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Fragments of Citrullinated and MMP-degraded Vimentin and MMP-degraded Type III Collagen Are Novel Serological Biomarkers to Differentiate Crohn's Disease from Ulcerative Colitis

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Abstract

Background and Aims: A hallmark of inflammatory bowel disease [IBD] is chronic inflammation, which leads to excessive extracellular matrix [ECM] remodelling and release of specific protein fragments, called neoepitopes. We speculated that the biomarker profile panel for ulcerative colitis [UC] and Crohn's disease [CD] represent a heterogeneous expression pattern, and may be applied as a tool to aid in the differentiation between UC and CD.

Methods: Serum biomarkers of degraded collagens I, III-IV [C1M, C3M, and C4M], collagen type 1 and IV formation [P1NP, P4NP], and citrullinated and MMP-degraded vimentin [VICM] were studied with a competitive ELISA assay system in a cohort including 164 subjects [CD $n = 72$, UC $n = 60$, and non-IBD controls $n = 32$] and a validation cohort of 61 subjects [CD $n = 46$, and UC $n = 15$]. Receiver operating characteristic curve analysis and logistic regression modelling were carried out to evaluate the discriminative power of the biomarkers.

Results: All biomarkers were corrected for confounding factors. VICM and C3M demonstrated the highest diagnostic power, alone, to differentiate CD from UC with an area under the curve [AUC] of 0.77 and 0.69, respectively. Furthermore, the biomarkers C1M [AUC = 0.81], C3M [AUC = 0.83], VICM [AUC = 0.83], and P1NP [AUC = 0.77] were best to differentiate UC from non-IBD. The best combinations of biomarkers to differentiate CD from UC and UC from non-IBD were VICM, C3M, C4M [AUC = 0.90] and VICM, C3M [AUC = 0.98] respectively.

Conclusions: Specific extracellular matrix degradation markers are elevated in IBD and can discriminate CD from UC and UC from non-IBD controls with a high diagnostic accuracy.

Keywords: Inflammatory bowel disease; serological biomarkers; extracellular matrix remodelling

1. Introduction

Inflammatory bowel diseases [IBD] are a group of idiopathic gastrointestinal chronic inflammatory conditions. A firm diagnosis necessitates clinical evaluations including endoscopies and histology, making the diagnosis tedious and uncomfortable for the patients and a challenge for the clinicians. Consequently, there is a medical need for improved IBD diagnostics.[1,2,3] Serological biomarkers may aid the clinicians in the diagnosis of IBD. However, there are only few biomarkers that are applicable for this purpose, thus there is a need for development and testing of novel serological markers. Examples of biomarkers are the cyclic citrullinated peptides and anti-citrullinated protein antibodies, which have been used as routine diagnostic tests for rheumatoid arthritis.[4,5,6] Currently the most well-studied serum biomarkers for differentiating CD and UC are the anti-glycan biomarker IgG anti-*Saccharomyces cerevisiae* antibodies [ASCA] and anti-neutrophil cytoplasmic antibodies [ANCA].[7] The ASCA is often associated with CD, whereas the ANCA assay is often associated with UC. Other biomarkers have also been investigated for use in IBD diagnostics; however, none of these biomarkers did report any clinical relevance to differentiate CD from UC.[8,9]

In IBD, the extracellular matrix [ECM] of the intestine is highly affected by chronic inflammation that leads to imbalanced tissue remodelling[10] which is a result of increased expression of both degenerative proteases, such as matrix metalloproteinases [MMPs] and ECM protein.[11,12] In IBD, several MMPs have been reported to be upregulated during inflammation, including MMP-2,[13,19] 8,[14,15,16] and 9.[13,14,15] The altered tissue remodelling, led by the MMPs, results in the release of small protein fragments into the surrounding tissue, where these ECM cleavage products may act as chemokines, which in turn can contribute to increased inflammation by increased infiltration by leukocytes, including neutrophils.[16] These protein fragments, also referred to as neo-epitopes,[17] can also be released into the circulation, and may be used as biomarkers for diagnostic, disease monitoring, and prognostic purposes.[18]

The main collagens found in the ECM of intestinal wall are collagen types I, III, and IV.[20,21] Collagen type I and III are fibrillar collagens and are often co-localised in the ECM where they play a major role in upholding the ECM structure and rigidity. Collagen type IV is a basement membrane protein.[22] Another interesting protein is vimentin, a type III intermediate filament protein, which has recently been demonstrated to be involved in the pathogenesis of IBD.[23] Furthermore, vimentin have shown diagnostic relevance in other inflammatory diseases including rheumatoid arthritis[24] and ankylosing spondylitis,[25] as well as in several forms of cancer.[26] Biomarker assays measuring specific neo-epitopes of vimentin (MMP-2 and MMP-8 degraded and citrullinated-vimentin [VICM]),[27] type I (MMP-9 degraded collagen type I [C1M]),[28] III (MMP-9 degraded collagen type III [C3M])[29] and IV [MMP-9 degraded collagen type IV [C4M]),[30] collagens and formation of collagen type I [P1NP] and IV [P4NP],[31,32] have shown promise as diagnostic tools in several autoimmune diseases[24,25,33,34,35] and in fibrosis.[32] These biomarkers have been validated by various different proteomic techniques, including mass spectrometry, for specific ECM MMP cleavages.

The aim of the current study was to explore the diagnostic potential of serum neo-epitope ECM biomarkers including the collagen degradation biomarkers [C1M, C3M, C4M], the collagen formation biomarkers [P1NP, P4NP], and the citrullinated vimentin biomarker VICM, for IBD.

2. Materials and Methods

2.1. Patient data

2.1.1. The Odense University Hospital cohort

This cohort comprises two independent studies, a CD study and a UC study, with a total of 164 serum samples.

The CD study was a prospective blinded multicentre study evaluating three small bowel imaging techniques [ClinicalTrials.gov Identifier NCT01019460], including 104 serum samples. The inclusion and exclusion criteria for the study have been described previously.[36] Out of the 104 patients: 72 patients were diagnosed with CD, and location and disease behaviour were assessed by the Montreal classification; 32 patients were diagnosed with non-IBD gastrointestinal diseases with no small bowel involvement, including irritable bowel syndrome [n = 22], functional diarrhoea [n = 5], undetermined abdominal pain [n = 2], chronic appendicitis [n = 1], coeliac disease [n = 1], and unclassified histocytic inflammation in the colon and megacolon [n = 1]. This population was referred to as non-IBD, and was applied as reference group. Patients had a standardised work-up including medical history, physical examination, blood samples, ileocolonoscopy, capsule endoscopy, MR enterography Crohn's disease activity index (CDAI) for assessing diease activity, and CT enterography. Patients were recruited from October 2007 to August 2009. Blood samples were drawn on the day of small bowel examinations and stored at ≤ -20 °C. C-reactive protein [CRP] was measured at the time of sampling.

The UC study is a randomised placebo-controlled, double-blind trial evaluating probiotic treatment for active ulcerative colitis [ClinicalTrials.gov Identifier NCT00374725]. Serum samples from 60 patients with a flare of UC were included. Inclusion criteria for patients were as follows: patients with known UC; at least one previous flare of clinical and endoscopic active disease; age 18 and above. Exclusion criteria were: changes in azathioprine dosage within the past 3 months; patients with toxic megacolon; peritonitis or severe colonic bleeding; known immunodeficiency; ongoing infectious disease; ongoing treatment with non-steroidal anti-inflammatory drugs [NSAID] or cholestyramine; pregnant or lactating women. Treatment was adjusted according to the physician's standard guidelines in order to achieve remission. The probiotic used in the trial was combination of *Lactobacillus rhamnosus* strain 19070–2 and *Lactobacillus acidophilus* strain 18911–2 [10EE9 colony forming unit/ml] 1 ml daily in the evening. At inclusion, patients entered a 6-month maintenance phase when the disease was in remission and treatment had been reduced to maintenance level. Study treatment was started at inclusion, and continued until the end of the maintenance phase. Patients had a standardised work-up at: 1] inclusion; 2] when entering the maintenance phase; and 3] either at study completion or at time of exclusion from the trial. The work-up included medical history, physical examination, clinical activity index, endoscopic evaluation, and C-reactive protein. Blood samples were drawn at each of these visits and stored at ≤ -20°C; CRP was measured at the time of sampling. However, the included samples were the baseline serum samples, hence before the initiation of the probiotic treatment.

2.1.2. The validation cohort

The validation cohort comprises two independent cohorts obtained from University Medical Center Groningen [UMCG, The Netherlands] and from the commercial vendor Asterand [Detroit, MI].

The serum samples obtained from Groningen are from patients diagnosed with ileocolonoscopy biopsy-confirmed CD [n = 46], which are included in the UMCG IBD centre database. This database contains serum samples at time of admission, independent of the state

of disease at that moment. Disease location and disease behaviour were assessed by the Montreal classification [Table 1]. Patients underwent standard work-up, including medical history, Harvey Bradshaw index [HBI] for assessing disease activity, physical examination, pharmacological treatment, blood samples, and ileocolonoscopy.

The serum samples obtained from Asterand were from 15 UC patients with chronic moderate to severe disease activity. Endoscopic evaluation was done to assess the extent of disease. All patients had signed an informed consent and, in accordance with Danish law, no additional ethical approval was obtained when measuring biochemical biomarkers in previously collected serum samples. Samples were stored at ≤ -20°C until analysed.

2.2. Biomarker assay

The neo-epitope fragments of ECM synthesis and degradation were assessed by solid phase competitive enzyme linked immunosorbent assays [ELISAs]. The antibodies only react against the degraded protein, not the intact protein, and thus are a measure of protein degradation. The biomarkers included in this study are VICM, C1M, C3M, C4M, PINP, and P4NP. [27,28,29,30,31,32]

Briefly, streptavidin pre-coated 96-well plates [Roche Diagnostics cat. No. 11940279, Hvidovre, Denmark] were coated with a biotinylated antigen for 30 min at room temperature. All samples were diluted in incubation buffer. The incubation buffer contains 1% bovine serum albumin [Sigma Aldrich, cat. No. A-7906, ≥ 98 purity] for protein stability and blocking purposes. Standard kit controls and serum samples were incubated horseradish peroxidase-conjugated monoclonal antibodies for 1–3 h at 4°C/20°C or for 20 h at 4°C, depending on the specific assay, and agitated at 300 rpm. Tetramethylbenzidine [TMB, Kem-En-Tec cat. No. 438OH, Taastrup, Denmark] was added [100 µl/well] and the plate incubated for 15 min at room temperature, and agitated at 300 rpm. The TMB reaction was stopped by addition of a stopping buffer [1% H_2SO_4]. Wells were thoroughly washed after each incubation step with buffer [25 mM TRIZMA, 50 mM NaCl, 0.036 % Bronidox L5, 0.1 % Tween 20] using a standardised ELISA plate-washing machine [BioTek® Instruments, Microplate washer, ELx405 Select CW, Winooski, USA]. The optical densities were read at 450 nm and 650 nm as reference using an ELISA reader [VersaMAX; Molecular Devices, Wokingham, UK]. A standard curve was plotted using a 4-parametric mathematical fit model.

2.3. Statistical analyses

Some of the biomarker levels were not normal-distributed, not even after log-tranformation. Therefore the Kruskal-Wallis test and Mann-Whitney U-test were applied. For some of the demographic statistics, the Chi-square test and Fisher's exact test were applied. The biomarker levels were presented as means with standard error of the mean [SEM] for figures and as means with confidence interval [CI] for tables. To evaluate the discriminative power of the biomarker, a receiver operating characteristic curve [ROC curve] was calculated with DeLong et al. methodology and backwards logistic regression analyses were carried out, to calculate the best diagnostic value by combining all the markers and to correct for confounding factors (age, body mass index [BMI], gender, smoking, use of immunosuppressant drugs, disease activity, and Montreal classification). The cut off values were determined by taking into account the cost of false and true positive. The diagnostic accuracy was calculated by the following equation: Diagnostic accuracy = ([True negatives + true positives]/[true negatives + true positives + false negatives + false positives]). A p-value of ≤ 0.05 was accepted as being statistically significant. GraphPad Prism 6 was applied to calculate the Kruskal-Wallis and Mann-Whitney U-test statistics and figure editing. MedCalc was applied to for ROC-curve and logistic regression model statistics.

3. Results

3.1. Cohort description

This study included in total 225 subjects. The variation between the cohort demographics was analysed and showed that several demographic differences exist between the cohorts, especially for gender, age, BMI, smoking, use of immunosuppressants, disease activity, Montreal classification, bowel resection, and CRP [Table 1].

3.2. Levels of biomarkers in patients with Crohn's disease, ulcerative colitis, and non-IBD

VICM and C3M were the only biomarkers that demonstrated significant differences between CD and UC [Figure 1A, B]. Levels of VICM were significantly lower in UC patients compared with either CD or non-IBD [P<0.001] [Figure 1A]. In contrast, serum levels of C3M were significantly elevated in the UC patients compared with CD patients [p = 0.008] and the non-IBD patients [p < 0.001], and CD vs non-IBD [p = 0.044] [Figure 1B]. In addition, C1M was significantly elevated in UC compared with non-IBD [p = 0.029] [Figure 1C]. P4NP was significantly higher in CD compared with non-IBD [p < 0.046] [Figure 1F]. The remaining biomarkers, C4M [Figure 1D] and PINP [Figure 1E], failed to show any statistical significance or trend difference between CD, UC, and non-IBD.

3.3. The diagnostic value of biomarker assays in Crohn's disease, ulcerative colitis, and non-IBD cohorts

The biomarkers VICM and C3M had the highest diagnostic power for differentiating CD from UC. The VICM assay had the best diagnostic value, and when adjusted for demographic variations (only gender and smoking were significant confounders), the area under the curve [AUC] was increased from 0.76 [p < 0.001] to 0.79 [p < 0.001] [Table 2]. In comparison, the C3M assay showed less diagnostic value; however, when C3M was adjusted for demographic confounding factors, the AUC increased from 0.62 [p = 0.002] to 0.69 [p < 0.001] [Table 2].

The biomarkers C1M and C3M were the best biomarkers to differentiate CD patients from non-IBD patients. The C3M assay had the best diagnostic value, with an AUC of 0.65 [p = 0.012], and was not affected by any demographic confounding factors [Table 2]. The diagnostic value of C1M was increased when adjusted for demographic confounding factors from an AUC of 0.60 [p = 0.041] to 0.68 [p = 0.018] [Table 2].

The biomarkers that performed best in differentiating UC from non-IBD were C1M, C3M, and VICM assays. When adjusted for demographic confounding factors (only gender and smoking were significant confounders), all three biomarkers performed similarly, and the AUC was increased in C1M, C3M, and VICM from 0.66 [p = 0.01], 0.81 [p < 0.001], and 0.72 [p < 0.001] to 0.81 [p < 0.001], 0.83 [p < 0.001], and 0.83 [p <0.001], respectively [Table 2]. For each biomarker, the optimal cut-off value was determined by ROC curve analyses and the odds ratio was calculated for the specified cut-off value [Table 3].

3.4. Combination of biomarkers increases the diagnostic value in CD vs UC and non-IBD

The optimal combination of biomarkers to differentiate CD from UC was a combination of VICM, C3M, and C4M with an AUC of 0.86, a specificity of 90%, and sensitivity of 75%. This combination of markers had a diagnostic accuracy of 79% [Table 4]. Adjusting for demographic confounding factors increased the AUC, sensitivity,

Table 1. Cohort demographics.

	Odense University Hospital cohort			Validation cohort		CD1 vs UC1	IBD1 vs non-IBD	CD1 vs CD2	UC1 vs UC2
	CD1 [*n* = 72]	UC1 [*n* = 60]	Non-IBD [*n* = 32]	CD2 [*n* = 46]	UC2 [*n* = 15]				
Gender						*p* = 0.22	*p* = 0.004	p = 0.015	p = 0.255
• Male, [%]	27 [37]	29 [48]	5 [16]	28 [61]	10 [67]				
• Female, [%]	45 [63]	31 [52]	27 [84]	18 [39]	5 [33]				
Age, mean years [range]	35.8 [15–76]	37.3 [21–70]	35.2 [15–75]	42 [16–77]	39.8 [18–72]	*p* = 0.99	*p* = 0.999	*p* = 0.999	*p* = 0.999
BMI, mean kg/m^2 [range]	25.8 [16.2–45.1]	26 [18.6–36.4]	26.4 [14.7–44.1]	23.3 [17.3–32.7]		*p* = 0.99	*p* = 0.999	*p* = 0.114	n/a
Smoking						*p* = 0.86	*p* < 0.001	*p* = 0.023	*p* < 0.001
• Yes, [%]	40 [56]	35 [58]	12 [37]	15 [33]	0				
• No, [%]	32 [44]	25 [42]	20 [63]	31 [67]	15				
Use of immunosuppressants						*p* = 0.22	*p* < 0.001	*p* = 0.074	*p* < 0.001
• Yes, [%]	30 [42]	32 [53]	1 [3.0]	11 [24]	15 [100]				
• No, [%]	42 [58]	28 [47]	31 [97]	34 [66]	0 [0]				
Clinical disease activity	CDAI score[a,d]	St Marks score[b]		HBI[c]		*p* < 0.001	n/a	*p* < 0.001	*p* = 0.005
• Active disease, [%]	37 [51]	54 [90]		10 [22]	15				
• Remission, [%]	30 [42]	6 [10]		36 [78]	0				
Endoscopic disease activity evaluation						n/a	n/a	n/a	*p* = 0.33
• Active disease, [%]	58 [81]	54 [90]			15 [100]				
• Remission, [%]	14 [19]	6 [10			0 [0]				
Montreal classification: Location						n/a	n/a	*p* = 0.017	n/a
• L1	24			24					
• L2	18			2					
• L3	29			20					
• L4	1			0					
Behaviour						n/a	n/a	*p* = 0.613	n/a
• B1	45			25					
• B2	22			16					
• B3	5			5					
Extent of disease						n/a	n/a	n/a	*p* = 0.43
• E1		18 [30]			7 [47]				
• E2		30 [50]			5 [33]				
• E3		12 [18]			3 [20]				
Bowel resection prior to inclusion	20	0	0	2	0	n/a	n/a	*p* = 0.001	n/a
c-reactive protein									
• >5mg/l	36 [50]	36 [60]	18 [56]	16 [35]					

CD, Crohn's disease; UC, ulcerative colitis; IBD, inflammatory bowel disease; BMI, body mass index; n/a, not available; CDAI, Crohn's Disease Activity Index; HBI, Harvey Bradshaw Index; St. Marks score, Saint Marks score; CD1, Crohn's disease patients from Odense University Hospital cohort; UC1, ulcerative colitis patients from Odense University Hospital cohort; CD2, Crohn's disease patients from the validation cohort; UC2, ulcerative colitis patients from the validation cohort.

[a]Crohn's disease activity index [active disease.>.150].

[b]St Marks score [active disease>2].

[c]Harvey Bradshaw index [active disease>5].

[d]CDAI was assessed in 67 patients.

[e]*p*-Values depict statistical differences between the Odense University Cohort and the validation cohort. Chi-square test, Fisher's exact test, Mann-Whitney U test, or Kruskal-Wallis test was applied when appropriate.

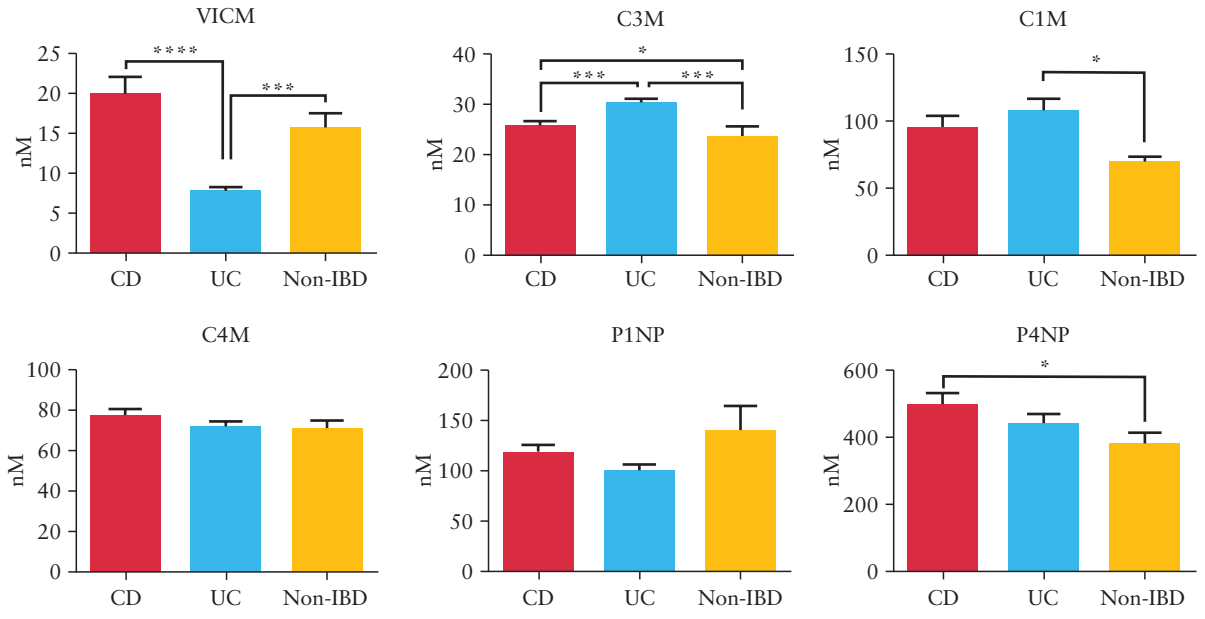

Figure 1. Difference in serum biomarkers [C1M, C3M, C4M, VICM, P1NP, P4NP], between the total population of CD [$n = 72$], UC [$n = 60$], and non-IBD [$n = 32$]. A] VICM; B]C3M; C]C1M; D]C4M; E]P1NP; F]P4NP; KruskalWallis test was carried out, and asterisks [*] depicts the significance level: $P < 0.05$,**$P < 0.01$,***$P < 0.001$. Values are depicted as mean + standard error of the mean [SEM]. CD, Crohn's disease; UC, ulcerative colitis; IBD, inflammatory bowel disease.

Table 2. AUC ROC curve analysis of the individual biomarkers.

	AUC [CI] [sensitivity; specificity]	*p*-Value	AUC adjusted [CI] [sensitivity; specificity][a]	*p*-Value adjusted
Biomarker: CD vs UC				
• VICM	0.76 [0.66–0.85] [71;75]	<0.001	0.79 [0.66–0.86] [56;93]	<0.001
• C3M	0.62 [0.52–0.73] [53;77]	0.002	0.69 [0.57–0.78] [65;66]	<0.001
• PINP	0.60 [0.49–0.70] [51;70]	0.11	0.60 [0.49–0.70] [51;70]	0.11
• C4M	0.54 [0.43–0.65] [38;80]	0.50	0.54 [0.43–0.65] [38;80]	0.50
• C1M	0.52 [0.41–0.63] [92;22]	0.69	0.52 [0.41–0.63] [92;22]	0.69
• P4NP	0.50 [0.39–0.61] [27;90]	0.85	0.50 [0.39–0.61] [27;90]	0.85
Biomarker: CD vs non-IBD				
• VICM	0.51 [0.39–0.63] [40;52]	0.92	0.51 [0.39–0.63] [40;52]	0.92
• C3M	0.65 [0.57–0.79] [74;24]	0.012	0.65 [0.57–0.79] [80;24]	0.012
• P1NP	0.50 [0.38–0.62] [52;41]	1.00	0.50 [0.38–0.62] [52;41]	1.00
• C4M	0.58 [0.47–0.71] [60;48]	0.14	0.60 [0.47–0.71] [60;48]	0.14
• C1M	0.60 [0.49–0.69] [69;46]	0.041	0.68 [0.56–0.75] [68;53]	0.018
• P4NP	0.57 [0.44–0.68] [63;39]	0.32	0.57 [0.44–0.68] [63;39]	0.32
Biomarker: UC vs non-IBD				
• VICM	0.72 [0.62–0.81] [91;50]	<0.001	0.83 [0.73–0.91] [95;59]	<0.001
• C3M	0.81 [0.71–0.88] [93;62]	<0.001	0.83 [0.73–0.90] [97;50]	<0.001
• P1NP	0.61 [0.50–0.71] [66;59]	0.11	0.77 [0.66–0.86] [62;88]	<0.001
• C4M	0.56 [0.45–0.66] [44;77]	0.54	0.56 [0.45–0.66] [44;77]	0.54
• C1M	0.66 [0.55–0.76] [98;11]	0.01	0.81 [0.71–0.89] [93;50]	<0.001
• P4NP	0.58 [0.47–0.68] [40;77]	0.62	0.58 [0.47–0.68] [40;77]	0.62

AUC, area under the curve; CI, confidence interval; CD, Crohn's disease; UC, ulcerative colitis; IBD, inflammatory bowel disease.
[a]The logistic regression model was adjusted for confounding factors.

and specificity to 88% and 72%, respectively. In addition, the diagnostic accuracy increased to 81%. To investigate the discriminative power of the regression models in the IBD patients, the model was carried out only including samples with CRP level > 5 mg/l [CD, $n = 36$; and UC, $n = 36$]. The logistic regression model did improve, with an AUC, specificity, and sensitivity of 0.90 [Figure 2A], 89%, and 83%, respectively, and the diagnostic accuracy was increased to 84% [Table 4].

The combination of C1M and C3M in a logistic regression model proved to be the best combination of markers to differentiate CD from non-IBD when adjusted for demographic confounding factors. This model had an AUC [Figure 2B] of 0.70 with a specificity and sensitivity of 60% and 79%, respectively [Table 4]. To investigate the pathological differences with regard to elevated CRP, level of > 5 mg/l was included [CD, $n = 36$; non-IBD, $n = 18$]. This did not, however, improve the logistic regression model [Table 4].

Table 3. Cut-off values determined by ROC curve analyses, and odds ratios for the specified cut-off values.

	CD vs UC		UC vs non-IBD		CD vs non-IBD	
Markers	Cut-off value [nM][a]	OR [*p*-value]	Cut-off value [nM][a]	OR [*p*-value]	Cut-off value [nM][a]	OR [*p*-value]
VICM	**9.66**	**5.62 [<0.001]**	**13.3**[c]	**7.43 [<0.001]**	19.8	0.53 [0.16]
C3M	**23.3**	**4.06 [0.002]**[b]	**23.3**	**17.4 [<0.001]**	**23.3**	**3.04 [0.01]**
C4M	77.9	1.59 [0.225]	74.3	2.05 [0.18]	**70.7**	**3.45 [0.011]**
C1M	159	0.63 [0.378]	**96.2**	**4.57 [0.006]**	**61.1**	**2.45 [0.046]**
P1NP	105	1.44 [0.302]	113	0.62 [0.29]	45.3	0 [0.99]
P4NP	639	2.38 [0.063]	**206**	**4.23 [0.028]**	**433**	**2.4 [0.049]**

ROC, receiver operating characteristic; OR, odds ratios; CD, Crohn's disease; UC, ulcerative colitis; IBD, inflammatory bowel disease; emboldened entries indicates statistical significance.

[a]Cut-off values from the specificity and sensitivity shown in Table 2.

[b]UC vs CD.

[c]Non-IBD vs UC.

Table 4. Combination of biomarkers by logistic regression. The AUC, sensitivity, and specificity, of each ROC analysis and diagnostic accuracy.

	AUC [CI]	Sensitivity %	Specificity %	Diagnostic accuracy %
Biomarkers: CD vs UC				
• [C3M, VICM, C4M]	0.86 [0.80–0.92]	75	90	79
• [C3M, VICM, C4M][a]	0.88 [0.82–0.94]	88	72	81
• [C3M, VICM, C4M][a,b]	0.90 [0.79–0.96]	89	83	84
Biomarkers: CD vs non-IBD				
• [C1M, C3M]	0.65 [0.57–0.80]	50	82	55
• [C1M, C3M][a]	0.70 [0.58–0.81]	60	79	67
• [C1M, C3M][a,b]	0.65 [0.50–0.77]	56	82	63
Biomarkers: UC vs non-IBD				
• [C3M, VICM]	0.93 [0.85–0.97]	80	94	85
• [C3M, VICM][a]	0.94 [0.87–0.98]	77	97	88
• [C3M, VICM][a,b]	0.98 [90–1.0]	96	94	95

AUC, area under the curve; ROC, receiver operating characteristic; CI:, confidence interval; CD, Crohn's disease; UC, ulcerative colitis; IBD, inflammatory bowel disease.

[a]The logistic regression model was adjusted for confounding factors.

[b]Patient with active disease, and CRP levels > 5mg/l.

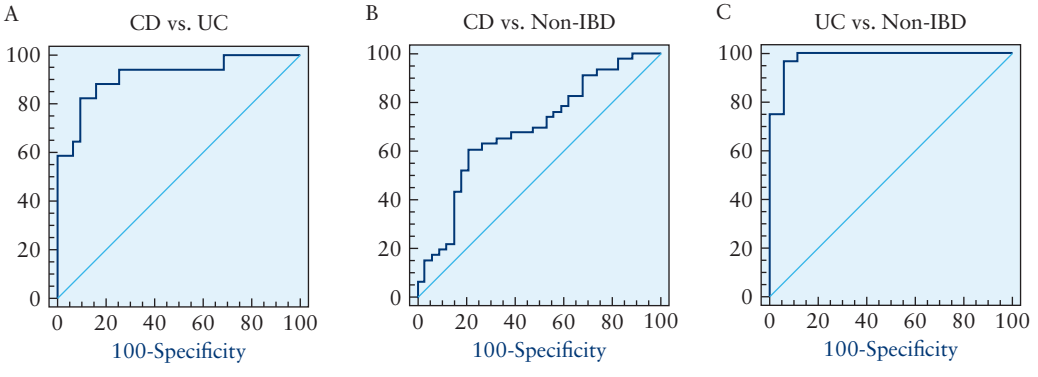

Figure 2. Diagnostic power of the biomarkers in combination by receiver operating characteristic curve [ROC curve], based on the logistic regression model adjusted for demographic confounding factors and CRP level > 5 mg/l. A] CD vs UC [VICM, C3M, and C4M], B] CD vs non-IBD [C1M and C3M], and C] UC vs non-IBD [VICM and C3M]. CD, Crohn's disease; UC, ulcerative colitis; IBD, inflammatory bowel disease.

The best combination of biomarkers to distinguish between UC and non-IBD was C3M and VICM. This model had an AUC, sensitivity, and specificity of 0.93, 80 %, and 94 %, respectively [Table 4]. Furthermore, the demographic confounding factors had little effect on the logistic regression model [Table 4]. However, when data were corrected for confounding factors and including patients with CRP level > 5 mg/l [UC, n = 36; non-IBD, n = 36], this model had a diagnostic accuracy of 95% [Table 4]. The AUC [Figure 2C], specificity, and sensitivity increased to 0.98, 96 %, and 94 %, respectively [Table 4].

3.5. Validation cohort

To corroborate current findings, the significant biomarkers [VICM, C1M, C3M, and C4M] were tested in a sample set including serum samples from the validation cohort;:CD [n = 46] and UC [n = 15] patients [Table 1]. There were no significant differences in the mean levels of the biomarkers between the two cohorts [Table 5]. As with the Odense University Hospital cohort, a significant difference between UC and CD was observed with VICM [$p < 0.01$] and C3M [$p < 0.001$] [Table 5], and ROC curve analyses of VICM, C3M, and combination of VCIM and C3M was comparable to the Odense University Hospital cohort [Table 6].

4. Discussion

The present study is to our knowledge the first study to evaluate the differences in the ECM remodelling of CD, UC, and non-IBD in a competitive ELISA system based on the neo-epitope technology. Here we assessed a panel of ECM serum biomarkers. The current study demonstrates that the remodelling of the ECM is different in UC and CD, and is significantly affected by the chronic inflammation that leads to continuous remodelling of the ECM. Hence we observed that a number of ECM degraded neo-epitope biomarkers were significantly different between the diseases in two different patient cohorts. The best combinations of biomarkers to differentiate were: CD from UC, VICM, C3M, and C4M; CD from non-IBD, C1M and C3M; and UC from non-IBD:, C1M and C3M [Table 4], all with high diagnostic accuracies. In addition, the logistic regression model was expanded to only include UC and CD patients with a CRP level > 5 mg/l. Interestingly, the logistic regression model was improved [Table 4]. These findings were validated in two independent cohorts of UC and CD patients, thus the ECM biomarkers demonstrate proof of concept that a panel of ECM biomarkers can be used to differentiate CD from UC [Tables 5 and 6].

In about 10% of all IBD cases, a definite diagnosis of either CD or UC cannot be made and is determined IBD unclassified [IBDU][37]; hence non-invasive serum biomarkers might improve the overall diagnosis of IBD and IBDU. The biomarker ASCA performed best among an anti-glycan antibody panel in terms of diagnostic value to discriminate CD from UC and other bowel diseases.[7,38,39] A meta-analysis of anti-glycan antibodies in IBD diagnostics have proven these to be promising biomarkers in the diagnosis of especially CD.[7] ASCA had the highest sensitivity of the anti-glycan biomarker panel; however, the specificity was equal to the other biomarkers' specificities.[7] The ASCA assay is currently widely accepted as the best serum-based biomarker in terms of CD diagnosis.[7,37]

It is interesting that the biomarker profiling differs in the UC patients [increased level of C3M] and CD patients [increased level of VICM]; thus different ECM remodelling processes are present in the two diseases. The difference in the ECM remodelling that was observed during this study may be explained by the differences in leukocyte and MMP expression between CD and UC. MMP-2, 8, and 9 are upregulated in IBD,[16] in particular MMP-2 activity, and expression is higher in CD than in UC.[40] In addition, MMP-2 is expressed by macrophages of CD tissue.[41] Interestingly, a study by Kamada *et al.* demonstrated that the macrophage expression profile was different in CD and UC, favouring an alternate macrophage expression pattern of CD14+ and CD33+ macrophages in CD patients, which further contributes to the notion that macrophage activation is a contributing factor of CD pathogenesis.[42] These different pathogenic features of CD and UC may explain our results. Granulomas are part of the pathological features of CD and macrophages are the cells that define the granuloma. VICM

Table 5 Biomarker values for the Odensen University Hospital cohort and the validation cohorts.

	Odensen University Hospital cohort		Validation cohort				Reference values
Biomarker	CD: mean nM [CI]	UC: mean nM [CI]	CD: Mean nM [CI]	UC: Mean nM [CI]	p-Values[a]	p-Values[b]	p-Values[c]	Healthy controls: mean nM
VICM	18.0 [14.0–21.9]	8.01 [6.54–9.49]	17.4 [12.4–22.4]	6.45[2.43–10.4]	0.003	0.468	0.346	6.60[25]
C1M	102 [69.8–121]	115 [93.7–123]	134 [108–161]	178 [124–243]	>0.999	0.543	0.951	50.9[44]
C3M	26.1 [23.7–27.6]	30.9 [27.9–32.6]	25[22.3–26.8]	34.6 [28.5–39.3]	<0.001	0.0128	0.0509	17.9[44]
C4M	73.2 [69.9–77.0]	70.3 [67.26–80.37]	n/a	83.3 [71.2–94.4]	n/a	n/a	0.096	n/a

CD, Crohn's disease; UC, ulcerative colitis; IBD, inflammatory bowel disease, n/a, not available.
[a]Validation cohort CD vs UC.
[b]Odense cohort CD vs validation cohort CD.
[c]Odense cohort UC vs validation cohort UC.

Table 6. The AUC, sensitivity, and specificity, of each ROC analysis and diagnostic accuracy from the validation cohort.

CD vs. UC	AUC [CI]	Sensitivity %	Specificity %	Diagnostic accuracy
VICM	0.78 [0.66–0.88]	64	87	79%
C1M	0.61 [0.47–0.72]	60	62	74%
C3M	0.68 [0.55–0.80]	45	87	79%
VICM, C3M[a]	0.86 [0.75–0.94]	79	93	83%

AUC, area under the curve; ROC, receiver operating characteristic; CI, confidence interval; CD, Crohn's disease; UC, ulcerative colitis; IBD, inflammatory bowel disease.

[a]Combination of biomarkers.

is a cleavage product generated by MMP-2 and MMP-8 and may be a biomarker of activated macrophages. Type III collagen is most abundant in submucosal layers of the ileum and colon, and therefore the increased levels of C3M seen in UC could be explained by the fact that the inflammation in UC is more superficial than in CD.[11,20] The non-IBD control cohort also showed high serum levels of VICM. This was unexpected, and therefore future studies are warranted to reveal the biomarkers' relation to other gastrointestinal disorders.

The cut-off values were determined for the biomarkers and the odds ratio was calculated. Patients with VICM level > 9.66 nM are 5.62 times likely to have CD rather UC, and patients with C3M level > 23.3 nM are 4.06 times more likely to have UC rather than CD, 17.43 more likely to have UC rather than non-CD, and 3.08 more likely to have CD rather than non-IBD. These data further support that the biomarker VICM is a marker of CD and the biomarker C3M is a marker of UC. Interestingly, patients with VICM level >13.3 are 7.43 times more likely to have non-IBD than UC. These results demonstrate that VICM is heterogeneously expressed in gastrointestinal diseases; however, when patients are suspected to have IBD, high levels of VICM and low levels of C3M may indicate CD and vice versa UC. To rule out non-IBD gastrointestinal diseases, VICM, C3M, C1M, and C4M may be good biomarkers indicative of IBD [Tables 2–4].

It is important to emphasise that this study focuses on the diagnostic accuracy of neo-epitope serum biomarkers of ECM in IBD. The neo-epitope technology is designed to target specific MMP-degraded protein peptides,[22] and thus the levels of the neo-epitopes in serum reflect specific diseases and not just general inflammation such as CRP and orosomucoid, where the CRP levels in the current study did not differ significantly between CD, UC, or non-IBD patients groups. Thus MMP-degraded ECM biomarkers can give valuable information about the diseases and hopefully, in the future, biomarker-based diagnostics will become an integrated part of the gold standard in IBD diagnostics and in evaluation of disease progression.

There are several limitations to this study. The heterogeneity of the patient cohorts could potentially affect the results throughout the study. The samples from each cohort were collected by different personel, which could introduce practical variances between the samples and potentially lead to under/over estimations. Fortunately, a independent validation cohort confirmed the initial findings, and the observed differences are unlikely to be due to different handling by the laboratories. A limitation of assessing the diagnostic accuracy of serum biomarkers is that the performance of the biomarkers is determined on how close they come to the actual diagnosis based on the gold standard methods. Two related disease activity scoring systems (the Crohn's Disease Activity Index [CDAI] and the HBI were used to assess the CD patients' disease activity. The HBI is a simplified version of CDAI, and the CDAI correlates very well with HBI.[43] The two CD cohorts included in this study, are therefore comparable in terms of disease activity. Endoscopic scores for disease activity were not available for all cohorts, and studies have proven that there is poor correlation between endoscopic disease activity scoring and clinical disease activity indexes[45,46]. To fully elucidate the potential of ECM biomarkers, a study including total endoscopic evaluation of IBD patients is needed. This will allow investigation of the biomarkers' relation to complications and mucosal healing for IBD patients. Furthermore, the samples were heterogeneous regarding demographics, and these differences could influence the results. Consequently a logistic regression model was developed to control for confounding factors. Sub-analyses will decrease the number of patients and the power of the statistical analyses; therefore further studies are needed to confirm our findings.

In summary, we have demonstrated for the first time that the neo-epitopes VICM and C3M are potential biomarkers to differentiate CD from UC, and VICM, C3M, C1M, and C4M also have potential as biomarkers to differentiate IBD from non-IBD. The discriminative power of the biomarkers to differentiate between UC and CD was considerably improved when combining VICM and C3M in a logistic regression model with the addition of C4M. Furthermore, the combination of VICM and C3M in a logistic regression model was the best combination of the biomarkers to differentiate UC from non-IBD. The combination of these biomarkers could be incorporated into the diagnostic panel to improve IBD diagnostics. These findings warrant further studies to investigate the heterogeneity of ECM pathological remodelling in UC and CD.

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Conflicts of Interest

JHM, MAK, and ACB-J are full-time employees at Nordic Bioscience. MAK and ACB-J holds stocks in Nordic Bioscience. Nordic Bioscience is a privately owned small-medium sized enterprise, partly focused on the development of biomarkers for connective tissue disorders and rheumatic diseases. None of the authors received any kind of financial benefits or other bonuses for the work described in this manuscript.

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Author Contributions

Study design, data acquisition, data analysis, interpretation of data, drafting the manuscript, and revising critically for important intellectual content: JHM. Study design, interpretation of data, revising critically for important intellectual content: LEG, MDJ, WTVH, LGK, PO, GD, JK, ACBJ, MAK, AK. All authors approved the final draft before the manuscript was submitted.

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