Microproteomics and Immunohistochemistry Reveal Differences in Aldo-Keto Reductase Family 1 Member C3 in Tissue Specimens of Ulcerative Colitis and Crohn’s Disease

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Purpose: Differential diagnosis of ulcerative colitis (UC) and Crohn’s disease (CD) is of utmost importance for the decision making of respective therapeutic treatment strategies but in about 10–15% of cases, a clinical and histopathological assessment does not lead to a definite diagnosis. The aim of the study is to characterize proteomic differences between UC and CD.

Experimental Design: Microproteomics is performed on formalin-fixed paraffin-embedded colonic tissue specimens from 9 UC and 9 CD patients. Protein validation is performed using immunohistochemistry (IHC) (n\textsubscript{UC}=51, n\textsubscript{CD}=62, n\textsubscript{CTRL}=10) followed by digital analysis.

Results: Microproteomic analyses reveal eight proteins with higher abundance in CD compared to UC including proteins related to neutrophil activity and damage-associated molecular patterns. Moreover, one protein, Aldo-keto reductase family 1 member C3 (AKR1C3), is present in eight out of nine CD and absent in all UC samples. Digital IHC analysis reveal a higher percentage and an increased expression intensity of AKR1C3-positive epithelial cells in CD compared to UC and in controls compared to inflammatory bowel disease (IBD).

Conclusion and Clinical Relevance: Overall, the results suggest that microproteomics is an adequate tool to highlight protein patterns in IBD. IHC and digital pathology might support future differential diagnosis of UC and CD.

1. Introduction

Inflammatory bowel disease (IBD) is a group of chronic autoimmune disorders. The two main representatives are ulcerative colitis (UC) and Crohn’s disease (CD). Increasing incidence and prevalence has been reported, with about 2.5–3 million people affected in Europe.[1]

While both diseases show typical clinical features regarding localization, histology, and extra intestinal manifestations, there may be a considerable overlap of symptoms. In 10–15% of cases, the distinction between UC and CD cannot be achieved at initial colonoscopy, in colonic biopsies or at colectomy.[2] Therefore, diagnosis and/or treatment may be delayed or inadequate.[2] However, given the serious consequences of IBD on growth and development, early and accurate diagnosis especially of pediatric patients is of utmost importance.[1,4]

The precise subtyping of IBD can only be achieved interdisciplinary, where histologic examination of tissue specimens remains important in the diagnostic work-up of affected patients. Typical indications to take a biopsy are i) confirmation or diagnosis of IBD,
ii) distinction between UC and CD, iii) exclusion of dysplasia, co-existing conditions or complications, and iv) evaluation of IBD activity and extent.\(^5\) While typical microscopic features of UC and CD are well known (Table S1, Supporting Information), some, for example transmural inflammation, fibrosis, and fistulas, may be present only in deeper layers of the bowel wall and are not evaluable in biopsy specimens.\(^6\) The current recommendations include an extensive work-up that underline the difficulties associated with the examination of biopsies in IBD. According to the current consensus, a minimum of two biopsies from at least five sites along the colon, including the rectum and the terminal ileum, should be obtained in separate vials. In addition, two or three tissue levels, each consisting of five or more sections, should be assessed.\(^5,7\)

UC and CD are multifactorial disorders that result from complex interactions between epithelial cells and cells of the innate and adaptive immune system, and their secreted mediators.\(^8\) While UC is thought to be mediated by T-helper cell (Th) 2 response, there is some evidence that CD is a Th1- and Th17-mediated disease.\(^9\) In line with this finding, whole genome sequencing revealed susceptibility loci associated with the respective cytokine responses.\(^10\)

Despite the advances that have been achieved, reliable biomarkers are lacking so far and the understanding of both diseases is limited. Up to now, the proteomic landscape of UC and CD has only been studied on small cohorts of fresh-frozen tissue or in serum.\(^11\) As fresh-frozen tissue is not the standard diagnostic material, there is a need to identify additional biomarkers that are applicable for the detection in formalin-fixed paraffin-embedded (FFPE) tissue material which is routinely evaluated.

A method has been developed for microproteomic analyses using nanoLC liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) of limited amounts of FFPE tissue collected from sections by laser microdissection (LMD).\(^12,13\) LMD enables the collection of tissue pieces from which cell content is fully controlled before further processing. LMD-based microproteomics allows for proteomic biomarker discovery from samples containing down to 3000 cells.

In the present study, we used LMD-based microproteomics to analyze FFPE tissue sections and identify proteomic differences between UC and CD. One protein was validated by immunohistochemistry (IHC).

### Clinical Relevance

Ulcerative colitis (UC) and Crohn’s disease (CD) are of increasing incidence, with 2.5–3 million people affected in Europe. The distinction between UC and CD is important to adapt treatment. Diagnosis is interdisciplinary and includes histological examination of biopsies. Although microscopic features typical for UC and CD exist, some may only be present in deeper layers of the bowel, thus not visible in biopsies. Hence, molecular markers are of utmost importance for the distinction between UC and CD. In this study, a method of laser microdissection-based microproteomics was used for biomarker discovery. This allowed us to highlight a panel of nine proteins that distinguished UC and CD, revealing a possible differential neutrophil activity and different abundance of damage-associated molecular patterns. We validated Aldo-keto reductase family 1 member C3 by immunohistochemistry, which displayed differential staining between UC and CD specimens.

### 2. Experimental Section

#### 2.1. Samples

FFPE blocks of colonic tissue specimens from patients with UC and CD resected between 1992 and 2012, and colon control samples from resection margins from patients resected for adenocarcinomas of the colon from 2016 were retrieved from the Institute of Pathology of the University of Heidelberg (Germany) with the support of the tissue bank of the National Center for Tumor Diseases (NCT, Heidelberg, Germany, #2799). Tissues were used in accordance with the ethical regulations of the NCT tissue bank defined by the local ethics committee. 5-μm tissue sections were cut and deposited either on polyethylene naphthalene membrane slides for LMD (Leica microsystems, Wetzlar, Germany) or on Superfrost glass slides (Thermo Fischer Scientific, Waltham, MA, USA) for further hematoxylin and eosin (H&E) staining\(^11\) or IHC. Diagnoses were made according to the European consensus on the histopathology of IBD.\(^14\) Eighteen cases (n\(_{\text{UC}} = 9\) and n\(_{\text{CD}} = 9\) ) were selected for microproteomics and 113 separate cases (n\(_{\text{UC}} = 51\), n\(_{\text{CD}} = 62\), and n\(_{\text{CTRL}} = 10\) ) were selected for IHC validation.

#### 2.2. Cell Counting and Laser Microdissection

H&E stained serial sections were analyzed for cell recognition and counting before LMD.\(^12\) Stained tissue sections were scanned using a slide scanner Hamamatsu Nanozoomer 2.0HT (Hamamatsu Photonics, Hamamatsu, Japan) and analyzed with open source digital analysis software (QuPath)\(^14\) to detect regions with an equivalent number of epithelial and stromal cells (Figure 1). The goal was to identify areas containing about 6000 epithelial and 12 000 stromal cells mainly containing immune cells. A classifier was trained with 12 regions containing uniquely immune cells and 12 regions containing uniquely epithelial cells under the guidance of a pathologist. The results of the classifier were verified by a pathologist. The settings for cell and nuclei...
detection in QuPath are described in M1, Supporting Information. For LMD, tissue sections were deparaffinized as previously described.[12] Subsequently, a LMD 7000 laser microdissector (Leica microsystems, Wetzlar, Germany) was used to collect the identified tissue regions containing the targeted numbers of epithelial and stromal cells. Parameters for LMDs are described in M2, Supporting Information.

2.3. Sample Processing

Samples were processed using a previously described protocol for LMD-based microproteomics.[12,13,15,16] Briefly, the samples were immersed in 10 mM citric acid buffer at pH 6 (Sigma Aldrich, Darmstadt, Germany) and submitted to heat-induced antigen retrieval for 1 h at 99 °C, while shaken at 800 rpm using a Thermoshaker (Eppendorf, Hamburg, Germany). After antigen retrieval, samples were cooled down to room temperature and reduction/alkylation/reduction steps were performed. Two steps of digestion in highly concentrated trypsin solutions (Pierce, Thermo Fisher Scientific) were performed in the presence of 0.01% Rapigest (Waters, Milford, MA, USA). The first step of digestion was performed in a solution of trypsin at 60 µg mL⁻¹ prepared in 50 mM NH₄HCO₃ (Sigma Aldrich, Darmstadt, Germany). The second step was performed in a solution of 30 µg mL⁻¹ trypsin in 80% acetonitrile (ACN) (Biosolve, Dieuze, France). After digestion, the samples were dried by vacuum centrifugation (Savant™ SPD 121P, Thermo Fisher Scientific) and desalted using C₁₈ Solid Phase Extraction tips (ZipTip, Merck Millipore, Burlington, MA, USA). The peptides were eluted using 50% of ACN in acidified water (0.1% trifluoroacetic acid in final concentration).

2.4. Nanoflow Liquid Chromatography—Tandem Mass Spectrometry Analyses

NanoLC-MS/MS analyses were performed using an Ultimate 3000 nanoflow high performance LC system (Thermo Fisher Scientific), on-line coupled to a high-resolution tandem mass spectrometer (Q-Exactive, Thermo Fisher Scientific). The Q-Exactive mass spectrometer was calibrated in positive ion
mode using a high-grade purity protein standard (Pierce LTQ Velos ESI Positive Ion Calibration Solution, Thermo Fisher Scientific).

Chromeleon Xpress software (Thermo Fisher Scientific) was used to set up the nanoLC-MS/MS system, and Tune 2.5 and Excalibur 2.2 software (Thermo Fisher Scientific) to pilot the mass spectrometer. Method optimizations were performed before analyzing the UC and CD cohorts of microdissected tissues as described in the M3, Figures S1 and S2, and Tables S2 and S3, Supporting Information.

Reconstituted tryptic digests were analyzed by reversed phase chromatography on an Acclaim PepMap 100 C18 nanocolumn (75 µm internal diameter, 150 mm length, 3 µm granulometry and 100 Å porosity, Thermo Fisher Scientific) on-line with a concentration micro-precolumn C18 PepMap 100 (300 µm internal diameter, 3 µm granulometry and 100 Å porosity, Thermo Fisher Scientific). Peptides were resolved at 300 nL min$^{-1}$ (split ratio 1/1000) using a diphasic linear gradient with H$_2$O with 0.1% formic acid (FA, v/v) as mobile phase A and ACN with 0.1% FA as mobile phase B. The gradient started with a first step from 2% to 10% B in 10 min; then from 10% to 35% B in 160 min, followed by an increase to 85% B in 10 min prior to a plateau at 85% B for 5 min. The multistep gradient ended up with a return to the initial mobile phase condition (2% B) in 2 min before stabilizing the column for 15 min. NanoLC-MS/MS datasets were acquired in positive ion and data-dependent modes with analytical parameters summarized in M3, Table S4, Supporting Information.

2.5. Data Processing

Raw files were analyzed using MaxQuant version 1.5.2.8 (Max Planck Institute of Biochemistry, Planegg, Germany). Andromeda search engine (Max Planck Institute of Biochemistry) was used for the analysis of MS/MS spectra with settings described in M4, Supporting Information. Perseus version 1.5.0.15 (Max Planck Institute of Biochemistry) was used for statistical assessment. All “only identified by site” and “reverse” entries were removed prior to analysis. All data were log2 transformed. Two sample t tests (false discovery rate (FDR) < 0.05, S0 value = 1) were used to identify the significantly more abundant proteins in one group compared to the other. Among the t-test significant proteins, a minimum t-test difference of 1.77 was found and a minimum –log test difference of 1.63 were found.

2.6. Immunohistochemistry and Staining Evaluation

To validate the best differential marker, IHC staining was performed as previously described.$^{[17]}$ In brief, slides were deparaffinized, pre-treated with a pH 8.4 buffer made of 0.089 mM Tris, 0.089 mM Borat, and 0.002 mM Ethylenediaminetetraacetic acid (Thermo Fischer Scientific) for 40 min, incubated with anti-AKR1C3 antibody (polyclonal, 1:750, Abcam, Cambridge, UK) for 24 min, and stained using an automated device (Ventana Benchmark Ultra, Roche, Rotkreuz, Switzerland).

All samples (n = 123) were analyzed by QuPath. To evaluate the percentage of positive epithelial cells, representative areas exhibiting typical morphological changes related to UC and CD diseases were chosen and the H-score as well as the Allred staining intensity were exported. The H-score was calculated as follows: 1$^*$ (percentage of cells with weak staining intensity) + 2$^*$ (percentage of cells with moderate staining intensity) + 3$^*$ (percentage of cells with strong staining intensity). The Allred staining intensity is scored based on optical density as follows: 1 = weak, 2 = moderate, and 3 = strong. Software was trained for epithelial and stromal cells as for the cell counting for LMD, and only positivity of epithelial cells was extracted for statistical analyses (Figure 1). To test for differences in protein abundance between UC and CD using microproteomic and IHC data, student t-test was applied. p < 0.05 was considered significant.

3. Results

3.1. Laser Microdissection–Based Microproteomics Reveals Nine Proteins with Different Quantities between Ulcerative Colitis and Crohn’s Disease

In an initial test series, we defined the optimal parameters for the proteomic analysis of UC and CD cohorts of microdissected tissues using a tryptic digest of HeLa cells (see M3, Figures S1 and S2, and Tables S2 and S3, Supporting Information). Subsequently, all 18 microdissected UC and CD tissue samples were successfully processed, analyzed, and evaluated by LMD-based microproteomics. Using the nanoLC-MS/MS analytical method that provided the best performance, 701, 727, 750, 734, 773, 810, 833, 710, and 821 proteins were identified in UC, and 717, 729, 774, 800, 783, 795, 755, 740, and 827 proteins were identified in CD samples. Among the 1416 proteins we identified in the combined analysis, 666 proteins could be quantified in seven out of the nine biological replicates in at least one group (UC or CD) (Supporting Information). A total of 404 common proteins could be identified in all the 18 samples (no NaN value). In addition, the nanoLC-MS/MS datasets corresponding to the cleaning sequence of the nanocolumn did not reveal significant carry-over peptides between successive analyses of UC and CD samples.

t-test comparisons between UC and CD revealed eight proteins that were significantly more abundant in CD. From the highest to the lowest fold change between CD and UC, we found: Keratin 4 (KRT4), Neutrophil elastase (ELANE), Lactotransferrin (LTF), Lysozyme C (LYZ), Protein S100 A9 (S100A9), Protein S100 A8 (S100A8), Cathepsin G (CTSG) and the protein group Ig mu chain C region and Ig mu heavy chain disease protein (IGHM) (Table 1). As described in a previous work,$^{[13]}$ some proteins that are found not t-test significant can nonetheless be completely absent from samples of one condition and present in most of the samples from the other condition. We then verified if some quantified proteins were not t-test significant but were only present in most of the samples of one condition and absent in all samples of the other. We found that only the protein group Aldo-keto reductase family 1-member C3 and Aldo-keto reductase family 1-member C1 (AKR1C3; AKR1C1) was present in eight of nine samples of CD and absent in all the UC samples (Table 1). Because of the absence of this protein group in UC samples, the fold change between CD and UC could not be calculated (marked as “Not a Number”[NaN] in Table 1).
Table 1. Proteins revealed by microproteomics with differential abundances between UC and CD.

| Gene names       | t-test significant | −log t-test p-value | CD log2 mean LFQ | UC log2 mean LFQ | Fold change CD/UC | Peptides | Unique peptides |
|------------------|--------------------|---------------------|------------------|------------------|-------------------|----------|----------------|
| AKR1C3; AKR1C1   | No                 | 0.00                | 27.67            | 0.00             | NaN               | 8        | 8              |
| KRT4             | Yes                | 1.63                | 29.93            | 26.49            | 10.85             | 33       | 23             |
| ELANE            | Yes                | 1.77                | 29.44            | 26.06            | 10.41             | 8        | 8              |
| LTF              | Yes                | 1.89                | 30.27            | 29.37            | 7.08              | 55       | 53             |
| LYZ              | Yes                | 2.57                | 31.92            | 29.68            | 4.71              | 14       | 14             |
| S100A9           | Yes                | 2.24                | 32.82            | 30.46            | 5.14              | 11       | 11             |
| S100A8           | Yes                | 2.15                | 31.92            | 29.68            | 4.71              | 14       | 14             |
| CTSG             | Yes                | 2.77                | 30.87            | 28.68            | 4.58              | 10       | 10             |
| IGHM             | Yes                | 2.54                | 32.15            | 30.45            | 3.25              | 17       | 3              |

UC, ulcerative colitis; CD, Crohn’s disease; LFQ: Label-free quantification; AKR1C1, Aldo-ketoreductase family 1 member C1; AKR1C3, Aldo-ketoreductase family 1 member C3; KRT4, Keratin 4; ELANE, neutrophil elastase; LTF, lactotransferrin; LYZ, lysozyme C; CTSG, Cathepsin G; IGHM, protein group Ig mu chain C region and Ig mu heavy chain disease protein; NaN, not a number.

Table 2. AKR1C3 IHC staining results.

| Variable       | UC     | CD     | CT     | p-value UC versus CD | p-value CD versus CT | p-value UC versus CT |
|----------------|--------|--------|--------|----------------------|----------------------|----------------------|
| Number of studied cases [n] | 51     | 62     | 10     |                      |                      |                      |
| Number of analyzed cells [n] | 6.8(2.5–16.9) x 10^4 | 6.4(1.6–20.2) x 10^4 | 5.7(3.5–12.7) x 10^4 | 0.988⁴² | 0.698⁴² | 0.700⁴² |
| Analyzed area (µm²) | 9.8(3.8–22.4) x 10⁶ | 9.3(2.3–28.0) x 10⁶ | 8.4(5.7–20.8) x 10⁶ | 0.690⁴² | 0.922⁴² | 0.926⁴² |
| Overall        | 73.8(14.3–91.6) | 81.4(44.6–98.4) | 94.0(80.7–97.5) | 0.002⁴² | <0.001⁴¹ | <0.001⁴¹ |
| Mild           | 43.2(15.5–54.0) | 32.8(15.2–56.8) | 19.1(12.0–48.4) |                      |                      |                      |
| Moderate       | 18.4(0.7–30.4)  | 19.8(4.2–29.7)   | 24.2(19.4–41.1) |                      |                      |                      |
| Strong         | 8.5(0.2–41.9)   | 82.4(0.2–58.0)   | 48.4(6.1–65.1)  |                      |                      |                      |
| H-score        | 113(18–196)     | 156(49–240)      | 213(19–247)     | <0.001⁴¹ | 0.001⁴¹ | <0.001⁴¹ |

AKR1C3 IHC intensity, n [%]

| H-score | 1 | 2 | 3 |
|---------|---|---|---|
| Number  | 37(72.5) | 14(27.5) | 0 (0) |
| 1       | 15(24.2) | 44(71.0) | 3(4.8) |
| 2       | 1(10.0)  | 4(40.0)  | 5(50.0) |

Numbers are given as median (minimum–maximum) or absolute number (percentage); AKR1C3, Aldo-ketoreductase family 1 member C3; CD, Crohn’s disease; CT, control tissue; IHC, immunohistochemistry; UC, ulcerative colitis; ⁴²two-sided t-test with unequal variances; ⁴¹χ²-test (AKR1C3 intensity 1 vs 1).

3.2. Immunohistochemistry Confirms Higher Expression Levels of AKR1C3 in Crohn’s Disease

As AKR1C3 was found to be completely absent from UC samples and present in most of CD samples, all 123 cases of the confirmation cohort were stained for AKR1C3 by IHC and were evaluated by digital analysis. The number of cells and the analyzed areas were not different between UC and CD (p = 0.988 and 0.690). Staining was generally pronounced in epithelial cells and weak in stromal cells. AKR1C3 was present in both groups, but staining intensity of epithelial cells was significantly stronger in CD as compared to UC (p < 0.001) in Table 2. Staining intensity (H-score) of control colonic samples was significantly higher as compared to IBD samples. Representative examples of AKR1C3 IHC are displayed in Figure 2.

4. Discussion

LMD-based microproteomics is a promising approach to detect biomarkers in human tissue specimens.⁸¹ Although several proteomic studies have investigated IBD, most of these were performed from plasma⁸² and only few were dedicated to the proteomic comparison between UC and CD tissue specimens.⁸¹,²²,²³ LC-MS/MS-based proteomics have already been used in the past to compare protein profiles between UC and CD in tissues.²²,²³ However, no study aimed at performing sample collection guided by a fine histological evaluation from FFPE tissues. In our retrospective study, tissue pieces containing equivalent numbers of epithelial cells were collected using LMD from FFPE tissue sections. Sections serial to the one used for LMD collection were stained by HE and subjected to software-based cell counting to guide the collection. This allowed for a reliable comparison of tissues in respect of the cell content that can be highly heterogeneous between and within specimens of IBD. LMD tissue pieces were further processed and analyzed by LMD-based microproteomics that allows for proteomic analyses of small tissue pieces.

A previously described LMD-based microproteomic approach was used in the present study for the comparison of UC and CD tissues containing a finely controlled number of cells. This method was already successfully used for
biomarker discovery in patients with anal cancer,\textsuperscript{15} uterine cervical dysplasia,\textsuperscript{18} and cystic echinococcosis of the liver.\textsuperscript{16}

Applying this method to UC and CD tissue specimens revealed eight proteins to be significantly more abundant in CD compared to UC. Among these proteins, ELANE was more than tenfold more abundant in CD compared to UC. ELANE is a serine protease involved in physiological and pathological inflammation processes and was formerly reported as a potential marker of IBD disease activity.\textsuperscript{24} In this context, it is interesting that neutrophils have a dual role in IBD and their behavior may differentiate UC and CD.\textsuperscript{25} Our finding might suggest a differential release of ELANE granules between UC and CD. Another neutrophil serine protease, CTSG, was previously reported to be involved in the alteration of the colonic epithelial barrier in UC but no investigation was done in CD.\textsuperscript{26} In our study, we found significantly higher CTSG quantities in CD. Moreover, we identified two additional proteins related to neutrophil activity, LTF and LYZ, more than sevenfold and fivefold more abundant in CD com-
pared to UC. Both proteins were formerly reported as potential fecal markers of IBD.[27] Our results suggest that LTF and LYZ might also differentiate UC and CD tissues at a protein level.

The proteins of the calprotectin complex, S100A8 and S100A9, are damage-associated molecular patterns (DAMPs) that have been suggested to play a role in IBD’s etiology.[28] S100A8/S100A9 were found at high levels in serum and intestinal tissues during active disease, and were considered as potential markers of several colonic inflammatory diseases.[29] Our findings highlight approximately 5-fold higher quantities of these two proteins in CD compared to UC, suggesting possible different activity of these two DAMPs proteins between UC and CD.

Among the proteins that we found in different abundances between UC and CD, AKR1C3 was totally absent in all UC cases and present in eight out of nine CD cases. As markers bearing total positivity or negativity between entities would represent potential entity specific biomarkers, we evaluated its immunohistochemical staining in a larger cohort of UC and CD (Table 1). Although AKR1C3 was present in epithelial cells in both diseases, digital pathology evaluation revealed decreased AKR1C3 IHC staining intensities in UC as compared to CD. AKR1C3 is an enzyme that has been detected in various tissue types including tumor and non-tumor tissues and that is involved in the metabolism of sugars, steroids, prostaglandins, and other metabolites.[30] Specifically, AKR1C3 metabolizes oxidative stress-derived aldehydes[31] and converts prostaglandin D2 to 11-beta prostaglandin F2 (11\(\beta\)PGF2a) which enhances cell restitution and thereby wound closure.[32] AKR1C3 has been previously investigated in patients with CD in a study where it was shown that AKR1C3 mRNA expression was down regulated in CD patient cells as compared to healthy control cells, which resulted in decreased levels of 11\(\beta\)PGF2a.[32] It is tempting to speculate that the decreased AKR1C3 expression results from the defective immune response and therefore is at least partly responsible for the sustained mucosal damage observed in IBD. In this regard, it is also interesting that AKR1C3 staining intensity was lower in IBD as compared to controls. However, it is not clear whether secreted mediators after inflammation-induced mucosal damage promote down regulation of AKR1C3 or if reduced levels of AKR1C3 actively contribute to epithelial erosion and/or ulceration. The near-absence of AKR1C3 in epithelial cells from patients with UC as compared to CD was highlighted by LMD-based microproteomic results and confirmed by lower staining intensity in IHC. The underlying mechanism for the difference in staining intensity is unclear. AKR1C3 bears a large sequence homology with different other Aldo-keto reductase family 1 members such as AKR1C2 (87% homology), or AKR1C4 (84% homology). Thus, it is possible that the selected antibody also targets other Aldo-keto reductase family 1 members that displayed basal abundance in UC tissues. This would explain the basal staining intensity in both diseases on a technical level.

The differences in abundance of the nine proteins found in UC and CD increase our understanding of the pathological processes of the development of UC and CD. Although we carefully selected the same number of epithelial cells supported by digital image analysis, our results may also be explained by a differential neutrophil count within the epithelial cells or by a differential neutrophil activity in UC and CD. To further clarify this issue additional investigations including precise counting of neutrophils in UC and CD samples are necessary. Furthermore, the effect of different therapeutics has not been evaluated here due to the lack of clinical data in our study and should be taken into account in future studies.

To conclude, our LMD-based microproteomic study highlighted eight proteins with significantly different abundances between UC and CD that provide valuable information about differences of UC and CD. The proteins found suggest a differential neutrophil activity between UC and CD and a differential implication of DAMPs between the two entities. The results also suggest that proteins formerly proposed as markers of IBD may also differentiate UC and CD.

Differential quantities of AKR1C3 were found between UC and CD and confirmed by IHC. As AKR1C3 was immunohistochemically observed in both UC and CD, it is not well suited as a diagnostic marker. However, the biological role of AKR1C3 in IBD merits further investigation. Overall, the study gives an interesting snapshot of the molecular actors differentiating UC and CD and shows that LMD-based microproteomics and IHC represent a promising combination to support biomarker discovery in the context of IBD.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Author Contributions
K.A. and M.K. contributed equally as first authors and P.B. and R.L. contributed equally as senior authors. K.A., M.K., J.K., R.C., P.B., and R.L. developed the concept and designed the study, M.K., M.R., F.L., M.v.W., and J.K. evaluated the tissue sample quality, M.K., M.R., F.L., M.v.W., and J.K. carried out the evaluation of diagnoses, K.A., M.F., D.K., and R.L. prepared the tissue samples for mass spectrometry, K.A. and R.L. performed the MS analyses. K.K. and M.K. performed the IHC analyses. M.K., M.F., K.K., and R.L. performed the statistical analyses. K.A., M.K., M.F., K.K., B.G., P.B., and R.L. drafted the manuscript. All authors reviewed the manuscript for important intellectual content.

Keywords
Crohn’s disease, digital pathology, immunohistochemistry, laser microdissection, microproteomics, nanoLC-MS/MS, ulcerative colitis
