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TOPIC HIGHLIGHT

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Biomarkers in inflammatory bowel diseases: Current status and proteomics identification strategies

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Abstract

Unambiguous diagnosis of the two main forms of inflammatory bowel diseases (IBD): Ulcerative colitis (UC) and Crohn's disease (CD), represents a challenge in the early stages of the diseases. The diagnosis may be established several years after the debut of symptoms. Hence, protein biomarkers for early and accurate diagnostic could help clinicians improve treatment of the individual patients. Moreover, the biomarkers could aid physicians to predict disease courses and in this way, identify patients in need of intensive treatment. Patients with low risk of disease flares may avoid treatment with medications with the concomitant risk of adverse events. In addition, identification of disease and course specific biomarker profiles can be used to identify biological pathways involved in the disease development and treatment. Knowledge of disease mechanisms in general can lead to improved future development of preventive and treatment strategies. Thus, the clinical use of a panel of biomarkers represents a diagnostic and prognostic tool of potentially great value. The technological development in recent years within proteomic research (determination and quantification of the complete protein content) has made the discovery of novel biomarkers feasible. Several IBD-associated protein biomarkers are known, but none have been successfully implemented in daily use to distinguish CD and UC patients. The intestinal tissue remains an obvious place to search for novel biomarkers, which blood, urine or stool later can be screened for. When considering the protein complexity encountered in intestinal biopsy-samples and the recent development within the field of mass spectrometry driven quantitative proteomics, a more thorough and accurate biomarker discovery endeavor could today be performed than ever before. In this review, we report the current status of the proteomics IBD biomarkers and discuss various emerging proteomic strategies for identifying and characterizing novel biomarkers, as well as suggesting future targets for analysis.

Key words: Inflammatory bowel disease; Biomarker; Proteomics; Citrullination; Ulcerative colitis; Crohn's disease; Posttranslational modification

Core tip: Establishing the correct diagnose of Crohn's disease and ulcerative colitis (UC) patients remains troublesome, and correct and early medication is critical. No reliable biomarkes have been implemented in clinical usage, to distinguish between Crohn's disease patients and UC patients. Considering the protein complexity encountered in intestinal biopsy samples and the recent development within the field of quantitative proteomics, submitting the intestinal mucosa to a more thorough analysis has the potential to reveal new biomarkers.

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INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic gastrointestinal disorders. The two most common forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC). Both disorders have great impact on the life quality of the affected individuals and for society, measured on lost labor and expenses to the health care system. Furthermore, new epidemiological data published in 2013 found that the incidence and prevalence of the diseases are still increasing[1]. The etiologies of CD and UC remain unclear, but involve a complex interplay between genetic and environmental factors[2-7]. The diagnosis can be delayed several years and may be difficult to make even for trained physicians, as no biomarkers or commercial tests capable of discriminating CD from UC patients have been implemented in clinical use[8-10]. Furthermore, an early and accurate diagnosis of IBD-patients is crucial, as *e.g.,* CD patients with extensive and deep ulcerations have a 5-fold higher risk of requiring colectomy compared to CD patients without extensive and deep ulcerations[11]. From 357 CD patients analyzed with computed tomography enterography, penetrating disease was found in 21% of the patients and extraintestinal manifestations in 19%[12,13]. Hence, there is a need for reliable and usable biomarkers for the early and better diagnosis and prognosis of the IBD diseases[4,8,14-18].

GENOMIC, TRANSCRIPTOMIC AND PROTEOMIC BIOMARKERS

In 2001, an NIH group defined a biomarker as "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention."[19], usable for diagnostics, monitoring disease prognosis and disease monitoring and prediction. The human genome contains the code for the expressed gene products, including the proteins. Proteins function as the building blocks of the human cells and tissue, and are responsible for the majority of the biological functions[20]. Proteins, therefore, represent an obvious target for biomarker discovery studies. The human genome comprises approximately 20000 protein coding genes[21]. During protein synthesis, the DNA code is first transcribed into different RNA transcripts. Each gene can give rise to several RNA transcripts resulting in a total of roughly 100000 different RNA transcripts[22-24] (Figure 1), which in turn are translated into 100000 different proteins. After translation, most proteins are covalently modified at least once[25], and the final mature protein products are termed proteoforms. These so-called posttranslational modifications (PTMs) are often crucial to the correct physiological function of the given protein, and can determine activity state, localization, turnover and interaction with other proteins and substrates[23,25,26]. More than 200 distinct biologically relevant PTMs have been identified[27], so each RNA transcript can be more than 200 different proteoforms. The PTMs increases the complexity and diversity of the proteins tremendously (Figure 1). As a result, it is estimated that the human body contains more than one million different proteoforms[23], which constitutes the human proteome (all expressed proteins).

When searching for biomarkers, it is possible to analyze the target sample on the DNA level, the RNA transcript level or the protein level. Techniques for studying an organisms DNA code (genome) or RNA transcripts (transcriptome) have the advantage that entire genomes and transcriptomes can be sequenced and studied with great sensitivity, precision and coverage, and a number of biomarkers have been found for various diseases. Using genomic sequencing techniques, several CD and UC loci have been known for more than a decade, and the studies have greatly increased our knowledge of the IBDs[22,28,29]. Several cellular IBD-pathways have been identified, including pathways involved in barrier function, epithelial restitution, microbial defense, immune regulation, reactive oxygen species generation, autophagy, and finally various stress and metabolic pathways associated with cellular homeostasis, reviewed by Khor *et al*[3]. However, as mentioned no IBD biomarkes capable of differentiating CD from UC have been implemented in daily clinical usage, and the impact of the genomic studies on the treatment and diagnosis of the IBDs has been questioned[30-32].

Proteins represent an obvious target for biomarker discovery studies, and as PTMs dramatically increases the diversity and in many cases function of the mature proteins, they represent a promising area for IBD biomarker studies. PTMs are introduced after translation of the RNA transcripts (Figure 1), hence analyzing DNA and RNA transcripts does not directly provide information about the PTMs. A key technique capable of measuring absolute and relative protein quantification in complex protein mixtures in a high-throughput manner, as well as identify several PTMs, is bottom-up mass spectrometry (MS) based proteomics[24,33]. Proteomics is the large-scale identification of proteins, and can often cover the study of all expressed proteins by an organism (the proteome). The bottom-up MS strategy is based on measuring the mass-to-charge ratios (m/z) of peptides derived from proteins which have been enzymatically cleaved into minor peptides. From the measured m/z's the molecular weight of the intact peptides can be calculated[25]. In addition to calculating the intact masses, the peptides are collided with an inert gas which fragments the peptides, and the fragment m/z's are measured. The proteins in the sample are subsequently identified by searching the

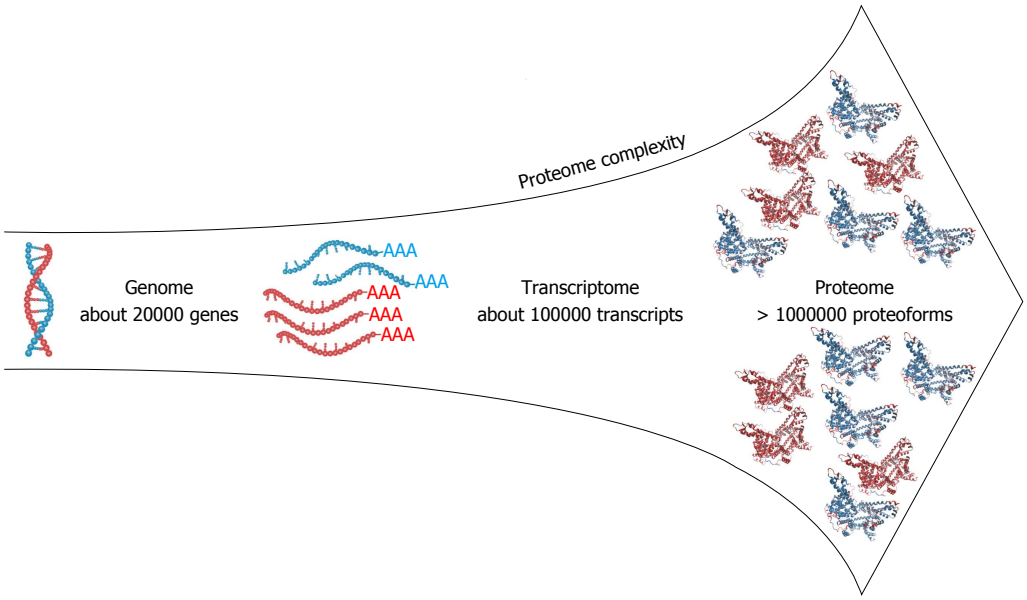

Figure 1 Major increase is encountered in the proteome complexity, from genes to RNA transcripts and finally to the mature, often posttranslational modification modified, proteins (proteoforms).

peptide masses and fragment m/z's against an *in silico* generated database, inferred from a reference database of protein sequences. By matching the *in silico* calculated peptide masses and fragment m/z's to the measured, the peptides and hence the proteins, are identified. For a more thorough description, we refer to the review by Steen *et al*[34]. The process can be performed in a quantitative manner to allow for relative or absolute quantitation of the proteins, using different strategies[34]. MS can in this way be used to identify proteins, as well as PTMs that change the molecular weight of the protein and can provide the amino acid position of the modification[25]. Previously, proteomics has been limited mainly by the speed and sensitivity of the mass spectrometers. However, recent development within the field of MS has allowed for the identification of nearly all expressed proteins of complex organisms, such as yeast, within a few hours of measuring time, identifying and quantifying several thousand proteins[33,35]. When considering the protein complexity encountered in the human intestinal tissue, an obvious place to search for biomarkers, and the recent development in the field of MS, a thorough analysis of PTMs and protein abundances in healthy and diseased state could be conducted. Biomarkers found in the intestine could then be searched for in more easily obtained sample material, such as blood or stool[6,10,31,36-39]. Antibodies to identified biomarkers for CD and UC found by proteomics can be generated for development of immunoassays and immunohistochemistry for evaluating the markers clinical use in routine tests less expensive than sequencing genomes, transcriptomes or MS driven proteomics.

This review reports known biomarkers for the IBDs, but will focus on the newly identified proteomics biomarkers and emerging proteomics strategies for identifying and characterizing novel IBD biomarkers.

DIAGNOSIS OF INFLAMMATORY BOWEL DISEASE AND KNOWN BIOMARKERS

Numerous biomarkers are known and used for the IBDs (Table 1); however, no single biomarker is able to diagnose IBD or to distinguish CD from UC patients with a high specificity and sensitivity[8-10,14]. CD is characterized by chronic inflammation in any part of the gastrointestinal tract. Most commonly the terminal ileum or the perianal region are inflamed, and in a non-continuous manner. Histologically, CD shows thickened submucosa, transmural inflammation, fissuring ulceration and non-caseating granulomas. UC, on the other hand, is characterized by inflammation limited to the colon, spreading continuously from the rectum and various distance proximal, and histology shows superficial inflammatory changes limited to the mucosa and submucosa with inflammation of crypts (cryptitis) and crypt abscesses[3]. There is currently no single "gold standard" diagnostic test or examination to differentiate CD and UC. Instead, diagnosis is based on a combination of symptoms, clinical examinations, laboratory findings, radiology, and endoscopy with histology, which also is used to assess severity and to predict the outcome of disease. Even when the tests are performed by expert clinicians they can result in diagnostic uncertainty[10,14,15,17,40]. This section will report some of the biomarkers commonly used to diagnose IBD. For a review of additional IBD biomarkers we refer to the work of Iskandar *et al*[4].

Antibodies and serum biomarkers

The two best-studied serological markers in IBD patients are anti-*Saccharomyces cerevisiae* antibodies (ASCA) and anti-neutrophil cytoplasmic antibody (ANCA)[41].

ASCA is an antibody with affinity for antigens in the

Table 1 Known common inflammatory bowel disease biomarkers

Biomarker	Specificity	Usability
Serum biomarkers		
ASCA	39%-79% of CD patients positive, 5%-15% UC patients[41-43]	14%-18% of controls tested positive, limiting the diagnostic value[44]
pANCA	20%-85% of UC patients positive, 2%-28% of the CD patients[41,42,45]	32% of controls tested positive, limiting the diagnostic value[44]
CRP	Marker for acute inflammation	Cannot differentiate CD from UC. However, usable for monitoring disease state[48-50]
Fecal biomarkers		
Calprotectin	Sensitive marker for intestinal inflammation[8,17,40]	Cannot differentiate CD from UC. Used to monitor disease state[17]
Lactoferrin	Can distinguish active IBD from inactive IBD and irritable bowel syndrome[60]	Unspecific for CD and UC. However, usable for monitoring disease state[60]

ASCA: Anti-*Saccharomyces cerevisiae* antibodies; ANCA: Anti-neutrophil cytoplasmic antibody; IBD: Inflammatory bowel diseases; UC: Ulcerative colitis; CD: Crohn's disease; CRP: C-reactive protein.

cell wall of the yeast *Saccharomyces cerevisiae*. In comparison to UC patients, CD patients are often positive for ASCA (Table 1)[41-43]. However, a substantial amount of healthy controls are also positive for ASCA positive[44], indicating that specificity and sensitivity for CD patients are relatively low; limiting the diagnostic value of the marker in differentiating CD from UC.

ANCAs are antibodies with affinity for neutrophil granules. The antibodies have been found in a variety of immune conditions, including Wegener's granulomatosis and rheumatoid arthritis (RA)[4]. When staining for ANCA, different patterns have been observed for UC and CD patients using immunofluorescence microscopy (Table 1), and mainly UC patients display perinuclear ANCA (pANCA) staining compared to CD patients[41,42,45]. Nonetheless, like the case of ASCA, a substantial amount of healthy controls are pANCA positive[44].

Lastly, C-reactive protein (CRP) is one of several proteins that increase in serum upon acute phase IBD. CRP is almost exclusively produced in the liver, upon stimulation by interleukin (IL)-6, tumor necrosis factor (TNF)-alpha and IL-1-beta produced at the site of inflammation. As such, an increased CRP-level is a marker for inflammation, but is not specific for CD or UC[8,40,46,47]. In some cases, but far from always, CD is associated with a strong CRP serum increase, whereas UC usually only results in a modest response. However, the difference insufficient to differentiate CD patients from UC patients[48-50], and the reason for the different responses remains to be thoroughly accounted for[40].

Other serum biomarkers used include white blood cell count, platelets, and albumin, which are all non-specific for IBD and can be seen in inflammatory diseases and cell stress[40]. More CD serologic markers are described in the review by Tamboli *et al*[51].

Fecal biomarkers

Stools are in direct contact with the inflamed intestinal area and site for the gut microbiome, both from which potential biomarkers are likely to originate. This is in contrast to serum biomarkers, which could increase on account of a variety of conditions, making stools an obvious place to search for biomarkers[40]. Fecal markers are especially useful for the diagnosis of CD patients, where the inflammation is patchy, may affect any part of the gastrointestinal tract, and therefore might be missed by colonoscopy[52]. The host-microbe interactions have been recognized as central for understanding human physiological diversity, and the human microbiome project has been launched to unravel the medical significance of the human microbiome[53]. Several studies have identified certain bacterial groups which are more abundant (*Enterobacteriaceae*, *Ruminococcus gnavus*, and *Desulfovibrio*) or less abundant (*Faecalibacterium prausnitzii*, *Lachnospiraceae*, *and Akkermansia*) in IBD[16], implicating that the host-microbe interaction might be involved, reviewed by Rosenstiel[54]. Novel biomarkers with high sensitivity and specificity may, therefore, be identified from stools.

The two most commonly used fecal markers for IBD screening are calprotectin and lactoferrin (Table 1)[8]. Calprotectin is a calcium- and zinc-binding protein occurring in large amounts in neutrophil granulocytes, where it accounts for 5% of the proteins. It is a very stable marker and is resistant to colonic bacterial degradation, and can be stored at room temperature for more than a week[55]. The concentration of fecal calprotectin is proportional to the neutrophil cell infiltrate in the bowel mucosa, and it is a very sensitive marker for intestinal inflammation[8,17,40]. However, calprotectin is not a specific marker for CD or UC, and increased levels can also be found with neoplasia, other forms of IBD, infections, and polyps[17], as well as with use of non-steroidal anti-inflammatory drugs, increasing age[56] and upper gastrointestinal disease, such as small bowel bacterial overgrowth[57].

Lactoferrin is an iron-binding glycoprotein expressed by activated neutrophils[58]. During inflammation, lactoferrin is released by the injured tissue and has been found to modulate inflammation and act in the defense against infections as a part of the innate immune system[59]. It is resistant to degradation and proteolysis, and unaffected by freeze thaw cycles, making it a useful biomarker[17]. As such, it is an ideal marker for intestinal inflammation. However, like calprotein it is unspecific for CD and UC, but can distinguish active IBD from inactive IBD and irritable bowel syndrome[60]. Several studies report similar performance of calprotectin and lactoferrin tests[6,60-64], and neither can be used to differentiate CD from UC with a high sensitivity and specificity.

To sum up, no reliable biomarkers exist usable as a single "gold standard". Therefore, to establish a diagnosis, histological examination of biopsies from the terminal ileum and colon is typically used in combination with

patient disease history and one or more of the above mentioned markers[17,65]. Hence, much effort is invested in analyzing the IBDs using various strategies, to identify usable biomarkers and explain the disease etiologies.

KNOWN PROTEOMICS BIOMARKERS FOR INFLAMMATORY BOWEL DISEASE

Proteomics studies can be performed in a discovery-based manner, where relative protein abundance levels between two or more samples are detected, and PTMs can be identified. Recent development of proteomics platforms has brought the technology to the point where several thousand proteins can be identified and (relatively) quantified in a single analysis or a subset by targeted approaches[6,10,31,36-39]. As inflammation takes place in the intestine, the gut-tissue represents an obvious place to look for novel biomarkers, which afterwards may be searched for in for example feces and blood and used as a disease marker. Several proteomic studies have successfully been aimed at identifying IBD biomarkers to investigate disease etiologies and aid in establishing the correct diagnose of UC and CD patients (Table 2). However, until now none of the identified biomarkers have been implemented in daily use[15].

The first group to publish a discovery-based proteomics study of the IBDs was Barceló-Batllori *et al*[66] in 2002. The aim of the study was to identify potential cytokine regulated proteins in colon epithelial cells isolated from IBD patients, which might be involved in the pathogenesis of IBDs. Human adenocarcinoma cells were *in vitro* exposed to known cytokines expressed in IBD, namely interferon-gamma, IL-1-beta and IL-6 (TNF-alpha was excluded as it is known to induce apoptosis in such cells). Using proteomics, the protein profiles of the cells were analyzed before and after exposure to the cytokines. All proteins from the cells were first separated using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). By staining all protein in the gels, different samples (gels) can be compared in terms of protein abundance based on the staining intensities, and differentiating protein spots can be visually identified. Spots of interest were cut from the gel with a knife and the proteins were enzymatically digested to specific peptides using the protease trypsin (in-gel digestion). The digestion of proteins is an essential step for protein identification, as no MS technique currently exist that can identify thousands of intact proteins in a complex sample in a high throughput manner. This is only possible when using digested proteins (peptides). The proteins were identified based on the peptides using MS, with the technique called matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS (Figure 2A). MALDI-TOF MS is a sensitive technique, but it involves placing a few drops of the sample on a plate which is left to dry prior to analysis. During analysis a laser is used to evaporate small spots from the dried droplet and ions in the produced gas are analyzed by MS. In the study, several cytokine regulated proteins were identified. Subsequently, human epithelial cells were isolated from UC patients and CD patients. Based on the findings, the samples were analyzed for the enzyme indoleamine-2,3-dioxygene using antibodies by western blotting. The group found an overabundance of the enzyme indoleamine-2,3-dioxygene in CD and UC compared to normal mucosa, hypothesizing an involvement of the Kynurenine pathway of tryptophan metabolism in the IBDs. Indoleamine-2,3-dioxygene activity has furthermore been found to be essential in dendritic cells to induce co-cultured T cell apoptosis[66].

When analyzing protein spots cut from gels the MALDI-TOF MS method is applicable, but the analysis of an entire 2D-PAGE gel is unfeasible, due to the commonly several thousand detectable spots. The technique is therefore less suitable for high-throughput identification of many thousand proteins. Therefore, when analyzing digested 2D-PAGE gels one usually only investigates changing protein spots and omits any information regarding non-changing protein spots. Information regarding non-changing proteins might prove equally important as changing proteins for studies seeking to describe disease etiologies. However, for biomarker studies 2D-PAGE strategies represent a feasible and proven way of identifying biomarker candidates. MALDI-TOF MS can also be conducted using intact proteins without prior enzymatic protein digestion. A variant of MALDI-TOF MS is to spot the protein mixture on a modified surface, to which the intact proteins bind and subsequently the intact masses of the proteins can be obtained by MS. This technique is called surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF MS) (Figure 2A). However, when studying intact proteins using MALDI-TOF MS or SELDI-TOF MS, one usually does not obtain identification of the detected signals.

Electrospray ionization (ESI) remains the only MS technique for identifying and quantifying several thousands of proteins in a high-throughput manner (Figure 2B). ESI involves spraying the digested proteins directly into the MS. By incorporating liquid chromatography (LC) with columns prior to the ESI process, the peptides can be separated and sequentially eluted over several hours. This gives the MS systems enough time to analyze a large proportion of the eluted peptides which subsequently can be identified. In this way, large-scale proteomic studies can be performed in a high-throughput manner using ESI LC-MS. These studies yield (relative) quantitative information of thousands of identified proteins in a single experiment, and thus might provide better information for explaining disease etiologies. In 2004, Hardwidge *et al*[67] published such a study, which was the first large scale proteomic analysis of a human cellular response to a pathogen. Discovery-based proteomics was applied to investigate the protein profiles (cellular response) of human Caco-2 intestinal epithelia cells before and after infection with *E. coli*. The group did not work directly with IBD, but the results are applicable to the diseases,

Table 2 Proteomics biomarker candidate studies and main findings

Ref.	Sample	Analysis	Findings and perspectives
Barcelo-Batllori *et al*[66], 2002	*In vitro* colon epithelial cells and purified epithelial cells from UC and CD patients	2D-PAGE protein quantitation, and in-gel digestion and MALDI-TOF MS and Western blot protein identification	The enzyme indoleamine-2,3-dioxygene was more abundant in cells from CD and UC patients compared to normal mucosa. Tryptophan and arginine metabolism may play a role in the IBDs
Hardwidge *et al*[67], 2004	Human Caco-2 intestinal epithelia cells before and after infection with *E. coli*	ESI LC-MS protein identification and quantitation, Western blot verification	125 proteins more abundant and 139 proteins less abundant after infection, some related to innate immune responses. These proteins might be relevant to look for in future biomarker studies
Hsieh *et al*[68], 2006	Colonic biopsies from UC, nonspecific infectious colitis patients and controls	2D-PAGE protein quantitation, and in-gel digestion and MALDI-TOF MS protein identification	6 proteins were found to be more abundant in UC and 13 less abundant. The result indicates that mitochondrial dysfunction might be involved in UC the etiology. Four biomarker candidates were identified, however, they require validation
Shkoda *et al*[69], 2007	Intestinal tissue cells purified from patients suffering from CD, UC, and colon cancer	2D-PAGE protein quantitation, and in-gel digestion and MALDI-TOF MS and Western blot identification	Proteins associated with signal transduction, stress response and energy metabolism were differently abundant in inflamed and non-inflamed tissue. 32% of the differentially regulated proteins were involved in energy metabolism
Meuwis *et al*[10], 2007	Serum from UC and CD patients	SELDI-TOF MS m/z signal profiling, MALDI-TOF MS and Western blot protein identification	Successful in differentiating CD from UC patients with a sensitivity of 85% and a specificity of 95% from several m/z signals. Four biomarker candidates were identified, all known acute inflammatory markers, limiting the diagnostic value. However, the feasibility of serum biomarker studies was demonstrated
Nanni *et al*[71], 2007	Serum from UC, CD patients and healthy controls	Solid-phase bulk protein extraction, MALDI-TOF MS signal profiling	Able to separate the three groups with 97% prediction results. The signals were not identified, but the feasibility of serum biomarker studies was demonstrated
Meuwis *et al*[70], 2008	Serum from responding and non-responding CD patients to infliximab	SELDI-TOF MS signal profiling, MALDI-TOF MS, Western blot and ELISA protein identification	Able to predict responders with a sensitivity of 79% and a specificity of 80%. Increased amount of PF4 was associated with non-response to infliximab with MS but not ELISA, so usability of PF4 as a biomarker seems limited
Nanni *et al*[72], 2009	Intestinal epithelial cells from CD patients and healthy controls	1D-PAGE and in-gel digestion, ESI LC-MS protein identification and quantitation	Proteins more abundant in CD patients include several proteins involved in inflammation processes, and less abundant include Annexin A1, involved in the anti-inflammatory action. Follow-up research is required to assess the feasibility of the biomarker candidates
Hatsugai *et al*[73], 2010	Peripheral blood mononuclear cells from UC and CD patients, and healthy controls	2D-PAGE quantitation, and in-gel digestion and MALDI-TOF MS protein identification	Successfully discriminated UC from CD based on seven differently present proteins, all associated with inflammation oxidation/reduction, the cytoskeleton, endocytotic trafficking and transcription. The biomarker candidates require validation using a larger number of patients, but seems promising
M'Koma *et al*[74], 2011	Mucosal and submucosal layers of samples originating from CC and UC patients	MALDI-TOF MS m/z signal characterization, no protein identification	Five m/z signals were detected in the submucosal layer, which could separate the two groups with an accuracy of 75 percent. The signals needs to be identified, however, the disease groups can be separated on basis of the mucosal and submucosal profiles
Presley *et al*[75], 2012	Microbes and human proteins at the intestinal mucosal-luminal interface from CD and UC patients, and healthy controls	Oligonucleotide ribosomal RNA fingerprinting, SELDI-TOF MS and MALDI-TOF MS identification	35% of the detected bacterial phylotypes were present in different amounts in the diseases, indicating the involvement of host-microbe interactions in IBD. The microbiome might prove useful as a target for therapy
Han *et al*[14], 2013	Colonic tissue biopsies of Korean IBD patients	ESI LC-MS protein identification with label-free quantitation	27 potential biomarkers were identified for UC, 37 biomarkers for CD and 11 proteins commonly associated with IBD. Three novel biomarkers were identified for active CD: Bone marrow proteoglycan, L-plastin and proteasome activator subunit 1. The biomarker candidates require validation, but might prove feasible as new diagnostic and therapeutic targets
Seeley *et al*[76], 2013	Histological tissue layers from UC and CC patients	MALDI-TOF MS m/z signal characterization, no protein identification	114 different m/z signals were found to be different between the two groups. The signals remain unidentified
Gazouli *et al*[77], 2013	Serum samples from responding and non-responding CD patients to infliximab treatment	2D-PAGE quantitation, and in-gel digestion and MALDI-TOF MS protein identification	15 differently abundant proteins between responders and non-responders to infliximab were identified. The biomarker candidates require further validation

IBD: Inflammatory bowel diseases; UC: Ulcerative colitis; CD: Crohn's disease; MALDI-TOF MS: Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry; 2D-PAGE: Two-dimensional polyacrylamide gel electrophoresis; ESI: Electrospray ionization; SELDI-TOF MS: Surface-enhanced laser desorption/ionization time of flight mass spectrometry; LC: Liquid chromatography.

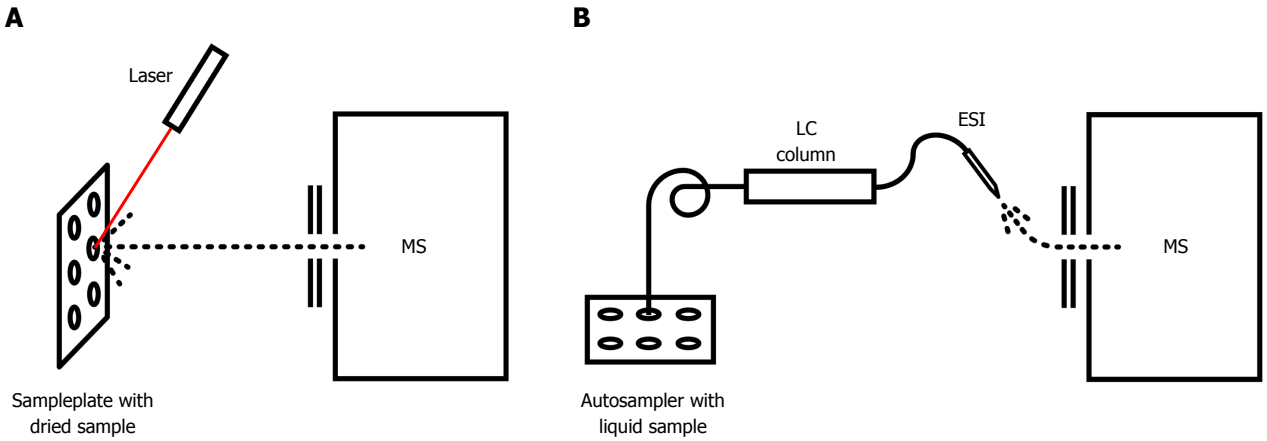

Figure 2 Two commonly used mass spectrometry techniques. A: Matrix-assisted laser desorption/surface-enhanced laser desorption/ionizatio time of flight mass spectrometry (MS), where the peptide or protein sample is dried on a target plate. Subsequently, a laser is used to evaporate the dried sample, and the generated gas phase ions are analyzed by the mass spectrometer; B: Liquid chromatography (LC)-electrospray ionization (ESI) MS, where the liquid peptide (or protein) sample is separated on a LC column, and sequentially eluted often over several hours. The eluted peptides are injected directly into the mass spectrometer by ESI and analyzed.

as the involvement of host-microbe interactions in the IBDs have been suggested[54]. The cells were lysed, and the lysates were chemically modified using chemical labels to allow for a relative comparison between the protein abundances measured by MS. Using ESI LC-MS, the group recorded 10921 peptide fragments mass spectra, from which they were able to identify 2000 proteins. Two hundred and sixty four proteins had a known biological function and were found to have at least a 2-fold abundance difference between infected and non-infected, roughly half were more abundant post infection. Some of the MS-findings were verified with western blots, and significant changes were found in amount of actin-related proteins before and after infection.

Even though ESI LC-MS has advantages in terms of high-throughput, many biomarker studies have successfully employed MALDI-TOF MS protein identification in IBDs. In 2006, Hsieh *et al*[68] applied discovery based proteomics using such a platform. The group analyzed the etiology and pathogenesis of UC using colonic biopsies to detect any significant difference in the protein profiles. The biopsies were obtained from four UC patients, three patients with nonspecific infectious colitis and five individuals with no obvious colonic disease. The proteins were separated by 2D-PAGE and a total of 1000 protein spots were compared visually between the diseased *vs* normal colon mucosa tissues. Forty protein-spots were found to be consistently different in intensity. Spots of interest were cut from the gel, tryptic digestion was performed and 19 proteins were identified using MALDI-TOF MS. Hereof, 13 identified proteins were less abundant in the UC-group and six proteins were more abundant. Eight of the less abundant proteins were identified as being mitochondrial proteins, suggesting that mitochondrial dysfunction might be involved in UC.

A year later in 2007, Shkoda *et al*[69] also identified a potential association between dysfunction in the energy metabolism and IBDs. The group applied a similar strategy and platform to investigate the loss of intestinal cell function, a critical component in the initiation and perturbation of chronic intestinal inflammation, and was the first to compare inflamed and non-inflamed tissue from the same patient. Intestinal cells were purified from intestinal tissue obtained from patients suffering from CD, UC, and colon cancer. The proteins were separated by 2D-PAGE and analyzed by MALDI-TOF MS and western blotting. 41 proteins were found to be differently abundant between inflamed and non-inflamed tissue, including proteins associated with signal transduction, stress response and energy metabolism. Thirty-two percent of all detected differentially regulated proteins associated with IBD were involved in energy metabolism. In 2007, Meuwis *et al*[10] published the first proteomic serum profiling study using SELDI-TOF MS in IBD, a variation of MALDI-TOF MS. The study included 30 patients with CD, 30 patients with UC, 30 inflammatory controls and 30 healthy controls. By characterizing the serum only by the m/z signals and not identified proteins with SELDI-TOF MS, the group was able to differentiate CD from UC with sensitivity of 85% (51/60) and specificity of 95% (57/60). Several of the unidentified signals were subsequently identified by MALDI-TOF MS, western blotting, and ELISA assay. Four biomarker candidates were identified: platelet aggregation factor 4 (PF4), myeloid related protein 8, fibrinopeptide A and haptoglobin alpha-2 subunit. All four proteins are known acute inflammatory markers to be expected in the IBDs, but the study succeeded in demonstrating that the separation of CD and UC patients based on serum markers is possible, highlighting the potential of serum profiling.

A year later, Meuwis *et al*[70] used the same platform and strategy to analyze if serum from 20 CD patients could be used to predict response to infliximab treatment. Infliximab is a monoclonal antibody against TNF-alpha, and was the first anti-TNF-alpha agent accepted for

IBD treatment. The protein profiles were characterized in serum prior to and post treatment with SELDI-TOF MS. The group verified the four previous biomarkers, and especially increased amount of PF4 was associated with non-response to infliximab. However, the association could not be confirmed by ELISA, and did not correlate significantly with other disease markers. Even so, the study was able to predict responders with a sensitivity of 79% (55/70) and a specificity of 80% (56/70). Even though the study did not succeed in identifying a usable biomarker for the prediction of responders, the study highlighted the potential in proteomic studies and response marker discovery.

In 2007, Nanni *et al*[71] optimized the methodological approach used to evaluate serum with MALDI-TOF MS. Using a solid-phase bulk protein extraction protocol followed by MALDI-TOF MS, they analyzed serum from 15 CD, 26 UC and 22 healthy individuals and were able to separate the three groups with 97% prediction results. Two years later, Nanni *et al*[72] conducted a study using high-throughput ESI LC-MS to investigate protein variations in the intestinal epithelial cells from CD patients. However, in contrast to Hardwidge *et al*[67] in 2004 who used chemical labelling of the peptides to measure the relative abundances, Nanni *et al*[72] employed a label-free strategy, and relied on the accurate detection of the peptide masses. In this way, significant savings can be achieved for large studies and the sample preparation protocols simplified. Intestinal epithelial cells were isolated from samples originating from two CD patients and two control patients. The cells were lysed and the proteins were separated by 1D-PAGE, where the proteins are separated only in one dimension in contrast to 2D-PAGE, which allowed the entire visualized gel lane to be cut into pieces and digested with trypsin. The resulting peptides were analyzed by ESI LC-MS and by comparing the peptide intensities, relative protein abundances could be calculated. Proteins which were found to be more abundant in the epithelial cells from CD patients include heat shock protein 70, tryptase alpha-1 precursor as well as several proteins involved in inflammation processes. The nuclear protein Annexin A1, involved in the anti-inflammatory action, and the malate dehydrogenase enzyme was found to be less abundant. The feasibility of the biomarker candidates remains to be validated. However, of great importance is the demonstration of the utility of label-free ESI LC-MS analysis for the identification of differences in protein abundances for IBD.

In 2010, Hatsugai *et al*[73] performed the first study which successfully discriminated UC from CD completely. The group analyzed peripheral blood mononuclear cells from 17 UC patients, 13 CD patients and 17 healthy controls. The proteins were separated by 2D-PAGE and more than 1000 protein spots were detected in each gel. Five hundred and forty-seven protein spots were selected for the quantitative analysis, and 34 protein spots were significantly different between the UC and CD groups. Using 58 protein spots, the UC and CD patients could be differentiated. The 58 protein spots were furthermore subjected to in-gel tryptic digestion followed by MALDI-TOF MS protein identification. Eleven of the proteins were successfully identified, and were found to be functionally related to inflammation, oxidation/reduction, the cytoskeleton, endocytotic trafficking and transcription. The profiles could, furthermore, predict disease severity and the UC patients' responses to treatment.

In 2011, M'Koma *et al*[74] analyzed mucosal and submucosal layers of samples originating from Crohn's colitis (CC) and UC, using MALDI-TOF MS. Five unknown m/z MS signals were detected, which could separate the two groups. The study did not identify the origin of the signals, but highlighted the possibility of finding biomarkers in the intestinal tissue.

As mentioned earlier, even though we are far from having a complete picture of the intestinal micro-biome, changes in the bacterial composition have been detected in IBD. In 2012, Presley *et al*[75] investigated the host-microbe interaction at the intestinal mucosal-luminal interface of 14 CD patients, 21 UC, and 16 healthy controls. The mucosa prevents microorganisms from entering the host tissue. Using a novel saline-lavage technique, saline was injected during colonscopy and extracted again to avoid interference from the intestinal layer contents resulting from a biopsy sample. The bacterial ribosomal RNA genes were analyzed by oligonucleotide fingerprinting and the proteins were analyzed by SELDI-TOF MS and MALDI-TOF MS. A combined proteome was constructed, constituting the proteomes from all detected organisms. Of the 3374 detected bacterial phylotypes, 35% significantly differentiated the diseases, indicating that host-microbe interactions might be involved in IBD, presenting new possibilities for diagnosis and therapy.

In 2013, Han *et al*[14] analyzed colonic tissue of Korean IBD patients in a high-throughput manner using ESI LC-MS and label-free quantitation. The study included four UC patients, three CD patients and two with inflammatory related polyps related to UC. The biopsies were homogenized and digested with trypsin without prior prefractionation and on average 324 proteins were identified for each group. Even though the number of identified proteins is relatively low considering the 2000 proteins Hardwidge *et al*[67] identified in 2004, 27 potential biomarkers were identified for UC, 37 biomarkers for CD and finally 11 proteins that were commonly associated with IBD. Three novel proteins, bone marrow proteoglycan, L-plastin and proteasome activator subunit 1 were identified as potential biomarkers for active CD. These biomarkes need validation, however, the feasibility of conducting high-throughput proteomics with label-free strategies in biomarker discovery was demonstrated.

A study published in 2013 by Seeley *et al*[76] investigated histological layers of 62 confirmed UC and CC tissues by MALDI-TOF MS. A total of 114 m/z MS signals were found to be statistically different between the two groups, however the signals have yet to be identified.

Finally, in 2013, Gazouli *et al*[77] published a study

where the response of 18 CD patients to infliximab treatment was correlated with known serum biomarkers. Serum samples were analyzed using 2D-PAGE, and 240 protein spot were selected for in-gel digestion and subsequent MALDI-TOF MS protein identification. The group was successful in identifying 15 proteins which were differentially present in the serum of CD patients depending on the response to infliximab. The proteins apolipoprotein A-I, apolipoprotein E, basic complement C4, plasminogen, serotransferrin, beta-2-glycoprotein 1, and clusterin were found to be more abundant in the patient groups with clinical and serological non-responders and responders, than in the group of patients with clinical and serological remission. Additionally, leucine-rich alpha-2-glycoprotein, vitamin D-binding protein, alpha-1B-glycoprotein and complement C1r subcomponent were found to be more abundant in the serum of the group of patients with remission. Interestingly, the group was unable to confirm the findings by Meuwis *et al*[70], that PF4 could be a biomarker for infliximab response, emphasizing that the biomarker candidates need further validation. Nonetheless, the study was successful in demonstrating the feasibility of identifying biomarkers in the serum usable to predict treatment outcome.

As apparent, many studies have successfully applied proteomic strategies to identify biomarkers, investigate IBD pathogenesis and identify prognostic markers in serum, stools, and tissue. Several biomarkers have been found (Table 2), most related to unspecific inflammation, and all biomarker candidates identified so far lacks follow-up validation studies. However, even though many of the identified biomarkers are related to inflammation, the studies have demonstrated the feasibility and potential of the proteomics platform in IBD, and given clues to the mechanisms of the IBDs. A few studies have successfully differentiated CD patients from UC patients. However, only based on unidentified m/z signals and not using identified protein or peptide biomarkers, from which the disease etiologies might be better explained. Nonetheless, these studies demonstrate the presence of usable biomarkers yet to be identified. Identified biomarkers hold the potential for designing diagnostic ELISA tests and protein array chips, where antibodies are used to detect the abundance of one or more antigens[78,79]. Such arrays could constitute new clinical tools for diagnosis, prognosis and identify novel targets for therapy.

The studies have demonstrated the presence of biomarkers, in serum, in the intestinal tissue and in stools. Many studies have aimed at performing global discovery-based proteomics in the intestinal tissue, and it has been demonstrated that high-throughput techniques such as ESI LC-MS, employing labelling or label-free quantitation are feasible ways to identify biomarkers in highly complex samples. The advantage of high-throughput protein identification and quantification strategies are especially apparent when disease etiologies are to be examined.

Furthermore, few studies have investigated the possible association between various PTMs and the IBD disease etiologies. Such an association is known from other inflammatory diseases; an example being the inflammatory joint-disease rheumatoid arthritis (RA) where the PTM citrullination is known to be involved in the etiology[80-83].

POSTTRANSLATIONAL MODIFICATIONS AS BIOMARKERS

Today, more than 200 distinct PTM's are known[84]. The PTMs are, to a large extend, important for the physiological function of the protein and the half-life of PTMs range from milliseconds to years[85]. Unfortunately, they are also often low abundance, highly diverse and complex, and thus can be challenging to detect and characterize[25,27,86]. Hence, PTMs represent promising targets for biomarker discovery studies. For a review on protein regulation by PTMs in the IBDs, we refer to the work by Ehrentraut *et al*[5]. Common *in vivo* PTMs include phosphorylation, which is a reversible modification of the amino acids tyrosine, serine and threonine. Phosphorylation is known to be involved in activation and inactivation of enzyme activity, modulation of molecular interactions and cell signaling through specific domains. Acetylation can target any N-terminal, and it is believed that 84% of all human proteins undergo this modification[87]. The PTM affects the protein stability, and histone acetylation is known to play a role in gene regulation. Glycosylation is another central PTM. It is reversible and known to be involved in cell-cell recognition and signaling, and regulation of proteins. Disulfide bond formation between two cysteines is a key element in the stabilization of proteins and protein complexes, such as, antibodies by forming intra- and intermolecular crosslinks. Deamidation of asparginine or glutamine is a possible regulator of protein-ligand and protein-protein interactions, and ubiquitination is a marker for protein recycling/destruction[25]. Several PTMs are known to be involved in the inflammatory responses, and PTMs could be involved in the IBD disease etiologies. Lastly, citrullination is the irreversible deimination of arginine into citrulline, *in vivo* catalyzed by the peptidylarginine deiminases, a calcium binding family of enzymes[88,89]. The exact role of the modification remains largely unknown, but the modification is believed to alter the fold of the proteins, change the protein polarity, and/or lead to denaturation in order to render the protein more prone to enzymatic degradation[80,88,89]. Citrullination has been associated with several diseases, including Alzheimer's disease[90], and RA where an anti-citrullinated protein antibody was identified[80-83]. Smoking has been associated with increased citrullination, and smoking is the best known environmental factor for the development of RA[91-95]. Several studies have, furthermore, associated smoking with an increased risk of developing CD and UC[96-100]. In RA, it is believed that citrullination of proteins results in the generation of new antigens being presented to the immune system, which in turn triggers an autoimmune response[83]. It therefore seems plausible that citrullination may have a similar role

in the IBDs as well as other inflammatory diseases. However, as with many PTMs the MS-driven detection of citrullinated proteins in a high-throughput manner is not straight forward[84,101-104]. Nonetheless, if disease-specific citrullinated proteins could be identified, these could be utilized in ELISA or protein array chips for prognostics and/or diagnostics. An example of the utilization of a similar biomarker is the diagnosis of RA patients, where the presence of anti-citrullinated protein antibodies in the serum is used to detect the disease with a sensitivity of 71% and specificity of 95%[80-83].

CONCLUSION

The diagnosis of UC and CD patients remains difficult, especially in the early stages of the diseases, and early and accurate diagnosis of IBD-patients is crucial. Several studies have successfully identified promising biomarkers in stools, serum and tissue, demonstrating the presence of IBD biomarkers. However, none of the identified biomarkers have been implemented in clinical daily use, and the diagnosis is based on a combination of disease history, colonscopy inflammation biomarkers and histological evaluation.

Few studies have aimed at investigating the global proteome of intestinal tissue using high-throughput techniques such as ESI LC-MS, and the potential of such analysis seems immense. The recent development within the field of high-throughput protein identification using MS, now allows for identifying and quantifying several thousand proteins in a few hours of analysis time. Besides protein abundances, PTMs represent promising targets for biomarker discovery studies. An analysis of tissue, serum or stools therefore seems promising to identify novel biomarkers. Such information could be used to make accurate diagnostic and prognostic tools to differentiate patient groups and predict treatment responses. Antibodies against one or more identified diagnostic targets could be used in ELISA or protein array chips, which in turn can be used to detect the abundance of the given antigen. Besides aiding physicians in making a correct diagnosis and treatment strategy, knowledge of disease specific proteins and PTMs might identify disease pathways and new targets for therapeutic agents, leading to improved pharmaceutical drugs.

Conclusively, protein identification and quantification using mass spectrometry holds great promise for the identification of novel diagnostic and prognostic biomarkers for the IBDs, and might help explain the disease etiologies, ultimately leading to improved treatment strategies.

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