



Original Article

# Identification of Chitinase-3-Like Protein 1 as a Novel Neutrophil Antigenic Target in Crohn's Disease

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## Abstract

**Background and Aims:** There is an increasing incidence of inflammatory bowel disease [IBD]. Autoimmune responses are involved in the pathophysiology of IBD, but their underlying pathways and target antigens have not yet been fully elucidated.

**Methods:** Autoantigenic targets in IBD were identified after separation of whole cell proteins isolated from neutrophils using two-dimensional electrophoresis and matrix assisted laser desorption ionization – time of flight mass spectrometry-based protein identification of the spots that displayed Western blotting signals with anti-neutrophil cytoplasmic antibody-positive sera. The prevalence of IgG, IgA and secretory IgA [sIgA] to chitinase 3-like protein 1 [CHI3L1] was analysed by enzyme-linked immunosorbent assays using recombinant CHI3L1 in 110 patients with Crohn's disease [CD], 95 with ulcerative colitis [UC], 126 with coeliac disease [CeD] and 86 healthy controls [HCs].

**Results:** The 18-glycosylhydrolase family member CHI3L1 was identified as a neutrophil autoantigenic target. CD patients displayed significantly higher levels of IgG to CHI3L1 than patients with UC and CeD ( $p < 0.0001$ , respectively). IgA and sIgA to CHI3L1 was significantly higher in CD than in UC, CeD and HCs [ $p < 0.0001$ , respectively]. IgA and sIgA to CHI3L1 demonstrated the highest prevalence in CD [25.5%, 28/110; and 41.8%, 46/110] compared to HCs [2.3%, 2/86; and 4.7%, 4/86;  $p = 0.0015$  and  $p < 0.0001$ ] and are associated with a more complicated progression of CD.

**Conclusion:** CHI3L1 is a novel neutrophil autoantigenic target in CD. IgA and sIgA to CHI3L1 may serve as novel markers for CD and may facilitate the serological diagnosis of IBD.

**Key Words:** Inflammatory bowel disease; chitinase-3 like protein 1; autoantibody; Crohn's disease; enzyme-linked immunosorbent assay

## 1. Introduction

Inflammatory bowel diseases [IBDs] are most prevalent in developed countries, affecting around one in 250 individuals. Their incidence in developing countries acquiring a Western lifestyle is rising.<sup>1</sup> However, the aetiology of IBD is not yet fully understood.<sup>2</sup> Based on clinical and pathological features, IBD can be divided into two main clinical entities: Crohn's disease [CD] and ulcerative colitis [UC]. The latter is locally limited to the colon and rectum and characterized by a diffuse mucosal inflammation. In contrast, CD can affect the whole gastrointestinal tract from the mouth to the rectum with transmural inflammation.<sup>3,4</sup> Environmental factors, genetic predisposition, dysfunction of the intestinal mucosal barrier and dysregulation of immune responses are involved in the manifestation of IBD.<sup>5,6</sup>

Autoimmune responses are considered as a part of IBD inflammation. Disease-specific autoantibodies [autoAbs] against neutrophils,<sup>7,8</sup> intestinal goblet cells<sup>9,10</sup> and exocrine pancreas<sup>11</sup> have been reported. Of note, the pancreatic major zymogen membrane glycoprotein 2 [GP2] was identified as the first autoantigenic target located at the site of CD inflammation<sup>11</sup> and exerting modulation of innate and acquired immune responses in CD.<sup>12,13</sup> Anti-neutrophil cytoplasmic antibodies [ANCA] and especially perinuclear ANCA [pANCA] have a prevalence of up to 70% in UC and up to 10% in CD patients.<sup>14</sup> Of interest, elevated levels of autoAbs to neutrophil proteinase 3 [PR3] detected by highly sensitive immunoassays have been reported in UC recently.<sup>15,16</sup> Thus, loss of tolerance to neutrophil antigens may play a pathophysiological role in IBD.

Neutrophils contribute to the first line of host defense, but are also involved in IBD inflammatory processes characterized by a concomitant dysregulated microbiota.<sup>17</sup> Phagocytosis and production of reactive oxygen species by neutrophils are defective in IBD

patients.<sup>18,19</sup> This can lead to bacterial accumulation and continuous recruitment of neutrophils to the inflamed mucosa. Thus, neutrophils may play an important role in the onset or perpetuation of IBD inflammation.

The aim of this study was to identify novel neutrophil autoantigenic targets possibly involved in sensing the intestinal microbiota as well as IBD inflammation. For the first time, we provide evidence that chitinase-3-like protein 1 [CHI3L1] overexpressed in enterocytes during inflammation of the large bowel and to support the uptake of pathogenic intestinal bacteria is a novel neutrophil autoantigenic target in CD.

## 2. Methods

### 2.1. Patient samples

In total, 331 patient samples were collected at the Pediatric Clinic [Technical University of Dresden, Germany]. Samples included 110 sera of patients with CD, 95 with UC and 126 with coeliac disease [CeD] [Table 1]. The diagnosis of IBD was established according to the Porto criteria of the IBD Working Group of the European Society for Pediatric Gastroenterology Hepatology and Nutrition and was based on clinical, radiological, endoscopic and histological evaluation. For serological analysis, serum samples taken around the date of diagnosis were used and retrospectively investigated. For the description of disease localization, disease behaviour and age of manifestation of our paediatric IBD cohort, we used the Montreal classification of IBD with its paediatric modification, the Paris classification [Table 2].

Eighty-six healthy controls [HCs] were obtained from in.vent Diagnostica [Hennigsdorf, Germany]. Additionally, eight serum samples with high ANCA titres [ $>1:320$ ] from patients with IBD were used for protein identification.

**Table 1.** Demographic data of the study population

	<i>n</i>	Male/female [%]	Median age, years [Q1; Q3]	Median CRP, mg/l [Q1; Q3]
CD	110	68/42 [61.5/38.5]	13 [10; 15]	22.7 [8.2; 50.8]
UC	95	43/52 [45.3/54.7]	14 [10; 15]	3.7 [1; 11]
CeD	126	46/80 [36.5/63.5]	8 [3; 14]	N/A
HC	86	52/34 [60.6/39.4]	28 [21; 41]	N/A

CRP, C-reactive protein; Q, quartile; CD, Crohn's disease; UC, ulcerative colitis; CeD, coeliac disease; HC, healthy controls.

**Table 2.** Characteristics of inflammatory bowel disease patients according to Paris classification

	CD [ <i>n</i> = 110]		UC [ <i>n</i> = 95]
<b>Age at diagnosis, <i>n</i> [%]</b>			
Below 10 years [A1a]	24 [21.8]		17 [17.9]
10–17 years [A1b]	84 [76.4]		69 [72.6]
17–40 years [A2]	1 [0.9]		9 [9.5]
Above 40 years [A3]	0		0
<b>Location, <i>n</i> [%]</b>			
Ileal [L1]	17 [15.5]	<b>Extent</b>	
Colonic [L2]	13 [11.8]	Proctitis [E1]	5 [5.3]
Ileocolonic [L3]	80 [72.7]	Left-sided UC [E2]	13 [13.7]
Upper disease, modifier [L4]	N/A	Extensive [E3]	7 [7.4]
<b>Behaviour, <i>n</i> [%]</b>			
Non-stricturing, non-penetrating [B1]	59 [53.6]	Pancolitis [E4]	70 [73.7]
Stricturing [B2]	36 [32.7]		
Penetrating [B3]	2 [1.8]		
Stricturing and penetrating [B2+B3]	13 [11.8]		
BMI – median, kg/m <sup>2</sup> [Q1/Q3]	16.5[14.7/18.2]		N/A

Location and behaviour of CD is defined as: L1, ileal involvement; L2, colonic involvement; L3, ileocolonic involvement; B1, non-stricturing and non-penetrating manifestation; B2, structuring manifestation [stenosis]; B3, penetrating manifestation; B2+B3, stricturing and penetrating manifestation. Extent of UC is defined as: E1, ulcerative proctitis; E2, left-sided UC [distal to splenic flexure]; E3, extensive [hepatic flexure distally]; E4, pancolitis [proximal to hepatic flexure]. BMI, body mass index; Q, quartile; N/A, not available.

## 2.2. Isolation of human neutrophils

Neutrophils were isolated as described previously.<sup>20</sup> In brief, anticoagulated blood [K2-EDTA] was layered over an equal amount of PolymorphPrep [Axis Shield] and centrifuged at 480 g for 30 min. The leukocyte band was harvested, resuspended in phosphate buffered saline with 0.2% bovine serum albumin [PBS-BSA] and centrifuged at 480 g for 10 min. Contaminating red blood cells were removed by adding lysing solution, 2 min of incubation at room temperature and centrifugation as before. Cells were washed twice with PBS-BSA and finally resuspended in PBS-BSA.

## 2.3. Two-dimensional electrophoresis and immunoblotting

Neutrophil proteins were extracted by sonication [pulse: 1 s, brake: 20 s, amplitude: 45%, on ice, Bandelin Sonoplus; Bandelin Electronic] in lysis buffer [50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5 mM EDTA, Protease Inhibitor Cocktail] with subsequent centrifugation for pelleting cell debris as described elsewhere.<sup>21</sup> Following acetone precipitation, neutrophil proteins were separated by two-dimensional gel electrophoresis [2DE] using isoelectric focusing [IEF] dry strips [Immobiline DryStrips pH 3–10], Ettan IPGphor 3 IEF System [GE Healthcare] and followed by vertical electrophoresis with the PerfectBlue Gel System Mini L [VWR].<sup>22</sup> Semi-dry blotting to PVDF membranes [Roth] was performed with samples for immunoblotting, followed by blocking with 5% skimmed milk powder in PBS and 0.1% Tween-20 [PBST]. Membranes were incubated with serum samples diluted 1:100 in 2% skimmed milk powder in PBST for 1 h, washed with PBST and subsequently incubated with horseradish peroxidase-conjugated anti-human immunoglobulin G [IgG]. Reactive spots were analysed with a UV-transilluminator [BioDocAnalyze, Biometra] by enhanced chemiluminescence [ECL].

For spot excision and protein identification, separate 2DE gels were performed and visualized by staining with Coomassie Brilliant Blue R250 [Roth].<sup>23</sup>

## 2.4. Protein identification using MALDI TOF-MS

Protein spots that displayed Western blot signals were excised from Coomassie-stained 2D gels and subjected to in-gel tryptic digestion as described elsewhere.<sup>21</sup> Proteins were identified using matrix-assisted laser desorption ionization time of flight mass spectrometry [MALDI TOF MS/MS; Ultraflex III TOF/TOF, Bruker Daltonics] as described.<sup>21</sup>

## 2.5. Expression of recombinant CHI3L1

For the recombinant expression of human CHI3L1, the Gateway Technology [Invitrogen] was used. In brief, primers flanking the full-length cDNA of CHI3L1 [accession number NM\_001276.2], adding a C-terminal polyhistidine-tag and Gateway recombination sites were designed to amplify CHI3L1 cDNA from the human intestinal cell line CaCo-2. The amplification product was further processed according to the manufacturer's guidelines. The recombinant vector pDEST8\_CHI3L1 was used to transform *Escherichia coli* DH10Bac generating recombinant bacmid DNA, which was used to transfect cultured Sf9 [*Spodoptera frugiperda*] insect cells to produce recombinant baculovirus expressing CHI3L1. The protein was harvested from the supernatant and purified by Ni-chelate chromatography and dialysed against 20 mM sodium phosphate buffer [pH 7.5] following sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE] and Coomassie staining to confirm the purity of CHI3L1.

## 2.6. Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay [ELISA] technique was used for detection of CHI3L1 autoAbs in serum samples by using recombinant human CHI3L1 as solid-phase antigen. Briefly, 2 µg/ml CHI3L1, diluted in bicarbonate buffer [pH 9.5], was coated on microtitre plates [Greiner Bio-one] overnight at 4°C. After blocking with 2% bovine serum albumin at room temperature for 1 h, the plates were incubated with serum samples [diluted 1:100 in sample buffer] for 1 h at room temperature. After washing, horseradish

peroxidase-conjugated anti-human IgA, IgG [Dianova] or anti-human IgA secretory component [antibodies-online] were added. Plates were developed with ready-to-use TMB substrate [Seramun Diagnostik] and stopped with 0.25 M sulphuric acid after 10 min. The optical density [OD] was read at 450 nm/620 nm in a microplate reader [Sunrise, Tecan Trading].

### 2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.00 for Windows [GraphPad Software]. Data were tested for normality by a Kolmogorov–Smirnov test. Statistically significant differences were determined by two-tailed, non-parametric Mann–Whitney U test for independent samples. Levels of significance of differences in prevalence were calculated in contingency tables by a two-tailed Fisher's exact test. The median and 95% confidence interval [CI] were used to express the measured data. On the basis of a significance level of 0.05, the Bonferroni correction was used to account for multiple comparison problems. MedCalc software version 12.4.0 [MedCalc] was used to perform receiver operating characteristic [ROC] curve analysis.

## 3. Results

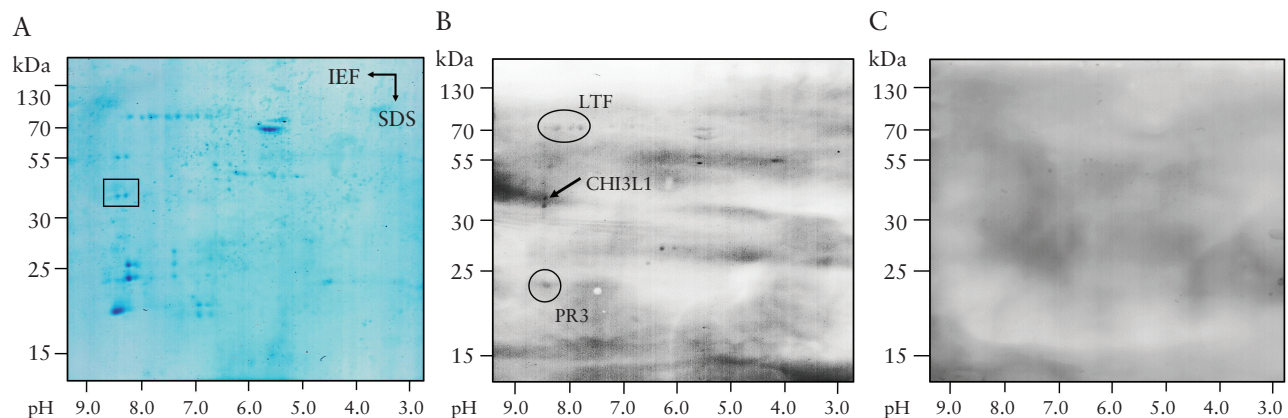
### 3.1. Identification of CHI3L1 as an autoantigenic target in neutrophils

Neutrophils isolated from the blood of three healthy donors were lysed and neutrophil proteins separated by 2DE [Figure 1A]. Separated 2DE gels were semi-dry blotted onto PVDF membranes and probed with ANCA-positive sera of patients diagnosed with IBD. Reactive spots were detected by ECL after incubation with peroxidase-conjugated human IgG [Figure 1B]. Among other known neutrophil antigenic targets such as PR3 or lactoferrin a reactive spot with an apparent molecular weight of 40 kDa and an isoelectric point of pH 8.5 was detected in four of eight high-titre ANCA sera. There was no reactivity when tested with control sera from healthy donors [Figure 1C]. Subsequent MALDI-TOF MS and database searches identified the reactive spot as CHI3L1 protein with a MOWSE [molecular weight search] score of 110 and sequence coverage of 37%.

### 3.2. Immunoglobulin reactivity to CHI3L1 of IBD sera by ELISA

Since CHI3L1 is expressed in colonic epithelial cells, the CaCo-2 cell line was used to amplify CHI3L1 cDNA for subsequent generation of recombinant baculovirus and expression of CHI3L1 in Sf9 cells. To reveal possible variations due to differences in post-translational glycosylation, the reactivity of the protein produced recombinantly in insect cells was compared with commercially available protein produced in human cells [Sino Biological, Cat. No. 11227-H08H] by means of ELISA. However, there was no difference of CHI3L1 reactivity to healthy controls or disease groups [Supplementary Figure 1], suggesting a comparable glycosylation and structural similarity among epitopes. Purified recombinant CHI3L1 was used as solid-phase antigen for the detection of CHI3L1 autoAbs in patient and control samples. The level of CHI3L1 autoAbs was read as the ratio of  $OD_{\text{sample}}/OD_{\text{cutoff}}$  [ $OD_{\text{cutoff}}$ , 3 sigma of HC times OD of internal calibration serum] for standardization. Sera from patients with CD showed higher IgG reactivity to CHI3L1 [median ratio, 0.532; 95% CI, 0.510–0.610] than sera from HC [median ratio, 0.468; 95% CI, 0.396–0.540;  $p = 0.0196$ ; Figure 2A]. Compared to patients with either UC [median ratio, 0.412; 95% CI, 0.382–0.451] or CeD [median ratio, 0.287; 95% CI, 0.260–0.304], IgG reactivity to CHI3L1 was significantly higher [both  $p < 0.0001$ ]. In contrast, IgG reactivity to CHI3L1 of UC and CeD patient sera was lower than in HC [ $p = 0.0155$  and  $p < 0.0001$ , respectively].

The same relationship was found when IgA reactivity to CHI3L1 was analysed in different patient cohorts. There was a significantly higher IgA reactivity in CD [median ratio, 0.658; 95% CI, 0.566–0.755] than in HC [median ratio, 0.384; 95% CI, 0.326–0.444;  $p < 0.0001$ ], UC [median ratio, 0.360; 95% CI, 0.307–0.408;  $p < 0.0001$ ] and CeD patients [median ratio, 0.294; 95% CI, 0.249–0.359;  $p < 0.0001$ , Figure 2B]. In addition, secretory IgA [sIgA] reactivity was the highest in CD patients [median ratio, 0.798; 95% CI, 0.689–1.027] and differed significantly from UC [median ratio, 0.516; 95% CI, 0.384–0.641;  $p < 0.0001$ ], CeD [median ratio, 0.424; 95% CI, 0.377–0.490;  $p < 0.0001$ ] and HC sera [median ratio, 0.437; 95% CI, 0.350–0.492;  $p < 0.0001$ ; Figure 2C, Supplementary Table 1].



**Figure 1.** Two-dimensional electrophoresis of neutrophil lysates. In the first dimension, proteins were separated by isoelectric focusing followed by SDS-PAGE in the second dimension and subsequently stained with Coomassie blue [A]. The rectangle marks the spot with an apparent isoelectric point of pH 8.5 and molecular weight of 40 kDa identified as chitinase-3-like protein 1 protein by MALDI-TOF mass spectrometry. Immunoblotting of neutrophil proteins separated by two-dimensional electrophoresis and incubated with patient sera [B] or sera from healthy controls [C] followed by enhanced chemiluminescence detection after incubation with peroxidase-conjugated IgG. Arrow marks the reactive spot that was identified as CHI3L1. Circles mark the known neutrophil antigenic targets proteinase 3 [PR3] and lactoferrin [LTF].



### 3.3. Prevalence of positive CHI3L1 autoAbs in IBD

After having determined cut-off values, we found 9.1% [10/110] of CD, none of UC, 2.4% [3/126] of CeD and 2.3% [2/86] of HC sera positive for IgG to CHI3L1, and there was no significant difference when comparing different disease groups and the HC group [Table 3 Supplementary Table 1]. There was a trend of higher prevalence in CD patients, which was significant when compared to UC patients [ $p = 0.002$ ]. We found a significantly higher prevalence of positive IgA and sIgA to CHI3L1 in patients with CD when compared to HC [28/110 vs 2/86,  $p < 0.0001$ ; 46/110 vs 4/86,  $p < 0.0001$ ]. In contrast, there was no significantly higher prevalence of these CHI3L1-reactive Ig classes in CeD patients and only a barely significantly elevated prevalence of positive sIgA to CHI3L1 in UC patients [17/95 vs 4/86;  $p = 0.0055$ ]. When comparing disease groups, we found a higher prevalence of positive IgA and sIgA to CHI3L1 in CD than in UC [both  $p < 0.0001$ ] or CeD patients [ $p = 0.0034$  and  $p = 0.0002$ , respectively] [Supplementary Table 1].

### 3.4. Diagnostic relevance of CHI3L1 autoAbs

ROC curve analysis was used to determine general cut-off values and to evaluate the diagnostic potential of CHI3L1 autoAbs, applying different clinical scenarios. For the identification of CD among healthy subjects, we performed ROC analysis with CD [ $n = 110$ ] vs healthy blood donors [negative criterion; HC;  $n = 86$ ] Figure 3A. The assay was performed with disparate outcomes when using anti-human IgG, IgA or sIgA, with area under the curve [AUC] values

between 0.597 and 0.781, and with better outcomes with the sIgA CHI3L1 autoAbs [Table 4].

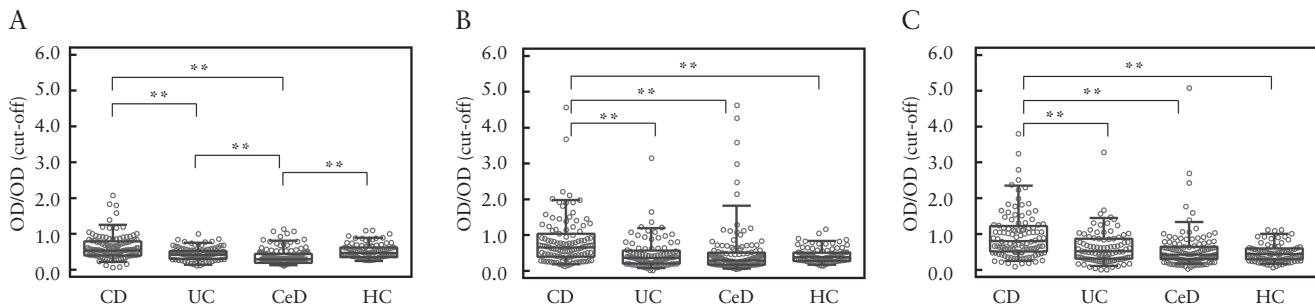
AUC values ranging from 0.694 to 0.712 were achieved for differentiation of CD vs UC [negative criterion;  $n = 95$ ] with best diagnostic utility for IgA [AUC 0.712] Figure 3B. However, the cut-off values at 95% specificity were higher than calculated for the identification of CD among healthy individuals.

In addition, we performed ROC analysis for CD vs disease and healthy controls [negative criterion; UC = 95, CeD = 126 and HC = 86] with AUC values ranging from 0.729 to 0.748. For this scenario, the assay performs best with IgG CHI3L1 autoAbs [AUC 0.748]. Similar or higher cut-off values at 95% specificity than for the differentiation of CD and UC were calculated [Supplementary Table 2, Supplementary Figure 2].

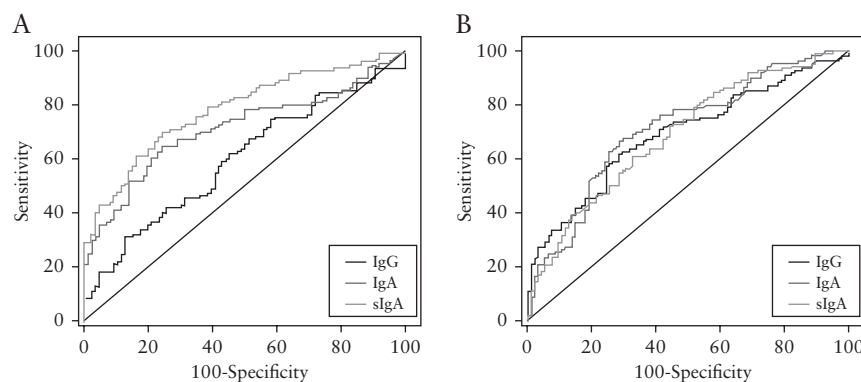
Furthermore, Ig reactivities were compared with other clinical parameters such as C-reactive protein [CRP] and anti-*Saccharomyces cerevisiae* antibodies [ASCA] [Table 5]. There was no correlation of Ig reactivity to CHI3L1 with CRP or ASCAs in CD, UC and CeD patients.

### 3.5. Association of CHI3L1 autoAbs with the localization of inflammation in CD patients

When comparing patients with ileal involvement [L1,  $n = 17$ ] and colonic involvement [L2,  $n = 13$ ], there was no significant difference but a trend towards higher reactivity to CHI3L1 of all Ig subclasses investigated in L1 patients [IgG,  $p = 0.2095$ ; IgA,  $p = 0.1806$ ; sIgA,



**Figure 2.** Median ratio of  $OD_{sample}/OD_{cutoff}$  of anti-CHI3L1 IgG, IgA and sIgA. [A] IgG reactivity to CHI3L1 was significantly higher in CD patients than in UC [ $p < 0.0001$ ], CeD [ $p < 0.0001$ ] and HC group sera [ $p < 0.05$ ]. UC and CeD group showed significantly lower IgG reactivity to CHI3L1 compared to HCs [ $p < 0.05$ ;  $p < 0.0001$ ]. There is a significantly higher IgA [B] and secretory component [C] reactivity to CHI3L1 in CD patients [ $p < 0.0001$ ] compared to UC, CeD and control groups. OD, optical density; CD, Crohn's disease; UC, ulcerative colitis; CeD, coeliac disease; HC, healthy control; \*\*  $p < 0.0001$ .



**Figure 3.** Receiver operating characteristic curve analysis of CHI3L1 autoAbs. [A] CD vs healthy controls. Disease group [positive criterion]: 110 patients with CD, control group [negative criterion]: 86 blood donors. [B] CD vs UC. Disease group [positive criterion]: 110 patients with CD, control group [negative criterion]: 95 patients with UC. CD, Crohn's disease; UC, ulcerative colitis.

**Table 3.** Prevalence of CHI3L1-reactive IgG, IgA and sIgA in patients with Crohn's disease, ulcerative colitis, coeliac disease and healthy controls determined by ELISA

	Patients [n]	IgG [n]	p	IgA [n]	p	sIgA [n]	p
CD	110	10 [9.1%]	0.070	28 [25.5%]	<0.0001	46 [41.8%]	<0.0001
UC	95	0	0.224	9 [9.5%]	0.061	17 [17.9%]	0.006
CeD	126	3 [2.4%]	1	12 [9.5%]	0.094	11 [8.7%]	0.309
HC	86	2 [2.3%]		2 [2.3%]		4 [4.7%]	

*p*-values were determined by two-tailed Fisher's exact test and describe differences in the prevalence of CHI3L1 autoAbs of disease groups compared to healthy controls. CHI3L1, chitinase-3-like protein 1; IgG, IgA and sIgA, immunoglobulin G, A and secretory component of IgA, respectively; ELISA, enzyme-linked immunosorbent assay; CD, Crohn's disease; UC, ulcerative colitis; CeD, coeliac disease; HC, healthy control.

**Table 4.** Cutoffs and performance for antibodies against CHI3L1 according to ROC curves for different clinical scenarios

	IgG	IgA	sIgA
<b>Identification of CD [CD vs HC]</b>			
Area under the ROC curve [AUC]	0.597	0.711	0.781
Standard error	0.0405	0.0369	0.0324
95% confidence interval	0.525–0.666	0.642–0.773	0.717–0.837
Cut-off at 95% specificity	>0.8626	>0.8123	>0.9567
Corresponding sensitivity at 95% specificity	18.18	35.45	42.73
95% Confidence interval	88.5–98.7	88.5–98.7	88.5–98.7
positive LR	3.91	7.62	9.19
negative LR	0.86	0.68	0.6
PPV	28.1	72.3	86.8
95% Confidence interval	12.2–52.4	49.2–87.5	71.2–94.6
NPV	92.1	81.2	69.9
95% Confidence interval	91.3–92.8	78.9–83.3	66.2–73.3
<b>Differentiation between CD and UC</b>			
Area under the ROC curve [AUC]	0.694	0.712	0.69
Standard error	0.0366	0.0362	0.0367
95% Confidence interval	0.626–0.756	0.645–0.773	0.622–0.753
Cut-off at 95% specificity	>0.7404	>1.1036	>1.3315
Corresponding sensitivity at 95% specificity	29.09	23.64	20.91
95% Confidence interval	88.1–98.3	88.1–98.3	88.1–98.3
positive LR	5.53	4.49	3.97
negative LR	0.75	0.81	0.83
PPV	35.6	60.6	74.0
95% Confidence interval	18.3–57.7	38.1–79.4	53.0–87.8
NPV	93.0	78.4	62.5
95% Confidence interval	92.1–93.8	76.4–80.2	60.0–65.0

For the identification of patients with CD among healthy controls, 110 CD patients [positive criterion] and 86 blood donors [negative criterion] were analysed. For the differentiation of patients with CD and UC, 110 CD patients [positive criterion] and 95 UC patients [negative criterion] were analysed. ROC, receiver operating characteristic; IgG, IgA, sIgA, immunoglobulins G, A and secretory IgA; LR, likelihood ratio; PPV, positive predictive value; NPV, negative predictive value.

$p = 0.2415$ ]. Independent of Ig type, there was no significant difference in Ig reactivity when comparing L1 and patients with ileocolonic involvement [L3,  $n = 80$ ] [IgG,  $p = 0.6097$ ; IgA,  $p = 0.3397$ ; sIgA,  $p = 0.8461$ ]. However, there was a higher IgG reactivity to CHI3L1 in L3 compared to L2 patients [ $p = 0.0250$ ] but only a trend for higher IgA and sIgA [ $p = 0.0856$  and  $0.0586$ , respectively] [Figure 4, Table 6].

Independent on anti-CHI3L1 Ig type, we found a higher prevalence in patients with both ileal and ileocolonic involvement, but the trends did not reach significance for either anti-CHI3L1 IgG, anti-CHI3L1 IgA or sIgA. Nevertheless, the highest prevalence of anti-CHI3L1 IgG and sIgA was found in L3 patients [11.3% and 46.3%, respectively], whereas L1 patients displayed the highest prevalence of anti-CHI3L1 IgA [29.4%] [Table 7, Supplementary Table 1].

### 3.6. Association of CHI3L1 autoAbs with disease behaviour in CD patients

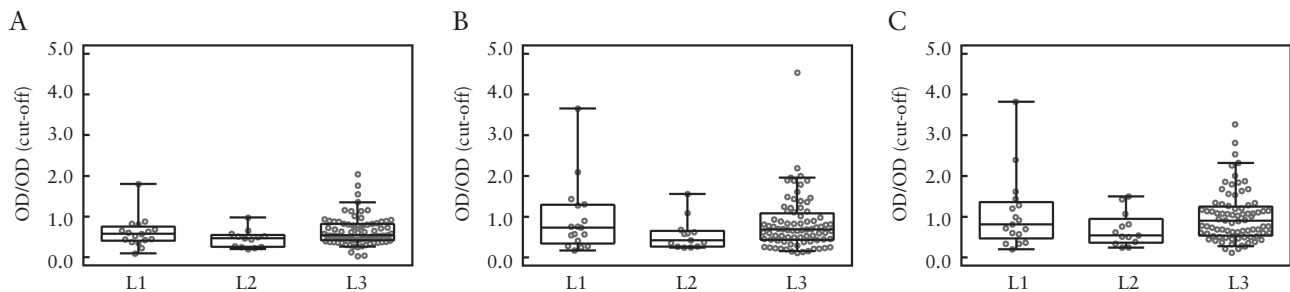
We found a trend of lower IgG reactivity to CHI3L1 in patients with the most severe manifestation [B2+B3, stricturing and penetrating;  $n = 13$ ] when compared to B2 patients [stricturing;  $n = 36$ ] but the trend did not reach significance [ $p = 0.3687$ ] [Figure 5A, Table 6]. There was a trend of higher IgA and sIgA reactivity to CHI3L1 in patients with more severe disease manifestation, but here as well the trends did not reach significance [Figure 5B and C, Table 6, Supplementary Table 1].

In accordance, the prevalence of CHI3L1 autoAbs was not significantly different, but there was a trend of higher prevalence of anti-CHI3L1 IgA and sIgA [38.5% and 53.8%, respectively] in B2+B3 patients compared with the low number of cases in B3 patients [Table 7, Supplementary Table 1].

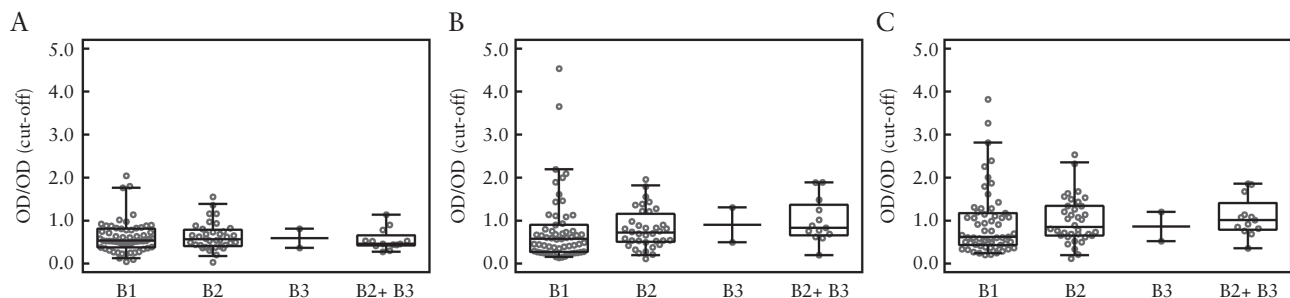
**Table 5.** Prevalence of CHI3L1-reactive IgG, IgA and sIgA in ASCA-positive and negative study patients

	<i>n</i>	aCHI3L1 IgG		aCHI3L1 IgA		aCHI3L1 sIgA	
		<i>n</i> [%]	<i>p</i>	<i>n</i> [%]	<i>p</i>	<i>n</i> [%]	<i>p</i>
CD		10		28		46	
ASCA+	50	7 [14.0]	0.1823	13 [26.0]	0.8279	23 [46]	0.4377
ASCA-	58	3 [5.2]		14 [24.1]		22 [37.9]	
ND	2	0		1 [2.1]		1 [2.1]	
UC		0		9		17	
ASCA+	5	0	1	1 [20.0]	0.3827	1 [20.0]	1
ASCA-	84	0		7 [8.3]		15 [17.9]	
ND	6	0		1 [16.7]		1 [16.7]	
CeD		3		12		11	
ASCA+	7	0	1	4 [57.1]	0.0015	4 [57.1]	0.001
ASCA-	119	3 [2.5]		8 [6.7]		7 [5.9]	

*p*-values were determined by two-tailed Fisher's exact test and describe differences in the prevalence of CHI3L1 autoAbs of ASCA-positive patient groups and ASCA-negative patients of the same group. ASCA, anti-*Saccharomyces cerevisiae* antibody; aCHI3L1 IgG/IgA/sIgA, immunoglobulin G, A and secretory component of IgA to chitinase-3 like 1 protein; ND, not determined.



**Figure 4.** Anti-CHI3L1 IgG, IgA and sIgA in patients with Crohn's disease according to disease localization. The median ratio of  $OD_{\text{sample}}/OD_{\text{cutoff}}$  of anti-CHI3L1 IgG [A], anti-CHI3L1 IgA [B] and anti-CHI3L1 sIgA [C]. There is a trend of higher reactivity of all immunoglobulins to CHI3L1 in patients with ileal [L1] and ileocolonic [L3] involvement in comparison to patients with solely colonic [L2] involvement. Disease localization is described according to the Paris classification and is defined as L1, ileal involvement; L2, colonic involvement; L3, ileocolonic involvement. OD, optical density.



**Figure 5.** Anti-CHI3L1 IgG, IgA and sIgA according to the behaviour of Crohn's disease. The median ratio of  $OD_{\text{sample}}/OD_{\text{cutoff}}$  of anti-CHI3L1 IgG [A] showed no significant differences when analysed according to disease behaviour, but showed a trend of lower reactivity in the most severe manifestation [B2+B3]. There was a trend of higher anti-CHI3L1 IgA [B] and anti-CHI3L1 sIgA [C] reactivity in the more severe manifestations of Crohn's disease [B2, B3 and B2+B3] when compared to mild manifestation [B1]. OD, optical density; B1, non-stricturing and non-penetrating manifestation; B2, stricturing manifestation [stenosis]; B3, penetrating manifestation; B2+B3, stricturing and penetrating manifestation.

#### 4. Discussion

Numerous efforts have been made to investigate the role of CHI3L1 as a possible biomarker for various diseases, including IBD.<sup>24</sup> However, research has focused mainly on the assessment of CHI3L1 as an analyte in patient material. This study demonstrates, for the first time, that CHI3L1 is an autoantigenic target in IBD as patients

with CD demonstrate significantly elevated levels of CHI3L1 autoAbs compared to patients with UC or CeD or healthy controls.

In contrast to other 18-glycosylhydrolase family members, CHI3L1, also referred to as HC gp-39 or YKL-40, lacks chitinase activity due to an amino acid change in the catalytic region.<sup>25,26</sup> Nevertheless, CHI3L1 binds to chitooligosaccharides with high affinity. It was suggested that

**Table 6.** Median ratio of OD<sub>sample</sub>/OD<sub>cutoff</sub> and 95% confidence interval of IgG, IgA and sIgA reactivity to CHI3L1 of Crohn's disease sera according to disease localization and behaviour as determined by ELISA and Mann-Whitney U test

	IgG		IgA		sIgA	
	OD	95% CI	OD	95% CI	OD	95% CI
L1	0.57	0.43–0.69	0.73	0.41–1.28	0.81	0.57–1.28
L2	0.47	0.25–0.58	0.424	0.26–0.68	0.54	0.34–1.07
L3	0.54	0.52–0.69	0.68	0.54–0.81	0.90	0.69–1.08
B1	0.53	0.51–0.63	0.58	0.41–0.68	0.62	0.51–0.99
B2	0.56	0.44–0.69	0.72	0.54–0.89	0.85	0.69–1.21
B3	0.59	0.38–0.81	0.90	0.49–1.31	0.86	0.52–1.20
B2+B3	0.46	0.42–0.78	0.83	0.62–1.49	1.01	0.76–1.68

OD, optical density; CI, confidence interval; IgG, IgA and sIgA, immunoglobulin G, A and secretory component of IgA, respectively; ELISA, enzyme-linked immunosorbent assay; L1, ileal involvement; L2, colonic involvement; L3, ileocolonic involvement; B1, non-stricturing and non-penetrating manifestation; B2, stricturing manifestation [stenosis]; B3, penetrating manifestation; B2+B3, stricturing and penetrating manifestation.

**Table 7.** Prevalence of CHI3L1-reactive IgG, IgA and sIgA in patients with Crohn's disease according to disease localization and behaviour determined by ELISA

	Patients [n]	IgG [n]	IgA [n]	sIgA [n]
Ileal [L1]	17	1 [5.9%]	5 [29.4%]	6 [35.3%]
Colonic [L2]	13	0	2 [15.4%]	3 [23.1%]
Ileocolonic [L3]	80	9 [11.3%]	21 [26.3%]	37 [46.3%]
Non-stricturing, non-penetrating [B1]	59	5 [8.5%]	13 [22.0%]	21 [35.6%]
Stricturing [B2]	36	4 [11.1%]	9 [25.0%]	17 [47.2%]
Penetrating [B3]	2	0	1 [50%]	1 [50.0%]
Stricturing and penetrating [B2+B3]	13	1 [7.7%]	5 [38.5%]	7 [53.8%]

Differences of the prevalence of CHI3L1 autoAbs were not significant [*p*-values 0.1655–1]. IgG, IgA and sIgA, immunoglobulin G, A and secretory component of IgA, respectively; ELISA, enzyme-linked immunosorbent assay; L1, ileal involvement; L2, colonic involvement; L3, ileocolonic involvement; B1, non-stricturing and non-penetrating manifestation; B2, structuring manifestation [stenosis]; B3, penetrating manifestation; B2+B3, stricturing and penetrating manifestation.

CHI3L1 may play a role in the innate immune system by responding to chitinous pathogens such as fungi and nematodes.<sup>27</sup> Besides being expressed in synovial cells, chondrocytes<sup>28,29</sup> and smooth muscle cells,<sup>30</sup> CHI3L1 can be found in macrophages, neutrophils<sup>25,31</sup> and colonic epithelial cells.<sup>32</sup> Thus, the latter sites of expression may support an immune function of CHI3L1 in the intestine.

The group of Mizoguchi demonstrated an up-regulated expression of CHI3L1 in lamina propria and colonic epithelial cells [CECs] of UC and CD patients as well as in a murine colitis model.<sup>32</sup> This upregulation enhances adhesion and invasion of *Salmonella* Typhimurium and adherent-invasive *E. coli* [AIEC] but not of non-pathogenic *E. coli* [DH5 $\alpha$ ] in CECs. Thus, CHI3L1 may act as a pathogenic mediator in acute colitis. In addition, it was shown that CHI3L1 could bind to chitin binding protein 21 [CBP21], which is expressed by *Serratia marcescens*.<sup>33</sup> In potentially pathogenic *E. coli*, a corresponding CBP21 homologue, ChiA, could be identified. ChiA has a unique amino acid motif in its chitin-binding domain, through which N-glycosylated CHI3L1 promotes the adhesion of AIECs to CECs.<sup>32–34</sup> This possibly CHI3L1-mediated, enhanced bacterial adhesion and infiltration of CECs could, in turn, be responsible for abscess development seen in patients with CD. These findings would be in accordance with our observation concerning the prevalence of CHI3L1 autoAbs in the more severe cases of CD. With respect to the low overall number of B2, B2+B3 and especially of B3 patients, we found a trend of higher CHI3L1 IgA reactivity in these patients. Later, it was shown that the carbohydrate [chitin] binding motif

[CBM] of CHI3L1, which is essential for the interaction with chitin-containing structures, activates protein kinase B [Akt] signalling. Akt signalling thus increases the secretion of pro-inflammatory cytokines tumour necrosis factor  $\alpha$  [TNF- $\alpha$ ] and interleukin 8 [IL-8] in the CECs,<sup>35</sup> which trigger neutrophil recruitment, accumulation and activation.<sup>36–38</sup> Additionally, TNF- $\alpha$  and IL-8 can also be induced by AIECs and mucosa-associated *E. coli*, respectively.<sup>39</sup>

In neutrophils, CHI3L1 is stored in specific granules and upon neutrophil recruitment and activation, CHI3L1 is co-mobilized with lactoferrin, a known antigenic target of atypical ANCA, frequently found in IBD patients. Of note, the tolerance break against CHI3L1 is more pronounced at the mucosal level with a higher frequency of sIgA reactivity to CHI3L1 in contrast to IgG. This might explain the difference to ANCA, which is an IgG reactivity to neutrophil targets and in general reported to be characteristic for patients with UC. Thus, CHI3L1 is probably not the main autoantigenic target of ANCA occurring in such patients.

During the acute phase of inflammation, neutrophils migrate to the site of inflammation and serve as a first line of immune response. A defective neutrophil regulation in IBD is thought to play an important role in the onset or manifestation of IBD, because it could lead to damage of the intestinal mucosal barrier by infiltration of neutrophils in the inflamed mucosa and the accumulation of pathogens (e.g. bacteria), which are not phagocytosed by neutrophils.<sup>18,19</sup>

Like neutrophils in the context of innate immune responses, IgA as an acquired immune response is involved in the defence of



the intestinal epithelium. Both may protect the intestinal epithelium from enteric toxins and pathogenic microorganisms in a non-inflammatory manner. In brief, IgA dimers secreted by intestinal B cells can be actively translocated to the epithelial cell surface as sIgA. Consequently, sIgA could interact with dietary antigens and microorganisms, reduce pro-inflammatory bacterial epitope expression, modulate attachment of microbes to epithelial cells and induce intra-epithelial neutralization of inflammatory products or pathogens.<sup>40,41</sup> Interestingly, IgA immune complexes trigger the IgA Fc receptor [FcαRI] on neutrophils, and thus it may initiate pro-inflammatory cellular processes.<sup>42</sup> Although this may explain the tolerance breach to CHI3L1 and the occurrence of IgA autoAbs to this particular target, the exact role and underlying mechanisms of CHI3L1 in the pathogenesis of IBD remain unclear.

Interestingly, the role of CHI3L1 in inflammatory conditions was first described in patients with rheumatoid arthritis, where elevated serum CHI3L1 levels were reported in active disease and additionally correlated with two other pro-inflammatory markers, IL-6 and CRP. In addition, an increased tissue expression was found in chondrocytes of arthritic cartilage as well as in lining and stromal cells [macrophages] in the synovium. Until now, rheumatoid arthritis was the only chronic inflammatory condition reportedly displaying autoantibodies to CHI3L1.<sup>43-46</sup>

Furthermore, CHI3L1 autoAbs may also be involved in the development of strictures and fistulas in patients with CD, as increased serum CHI3L1 was associated with stricture formation.<sup>47</sup> This might be underlined by the fact that CHI3L1 was reported to play a role in tissue remodelling and cell migration and adhesion in different cell types. In fact, CHI3L1 was shown to act as a mitogen or growth factor in, for example, chondrocytes and synovial cells, stimulating tissue remodelling by interaction with insulin-like growth factor 1 [IGF-1]. Additionally, it acts as a chemoattractant in vascular endothelial cells [HUVECs], stimulating cell migration and adhesion.<sup>30,48-51</sup>

In general, our observation is in accordance with other work, demonstrating that serum CHI3L1 levels correlate with disease activity in IBD patients.<sup>52</sup> About half of CD patients show increased serum CHI3L1, and these levels do not decrease when the disease becomes inactive. In contrast, although half of UC patients also display higher CHI3L1, these levels drop when the disease becomes inactive and correlate with the simple clinical colitis activity index [SCCAI].<sup>52,53</sup> A persistently high CHI3L1 level could increase the risk for a tolerance break in CD, resulting in the formation of autoAbs to CHI3L1 in these patients. In contrast, this tolerance break might not be seen in UC, due to disease activity-dependent variations in CHI3L1 levels. However, the difference of stable and varying CHI3L1 serum levels in IBD could explain the higher prevalence of IgA and sIgA CHI3L1 autoAbs in patients with CD compared to UC patients. In this context, IgA and/or sIgA CHI3L1 autoAbs could be a new marker for the differentiation of IBD.

Serum CHI3L1 may not be suitable for the differentiation of CD and UC and monitoring disease activity, but like CRP, faecal calprotectin and faecal lactoferrin, faecal CHI3L1 could be used to observe disease activity.<sup>54-56</sup> Faecal CHI3L1 appears to correlate with endoscopic activity in CD patients.<sup>57,58</sup> Although this work showed a higher prevalence of anti-CHI3L1 IgA and sIgA in CRP-positive compared to CRP-negative CD patients, there was no significant correlation of the CRP level with the autoAb reactivity to CHI3L1. Similar results were found for UC patients, in whom CRP levels also did not correlate with CHI3L1 reactivity.

The most extensively studied serological markers for differentiation are ANCA,<sup>7,59</sup> ASCA,<sup>14,60</sup> goblet cell antibodies [GAB]<sup>9,10</sup> and pancreatic antibodies [PAB],<sup>11</sup> which are utilized in different combinations for the differentiation of IBD. Regarding the combinations ANCA+/ASCA- and ANCA-/ASCA+, a specificity of 81–98% for UC and 92–97% for CD achieved.<sup>59</sup> Patients are also more likely to have CD when they were tested positive for ASCA and PAB and negative for ANCA and GAB.<sup>14</sup> In conclusion, integrating IgA and/or sIgA antibodies against CHI3L1 along with other novel CD-specific autoAbs such as antibodies to the zymogen granule GP2 into the laboratory screening of patients with a suspicion of CD might increase the serological sensitivity and the specificity of the screening.

Future work should examine whether there is diagnostic potential with CHI3L1 autoAbs and whether increased CHI3L1 levels and anti-CHI3L1 antibodies are an epiphenomenon particularly in CD or contribute to the onset or progress of IBD.

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## Conflict of Interest

DR is a shareholder of GA Generic Assays and Medipan GmbH and has a managerial position in both companies. All other authors have no conflict of interest.

## Author Contributions

Study concept and design: CD, NS, MP, DB, DR, PS. Acquisition of data: CD, JM, UR, NR, ML, KC. Analysis and interpretation of data: CD, MS, JM, NR, KC, DR, SR. Drafting of the manuscript: CD, DR, PS. Critical revision of the manuscript for important intellectual content: CD, JM, NR, KC, ML, MP, SR, DR, PS. Approval of the final manuscript: CD, MS, JM, UR, NR, KC, ML, NS, MP, DB, SR, PS. Guarantor of the article: CD.

## Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

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