IBD vs. all			Active IBD vs. IC			IBD vs. all			Active IBD vs. IC		
m/z	imp%	*P* value	*m/z*	imp%	*P* value	*m/z*	imp%	*P* value	*m/z*	imp%	*P* value
4636	21.4	0	15,856	57.1	7.0×10^{-7}	4213	12.9	5.0×10^{-10}	**7772***	**58.2**	**0**
6233	10.4	2.41×10^{-8}	4636	21.3	10^{-10}	4238	6.3	1.1×10^{-6}	5822	14.1	0
12,452	8.7	4.90×10^{-9}	16,836	4.9	0.59	3068	5.1	0	**2664***	10.3	**0**
4827	6.8	5.82×10^{-5}	1622	3.3	2.75×10^{-7}	24,097	3.7	3.0×10^{-10}	4215	4.3	0
1263	3.7	1.70×10^{-9}	15,121	2.9	3.63×10^{-5}	4289	3.1	0.048	2993	1.4	2.85×10^{-2}
1545	3.2	4.70×10^{-5}	4269	2.4	7.25×10^{-4}	3163	2.8	0.12	3688	1.2	7.25×10^{-5}
1469*	**3.0**	$\mathbf{9.6 \times 10^{-3}}$	1424	1.0	0.86	23,197	2.0	2.90×10^{-7}	4288	1.0	0.01
1453	2.9	8.49×10^{-5}	4479	1.0	9.5×10^{-9}	5753	2.0	4.19×10^{-7}	**15,870***	**0.9**	$\mathbf{4.65 \times 10^{-4}}$
13,889	2.3	0.09	1439	0.9	1.5×10^{-3}	1741	1.9	1.94×10^{-6}	3821	0.9	0
4479	2.1	4.19×10^{-7}	1523	1.0	2.55×10^{-5}	1945	1.9	2.24×10^{-4}	2543	0.8	0.28

Peaks detected on Q10						Peaks detected on CM10					
UC vs. CD			Active UC vs. active CD			UC vs. CD			Active UC vs. active CD		
m/z	imp%	*P* value	*m/z*	imp%	*P* value	*m/z*	imp%	*P* value	*m/z*	imp%	*P* value
15,856	12.3	0.56	12,608	10.6	1.8×10^{-4}	5822	8.0	5.0×10^{-4}	2681	18.8	0
12,608	10.8	0.03	13,889	8.9	8.05×10^{-4}	1898	6.0	1.63×10^{-5}	**2664***	**7.6**	**0**
4827	7.3	1.19×10^{-5}	4636	7.8	7.51×10^{-2}	2681	5.2	1.70×10^{-7}	4970	3.3	0.80
4164	6.7	0.03	**1469***	**6.8**	$\mathbf{5.6 \times 10^{-2}}$	4971	4.1	0.14	1888	3.7	10^{-10}
1469*	**5.9**	$\mathbf{1.69 \times 10^{-3}}$	20,844	6.7	0.71	**10,836***	**4.1**	$\mathbf{4.31 \times 10^{-5}}$	3332	3.4	2.7×10^{-8}
4636	5.6	0.01	4164	6.1	0.87	**2663***	**3.9**	$\mathbf{1.75 \times 10^{-6}}$	3247	3.3	10^{-8}
1439	4.4	1.23×10^{-4}	1453	4.5	0.01	2953	3.4	4.48×10^{-4}	1076	3.2	2.7×10^{-9}
22,148	3.3	2.24×10^{-5}	12457	3.9	0.16	1078	3.0	7.12×10^{-6}	1897	3.1	1.9×10^{-9}
4479	3.0	0.04	1439	2.9	0.32	1921	2.7	2.75×10^{-7}	8923	3.0	4.5×10^{-8}
8224	2.9	5.0×10^{-3}	4195	2.9	1	1888	2.7	6.8×10^{-6}	2298	3.0	0

FIBA or fibrinopeptide A
peptide sequence: DSGEGDFLAEGGGVR
(A)

Charged form	Comparison	P value	*imp%*
1465 1H+	IBD *vs* HC, IC	10^{-2}	3.03
	UC *vs* CD	$1.3\ 10^{-3}$	5.08

(B)

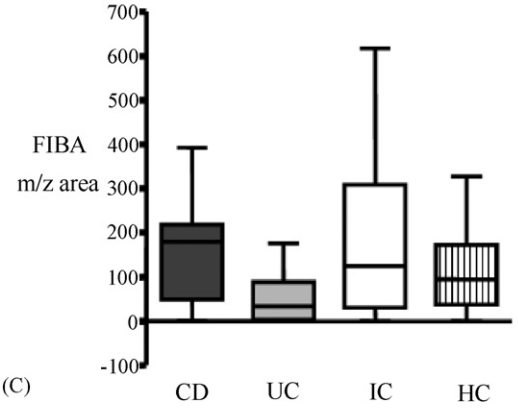


Fig. 1 – Identification of biomarker at *m/z* 1465, as FIBA. (A) Identification of peptide by MALDI TOF-TOF. Biomarker at *m/z* 1465 was purified by ion exchange chromatography on CM-Sephacryl and analyzed by MALDI TOF–TOF. Its fragmentation led to the sequence of FIBA or fibrinopeptide A. FIBA shows a theoretical MW of 1464.72 Da very close to value measured by MALDI: 1464.65 Da. (B) Statistical univariate and multivariate analysis of the biomarker at *m/z* 1465, on Q10. *P* value determined by Mann–Whitney and importance (imp%) derived from boosting are given for every diagnosis comparison in which FIBA is involved. (C) Distribution of FIBA in patient groups. The plot shows the distribution of FIBA peak area obtained on Q10 chip, for every patient group. Values of median, 25th and 75th percentiles, range were shown in plots.

FIBA in SELDI spectra (peak area) for every subject ($n = 120$). Fig. 4A shows FIBA's peak intensity distribution among the IBD group. We observed on spectra higher level of FIBA for inflammatory controls compared to other patients and lower levels in UC than in CD.

The most important biomarker in the boosting model discriminating active IBD from other inflammatory controls was the peak at *m/z* 7771 (Table 4). Fig. 2A shows SELDI peak at *m/z* 7771. Its peak intensity distribution for active disease is given in Fig. 4B. We used the program TagIdent (expasy.com) to hypothesize the identity of this protein, on the bases of its MW (7771 Da with 0.3% mass variation) and possible isoelectric point (>4) as platelet aggregation factor 4 (PF4) or CXCL4. We confirmed this hypothesis by specific immunodepletion of PF4 in IC sera and subsequent analysis of corresponding peak by protein chip (Fig. 2B). PF4 was detected in sera of active patients by Western blotting (Fig. 2C). In addition, specific PF4 ELISA was performed on the entire cohort of patients to compare and confirm SELDI results (Fig. 2D). Means, medians and ranges for all the patient groups revealed an increased level of PF4 for IC compared to active IBD patients. The correlation coefficients between PF4 measured by ELISA and by SELDI were statistically relevant and were 0.56 for active diseases, 0.41 for active IBD, 0.6 for active CD, 0.56 for IC and 0.48 for active UC.

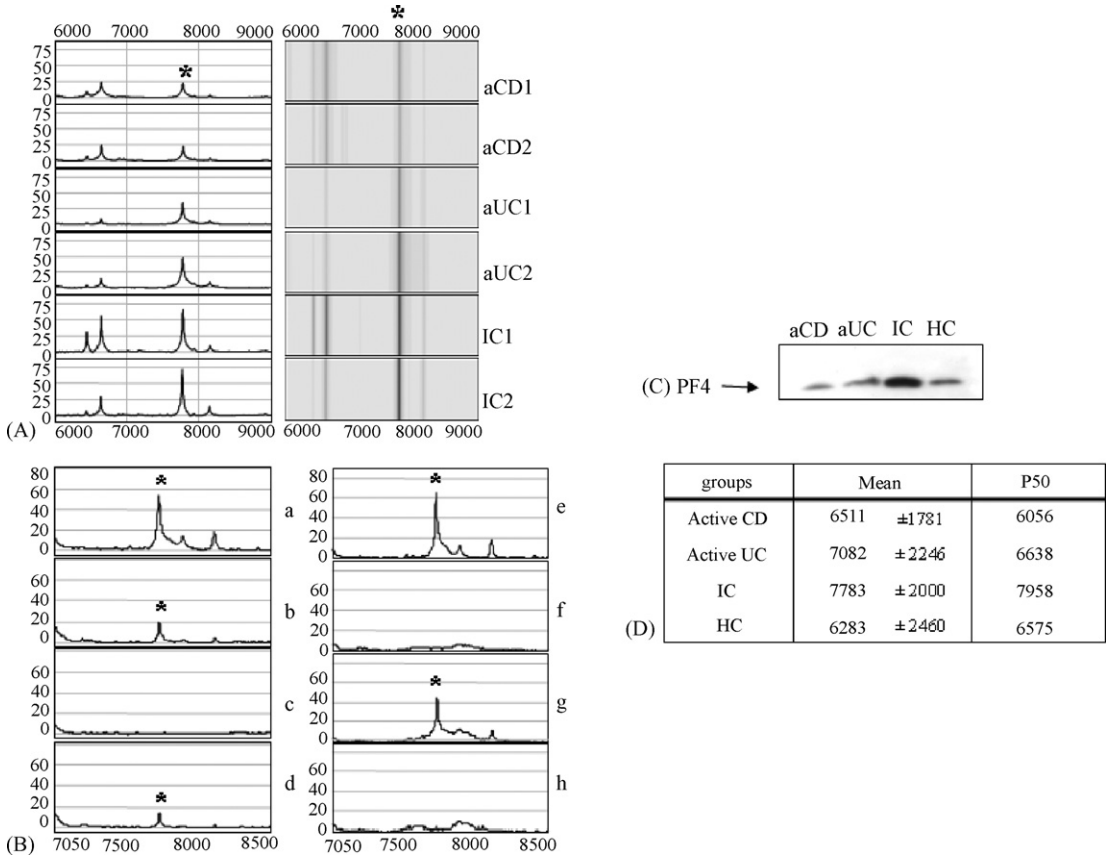
The same methodology was applied to identify the biomarker at *m/z* 10,838 as MRP8: myeloid related protein 8, as described previously by de Seny et al. [34]. In our SELDI profiles, the peak corresponding to MRP8 can only be detected for IC and UC patients (data not shown), thus MRP8 appeared in this study statistically relevant for discrimination of CD versus UC, as reported in Table 4.

As noted by Ciphergen software peak clustering, many forms of the same protein can be observed on CM10 spectra as statistically relevant: *m/z* at 2663 ($6 \times H^+$) and *m/z* at 15,870 ($1 \times H^+$).

Fig. 3A shows SELDI peaks at *m/z* 2663 in active diseases which is the six times charged form of the one at *m/z* value 15,870. We identified this protein using separation on 12% SDS-MES Nu PAGE and in gel trypsin digestion of the corresponding band. This band localized around 16 kDa and was found increased in IC samples as observed on SELDI spectra. The trypsin digested band was analyzed by nanoLC–MS–MS and revealed two peptidic sequences corresponding to Haptoglobin precursor, HpP. We finally identified this biomarker as Haptoglobin α2 (Hpα2) and Fig. 3C shows the complete sequence of Hpα2 and the percentage of peptide coverage obtained by MS–MS analysis. As observed in silver stained gel and specific Western blotting for Hp, Hpα2 is increased in IC compared to active IBD and HC (Fig. 3B(a and b)). Fig. 4C shows peak intensity distribution of Hpα2 ($6H^+$), among IC and IBD groups.

3.4. *Correlations and possible link between potential biomarkers and patients' characteristics*

We also analyzed, by univariate method (Mann–Withney test), links between selected biomarkers and characteristics of patients. The *P* value between each patient characteristic and every peak considered was calculated. Despite possible lack of homogeneity in age distribution, naturally encountered in IBD population, none of the selected biomarkers was significantly linked to age, neither to sex repartition. Moreover, we found that most of the potential biomarkers discriminating for CD were statistically linked to ASCA positive test, as for potential biomarkers linked to UC and pANCA status ($P < 5 \times 10^{-2}$). Among selected biomarkers, within CD patients, we could calculate a significant *P* value between carriage of *Card15* gene variant and PF4 ($P = 2.7 \times 10^{-2}$) and an unidentified peak at *m/z* 1570 ($P = 2.7 \times 10^{-2}$), on CM10 array. On Q10 array, two unidentified potential biomarkers at 13,889 Da ($P = 10^{-2}$) and 14,153 Da ($P = 1.9 \times 10^{-2}$) were significantly linked to *Card15* mutations. In addition, we found some potential biomarkers linked with disease clinical activity, among which PF4 and Hpα2. Finally, PF4 and FIBA found on SELDI spectra could significantly be correlated with serum CRP levels, measured by ELISA (Spearman test), in the active CD population ($r = 0.68$, $P = 5.8 \times 10^{-3}$ and $r = 0.62$, $P = 1.3 \times 10^{-2}$). A potential biomarker at *m/z* 10,253 on CM10 and Hpα2 ($6H^+$) were correlated to



(D)

groups	Mean		P50
Active CD	6511	±1781	6056
Active UC	7082	±2246	6638
IC	7783	±2000	7958
HC	6283	±2460	6575

Fig. 2 – Identification of biomarker at *m/z* 7771 as PF4. (A) CM10 spectra showing PF4 peak at *m/z* 7771. Two spectra of two patients per active disease groups are represented: aCD, aUC and IC. (B) Identification of PF4 with specific antibody for PF4 SELDI spectra of raw sera (a and e), depleted sera with protein G+ alone (b), with protein G+ coated with specific antibody for PF4 (c) and protein G+ coated with a non specific antibody (d) are represented. PF4 was clearly detectable in eluted fractions from protein G+ coated with PF4 antibody (g) and not from protein G+ alone (f) or protein G+ coated with a non-specific antibody (h). (C) Western blotting on raw sera (0.5 μl per lane) of four patients with specific antibody for PF4. (D) ELISA detection of PF4 in sera of all patients (*n* = 120). Median, mean and standard deviation of this assay are given for every group of patients. PF4 level are in ng/ml of raw sera.

CRP only for active UC patients ($r = 0.51$, $P = 5.2 \times 10^{-2}$ and $r = 0.57$, $P = 2.6 \times 10^{-2}$). Correlations between other biomarkers on spectra and CRP level were evaluated but did not reach statistical confidence.

4. Discussion

We report the first proteomic study using serum profiling with SELDI-TOF-MS in IBD. Our general aim was to select new biomarkers potentially involved in IBD pathophysiology and which might be useful for IBD diagnosis and management. The study was performed on 120 samples, all processed with cautions in regards of general criticisms and limitations often observed with the technique [46,47]. Patients were recruited in our University hospital and are representative of IBD populations affected in our region.

Our major goal was to achieve preliminary proteomic experiments on a wide panel of IBD cases in order to obtain general diagnostic tools as well as general information concerning pathophysiology of these diseases [1–3]. Therefore, we selected both clinically active and inactive IBD patients, as well as some digestive and non-digestive IC. We first analysed all the 120 patients and secondarily the 90 clinically active patients independently, to be closer to real diagnosis situation. The mass spectra analysis revealed a large number of potentially interesting protein peaks with significant *P* value. As IBD are complex and heterogeneous pathologies, combination of several biomarkers may be more relevant for diagnosis. Therefore, we ran a multivariate analysis model, with the boosting method aiming at selecting a limited number of biomarkers according to a robust statistical basis [42].

Using boosting algorithm, we built up classification models based on four comparisons and cross validation by leave-one-out to calculate sensitivity, specificity and accuracy. These were higher for most of the comparisons for the active cohort than for the entire cohort. This could be due to a greater homogeneity of the active patient subgroup, but also to the fact that important discriminating biomarkers, among which acute phase reactant proteins, might appear significant only in clinically active diseases. Despite this general tendency, the sensitivity observed in this active subgroup for the comparison CD versus

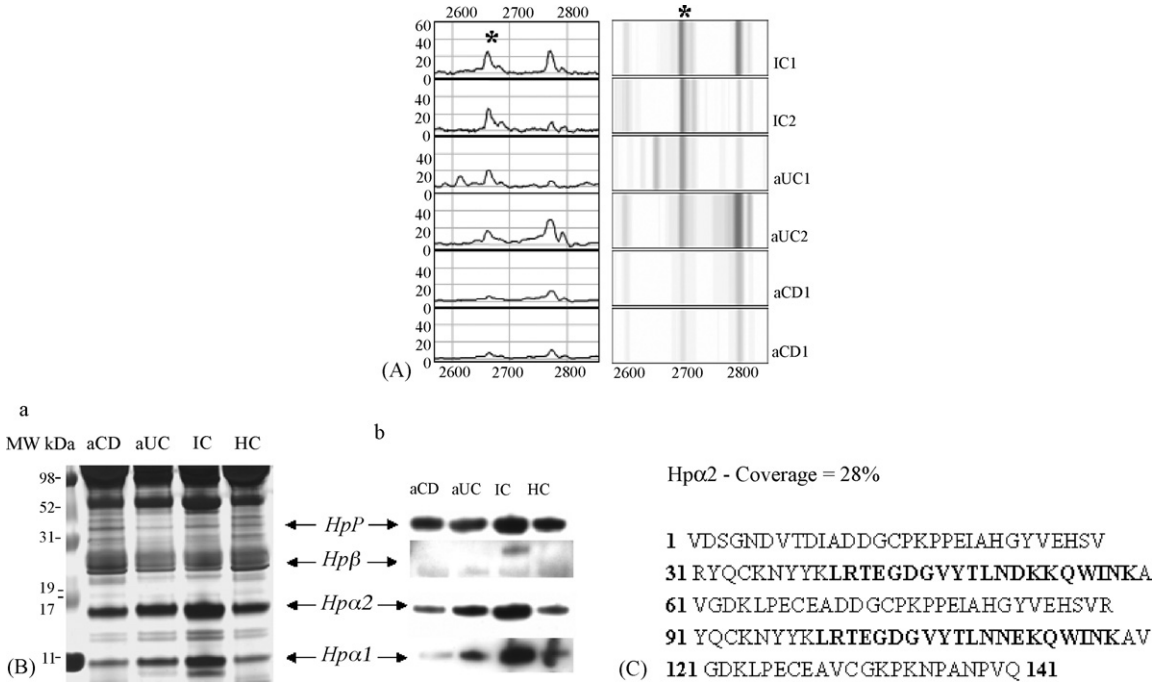


Fig. 3 – Identification of biomarkers at *m/z* 15,870 and 2663, as Hpα2. (A) Hpα2 main peak on SELDI profiles for active diseases. Two patients profiles recorded on CM10 chip array are given for each group of active diseases: aCD, aUC and IC. (B) Hp precursor and Hp subunits, on SDS-PAGE. (a) Silver stained corresponding gel with 0.5 μl of raw sera. (b) Western blotting anti-Hp on 0.5 μl of raw sera of patients, taken in active CD, active UC, IC and HC groups. Western blot signal was obtained with a specific antibody anti-Hp, recognising every allotypes allowing detection of precursor band (45 kDa), β subunit (27 kDa), Hpα2 (16 kDa) and Hpα1 (11 kDa). (C) Identification of Hpα2 by MS–MS. The gel band corresponding to HPα2 (B) was analyzed on ion-trap MS–MS. The two amino acid stretches deduced are represented here and belong to Hp precursor (HPT_HUMAN). The sequences obtained by MS–MS are amino acid in bold and present a percentage of coverage of 28% of the complete sequence of HPα2.

UC was somewhat lower (60 and 80% on Q10 and CM10 arrays, respectively), while specificity remained high (93% for both arrays). This might be consecutive to the fact that we were considering very similar inflammatory manifestations occurring in two very close pathologies. But, the efficiency of this model on active patients remained nevertheless very promising. Accuracies of models designed by boosting and cross validation by leave-one-out were similar or higher than the currently available serum biomarker tests, ANCA and ASCA [13–16]. In particular, when we tried to discriminate active IBD group from IC group, we obtained a higher accuracy than by combining ASCA and ANCA status. Globally, the step of cross validation by leave-one-out, the number of recorded patients and the use of replicates imply that the data obtained with our models are statistically relevant.

We achieved successful purification and identification of four relevant biomarkers (with good *P* value and high imp%): PF4, Hpα2, FIBA and MRP8. They are all known proteins of acute phase inflammation, but their differential distribution among patient groups could help to discriminate IBD from controls.

PF4 belongs to the CXCL chemokine family and is produced mainly by megakaryocytes, stocked in platelet α granules and released upon activation at site of injury [48–50]. Danese et al. have previously reported evidences that IBD patients present a higher platelet activation state than healthy controls and proposed that platelet dysfunction could be part of IBD pathophysiology [51]. We found a PF4 distribution on spectra different between inflammatory controls and IBD patients, this could reflect this platelet dysfunction. In addition, PF4 level in sera of our subjects were assessed by ELISA and correlated successfully to PF4 SELDI distribution for active diseases. But, PF4 distribution based on ELISA test failed to provide the nice separation among IBD patients obtained with SELDI profiles. PF4 distribution was also studied by others using ELISA technique [52,53]. Interestingly many variant forms of PF4 exist and are generated by proteolytic processing of the mature form, as well as genetic ones [54–56]. In this work, the major form of PF4 detected is precisely the one at 7771 Da. Unlike ELISA, SELDI-TOF-MS technology is able to differentiate PF4 variants allowing here an efficient discrimination of active IBD from other inflammatory digestive pathologies.

FIBA is a peptide released during clotting from fibrinogen precursor to produce fibrin and is also released during inflammation [57]. FIBA release is associated with a prethrombotic state which characterizes IBD [58,59]. In this context, we confirm results from other studies reporting an increase of FIBA in IBD compared to healthy controls and also in active CD compared to CD in remission. As Knot et al., we observed no difference in FIBA in IBD patients in stable remission compared to healthy controls [52].

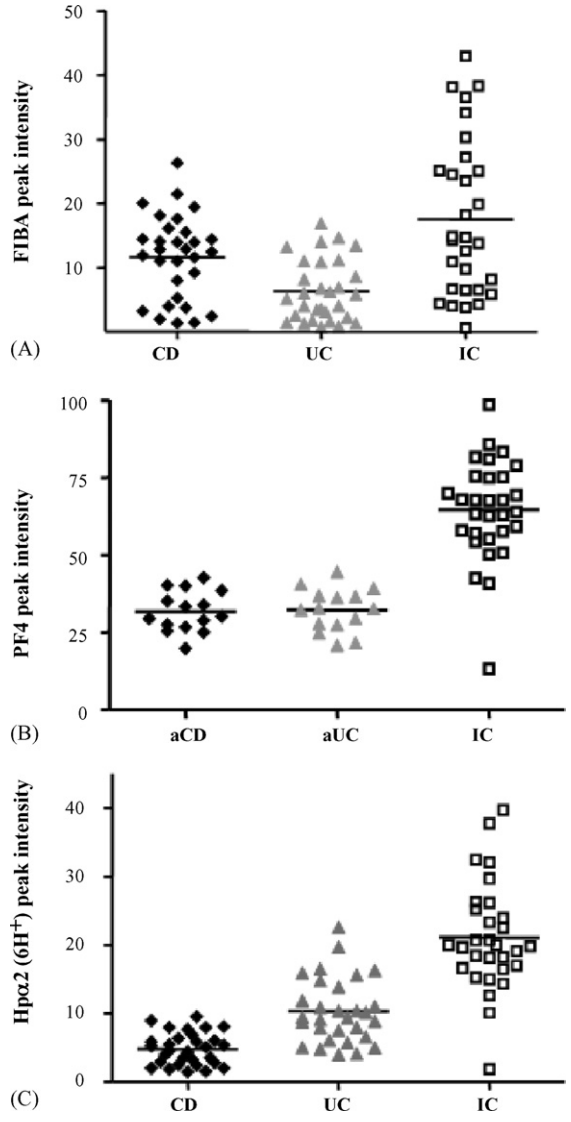


Fig. 4 – Distribution of biomarkers FIBA, PF4 and Hpα2 (6H+) (peak intensity) among patient groups. (A) Distribution of the FIBA peak intensity among IC and IBD patients: CD and UC. (B) Distribution of the PF4 peak intensity among active patient groups: aCD, aUC and IC. (C) Distribution of the Hpα2 (6H+) peak intensity among patient groups: CD, UC and IC. Every dot corresponds to the mean calculated from quadruplicates recorded for each patient. Cluster plots corresponding to patient groups are given only when the peak of interest is significant. Lines represent population distribution mean.

In this work, MRP8 was identified as specific marker for UC and is an important protein for many chronic inflammatory diseases as reported for rheumatoid arthritis and psoriasis [60–62]. MRP8 is also part of the calprotectin complex, a biomarker of clinical activity in IBD when quantified in the stool. Calprotectin is also detected in blood but without reaching the same diagnosis efficiency [63]. In this study MRP8, appears valuable to help in discriminating UC from CD, taking into account that only 50% of UC patients present the typical peak in their profiles.

Haptoglobin (Hp) is a protein existing in blood as a multimeric protein complexes of 2α and 2β subunits. Two haplotypes of Hpα exist: Hpα1 and Hpα2, giving phenotypes Hp1-1, Hp1-2 and Hp2-2. Hp is generally quantified independently of its subunits repartition and is used as a general marker of inflammation or haemolysis and fluctuates with types and disease evolution. Indeed, a previous study reported general increases in Hp level for active IBD, correlated with CDAI [64]. Moreover, Hp was reported as globally decreased after successful treatment with anti-TNF therapy for CD [65]. In this work, we demonstrate that the Hpα2 subunit independently of Hpα1 and β could be considered, as a new biomarker specific for IBD. We also observed a lower level of Hpα2 on spectra, for patients in remission compared to active one, some of them treated with anti-TNF therapy (data not shown).

Finally, some selected biomarkers appeared linked to patient characteristics (*Card15** or ASCA seropositivity in CD and pANCA status for UC). Moreover, we did not find any association between biomarkers and any trivial characteristics like age, gender and treatment. Some biomarkers, particularly those selected in the subgroup of active patients, as PF4 and Hpα2, were correlated with the clinical activity of the disease, and to CRP in some subgroup. Hence classical markers of inflammation, such as CRP, are not perfectly correlated to either clinical or endoscopic activity of IBD, these new markers may reveal to be useful and complementary to characterize disease activity.

The biomarkers that were identified in our study are not completely new, nor surprising in the setting of IBD. However, the fact that they were selected through a robust statistical process among a huge range of potential serological markers highlight their potential importance for both IBD pathophysiology and diagnostic. Furthermore, the potential biomarkers selected here are small proteins or fragments of proteins or small proteins isoforms potentially with variable postranslational modifications, which would not necessarily be easily detected by strategies other than proteomic analysis and mass spectrometry.

In conclusion, this first proteomic study on sera of IBD patients provided preliminary promising results. Use of proteomic profiles gave high sensitivity and specificity for IBD diagnosis. Furthermore, purification and identification of some selected biomarkers could give information about their possible contribution to the pathophysiology of these diseases. Validating such results in confirmatory cohorts and in particular situations, such as in paediatric IBD cases would be of particular interest. The discovery of new biomarkers through ''without a priori hypothesis'' strategies such as proteomic profiling may help to better understand these diseases and improve diagnostic procedure.

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