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Serum Protein Biomarkers of Fibrosis Aid in Risk Stratification of Future Stricturing Complications in Pediatric Crohn's Disease.

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Abstract

BACKGROUND: Avoiding fibrostenotic complications is of paramount concern in the management of Crohn's disease (CD). We sought to investigate the association of candidate biomarkers of fibrosis collected at diagnosis with the future development fibrostenotic CD.

METHODS: Using the RISK cohort, a multicenter prospective observational pediatric inception cohort, subjects with an inflammatory phenotype (B1) at diagnosis who later converted to a stricturing phenotype (B2) within 3 years were compared to those who remained B1. Serum collected at diagnosis underwent both Parallel Reaction Monitoring (PRM) targeted proteomic analysis and conventional ELISA for 10 candidate biomarkers of intestinal fibrosis. Cox proportional hazard regression was used for multivariable analysis of time-dependent outcomes.

RESULTS: In 116 patients, 58 subjects with verified B1 phenotype at diagnosis who later converted to B2 disease were compared to 58 patients who remained B1 over 3 years of follow-up. Extracellular Matrix Protein 1 (ECM1) levels in the upper quartile (HR 3.43, 95%CL 1.33, 8.42) were associated with future fibrostenotic disease. ASCA IgA (HR 4.99, 95%CL 1.50, 16.68) and CBir levels (HR 5.19, 95%CL 1.83, 14.74) were also associated with future intestinal fibrostenosis, though ECM1 continued to demonstrate independent association with conversion to B2 even with adjustment for IBD serologies in multivariable analysis (HR 5.33, 95%CL 1.29, 22.13).

CONCLUSIONS: ECM1 and other biomarkers of fibrosis may aid in determining the risk of uncomplicated inflammatory disease converting to B2 stricturing phenotypes in children with CD. Prospective validation studies to verify test performance and optimize clinical utilization are needed prior to clinical implementation.

Keywords

Inflammatory Bowel Disease; Crohn's disease; fibrosis; extracellular matrix; prognostics; biomarkers; decision support; predictive modeling; proteomics

INTRODUCTION

Crohn's disease (CD), an idiopathic inflammatory bowel disease, is often complicated by the development of intestinal strictures and penetrating complications¹. While most children and adults present with inflammatory phenotypes (B1), cyclical intestinal inflammation and repeated episodes of aberrant wound healing result in nearly 50% of patients developing fibrostenotic complications (B2) within the first 10 years of disease, which are often refractory to medical therapy². Further, an estimated 10% of pediatric and adult patients CD appear to rapidly progress from an inflammatory to fibrostenotic phenotype within 3 years of diagnosis^{3,4}. The consequences of intestinal strictures can be severe, as they contribute to perforations, abscesses, and bowel obstruction, often requiring surgical resection or other mechanical maneuvers⁵. In the absence of anti-fibrotic therapies, avoidance of these complications is paramount. Prevention strategies focus on sufficiently suppressing inflammation to reduce the likelihood of new development or progression of existing intestinal fibrosis. Improved risk stratification tools are needed to select the appropriate therapeutic choice and timing for individual patients to maximize avoidance of complications while also protecting from overtreatment.

Objective CD assessments focus on biomarkers of inflammatory disease activity, yet no reliable measures of existing intestinal fibrosis are readily available. Fibrostenotic strictures are characterized by intestinal wall thickening, expansion and disorganization of the muscularis propria, and deposition of insoluble excessive extracellular matrix proteins⁶. Typically, fibrostenotic changes occur in tandem with inflammatory changes⁷. Prior biomarker discovery work has identified several glycoproteins with differential expression between inflammatory and fibrostenotic clinical phenotypes using high-resolution proteomic techniques⁸. Candidate biomarkers of intestinal fibrosis included extracellular matrix structural and remodeling proteins, along with their interface proteins. Extracellular Matrix Protein 1 (ECM1) and Fibronectin (FINC) are glycoproteins known to be involved in fibrotic skin diseases⁹. Evidence supports ECM1 as a linkage protein with epitopes for structural macromolecules including collagen, laminin, fibulin, and fibronectin^{10,11}. Matrix metalloproteinases (MMPs) are key enzymes responsible for degradation and remodeling of extracellular matrix with ability to cleave collagen, fibronectin and elastin^{12,13}. Cartilage oligomeric matrix protein (COMP) is a member of the thrombospondin glycoprotein family that is induced by Transforming growth factor- β (TGF- β), influences extracellular matrix structure, and has a role in myofibroblast proliferation and secretion of collagen¹⁴⁻¹⁷. COMP has been associated with connective tissue diseases including rheumatoid and osteoarthritis, scleroderma, idiopathic pulmonary fibrosis, and hepatic fibrosis¹⁸⁻²⁰.

We hypothesized that candidate biomarkers of fibrosis may be associated with the development of stricturing complications in children with Crohn's disease. While gold

standards for measuring intestinal fibrosis remain in development, the conversion from inflammatory (B1) to stricturing (B2) phenotypes are detected by conventional imaging and endoscopy. The Risk Stratification and Identification of Immunogenetic and Microbial Markers of Rapid Disease Progression in Children with Crohn's Disease (RISK) is a large multicenter cohort enrolling newly diagnosed patients with pediatric CD²¹. This prospective multi-year follow up of the RISK cohort provides an opportunity to better study the natural history of CD. Our aim was to determine if candidate fibrosis-related biomarkers measured at the time of diagnosis in patients with a B1 inflammatory phenotype were associated with the future development of fibrostenotic complications.

METHODS

Study Population and Patient Selection

Patients were selected for analysis from the RISK study, a prospective inception cohort of patients with suspected CD 18 years of age and younger recruited from 28 sites in the United States and Canada between 2008–2012 ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00790543) identifier NCT00790543). Participating subjects underwent disease activity assessment, capture of clinical data including medication use, endoscopy and imaging results, as well as blood samples collected at the time of diagnosis. Clinical management, including decisions on endoscopy and imaging, treatment, and surgery were at the discretion of the treating physician. The Montreal classification system was used for phenotyping²². Stricturing disease phenotype was defined by cross sectional imaging (CT or MR) where luminal narrowing and pre-stenotic dilation was present or endoscopically in the presence of an impassable stricture. The radiographic and endoscopic data were collected in a prospective manner by site radiologists and gastroenterologists who were not involved in assigning the B1 or B2 classification. Two audits of the data were completed by the site investigators to confirm consistent application of the B1 and B2 definitions. Treating clinicians and study team members adjudicating phenotypes were blinded to results of candidate biomarkers of fibrosis. Patients were followed prospectively every 6 months for 3 years.

Using a nested case-control study design, all 58 patients entering the study with a B1 phenotype who converted to B2 stricturing phenotype within 3 years of diagnosis were included. These patients were compared to a group of 58 subjects matched by disease phenotype and activity class at diagnosis but who remained B1 over the 3 year follow up. Using Breslow and Day methods, for 80% power and an alpha of 0.05, a sample size of 100 patients (50 pairs) is needed to detect an odds ratio of 3.0 and 60 patients (30 pairs) are required to detect an odds ratio of 4.0²³. Available covariates examined included age, gender, anthropomorphic characteristics (height and weight raw, Z-scores, percentiles and change over 6–12 months from diagnosis) disease location, clinical laboratory values including IBD serologies, and disease activity scores by pCDAI at diagnosis. Outcomes collected from both groups included the time from diagnosis to conversion to B2 disease and the need for surgical bowel resection.

Targeted Proteomics by Parallel Reaction Monitoring

Sera collected at diagnosis underwent parallel reaction monitoring (PRM), a targeted proteomic method, for quantitation of 10 candidate biomarkers of intestinal fibrosis identified in prior discovery work comparing inflammatory and fibrostenotic phenotypes⁸. Proteins targeted for quantitation included Extracellular matrix protein 1 (ECM1), Cartilage oligomeric matrix protein (COMP), Fibronectin (FINC), Matrix metalloprotease 9 (MMP9), procollagen C-endopeptidase (POCE), Galectin-3-binding protein (LG3BP), Hepatocyte growth factor activator (HGFA), Thrombospondin-1 (TSP1), collagenase type IV (MMP2), and Fibulin-1 (FBLN1)⁸. PRM measures ionization fragments using an Orbitrap mass analyzer for accurate and high-throughput quantification of target peptides, even in the setting of low protein abundance²⁴. Peptides used for protein quantification were based on sequences obtained from PeptideAtlas (<http://www.peptideatlas.org/>). The sequences were further filtered according to the following criteria: (1) peptides are unique to the proteins of interest, (2) peptide length is about 7–20 amino acids, (3) avoided missed cleavage sites and frequently modified amino acids such as methionine, tryptophan, N-terminal glutamine, and asparagine or glutamine followed by glycine²⁵. Peptides that satisfied the above criteria were selected for the PRM assay which was performed in duplicate independent runs. Proteins demonstrating significant differences by B2 conversion status underwent enzyme-linked immunosorbent assay (ELISA) in triplicate, a routine protein quantitation method available in most clinical laboratories. Additional detail on laboratory techniques are included in the Supplemental Methods.

Data Analysis

Raw mass spectrometry data were processed using Skyline software (v3.6.0)²⁶. For PRM peptide data, the averaged light/heavy area ratios were calculated between duplicates. Heavy peptide standards were analyzed by mass spectrometry to ensure no interference was present for detection of endogenous peptides. The linear correlation between the signature peptides of the same protein by PRM was calculated using the Pearson correlation coefficient. Due to the non-normality of the data, comparison peptide abundance by PRM was performed using the non-parametric Mann-Whitney U test. Multivariable modeling for an outcome of conversion from B1 to B2 stricturing disease was performed using Cox proportional hazard regression. Model building utilized a backward variable selection process with forced inclusion of covariates deemed clinically relevant *a priori*. Analysis of maximum likelihood estimates provided hazard ratios and confidence limits for each parameter within the model. Integrated time-dependent area under the receiver operating curve (AUROC) was calculated to determine model performance using Harrell's Concordance Statistic. The statistical analysis was performed in SAS 9.4 (Cary, NC).

RESULTS

Patient Characteristics

Patient demographics, anthropomorphics, disease location, pCDAI scores, and laboratory data between those remaining B1 and those converting to B2 disease are shown; at diagnosis patients were not using IBD medications (Table 1). The median time to B2 phenotype conversion was 15.8 months, with stricture development detected at 12, 24, and 36 months

from diagnosis in 37.9%, 39.6%, and 22.4% of those developing strictures. On univariate analysis, those converting to stricturing disease presented at an older age (12.8 vs. 11.3yrs, $p=0.003$). All subjects were shorter than adjusted population norms at diagnosis, though B1 and B2 phenotypes did not differ by standardized heights (height Z-scores -0.36 vs. -0.59 , $p=0.296$) or occurrence of severe growth retardation (3/58 vs 6/58, $p=0.227$). An ileocolonic disease distribution was the most frequent location among all patients, however those converting to B2 stricturing phenotype more frequently had disease restricted to the ileocecal region (32.8 vs. 15.6%, $p=0.039$). While white blood cell counts were lower in those converting to B2 disease (9.2 vs. 10.9 kcells/mL, $p=0.048$), no differences in CRP (14.3 vs. 14.2mg/L, $p=0.994$) or ESR (36.6 vs. 34.8mm/hr, $p=0.656$) were observed. Of the 45 patients undergoing bowel resection during the 3 year follow up period, 44 underwent surgery for obstructive symptoms due to stricture development, 1 underwent surgery for fistulizing disease.

Medication Use Characteristics Among Those Converting to Stricturing Disease

Anti-TNF use was common among all patients (82.2%) over the entire 3-year observation period with no difference in overall use between those converting to B2 disease and those remaining B1 (86.7% vs. 77.6%, $p=0.197$). Early anti-TNF use within 3 months of diagnosis occurred in 19.8% (23/116) of all subjects. Of those eventually converting to B2 stricturing disease, 56.9% (33/58) started an anti-TNF prior to stricture identification with 25.8% (15/58) of the B2 group starting within 3 months of diagnosis; early anti-TNF start did not differ by B2 conversion status ($p=0.1245$). Medication use and start time relative to diagnosis did not differ between groups for mesalamine, thiopurine, and methotrexate users.

Candidate Biomarkers of Intestinal Fibrosis

Peptide fragments of candidate biomarkers of intestinal fibrosis selected for quantification by PRM assay were based on optimal ionization characteristics (Supplemental Table 1). PRM peptide quantitation was accurate and reliable on replicate analysis, with a R-coefficient of >0.90 between peptides derived from the same protein and a R-coefficient of 0.99 between replicates (Supplemental Figure 1). Relative peptide abundance of Extracellular Matrix Protein 1 (ECM1), Cartilage oligomeric matrix protein (COMP), Matrix metalloproteinase-9 (MMP9) and Fibronectin (FINC) at diagnosis demonstrated significant differences between those remaining B1 and those converting to B2 stricturing disease (Figure 1). Comparing those converting to B2 disease to those remaining B1, no differences in serum procollagen C-endopeptidase (POCE), Galectin-3-binding protein (LG3BP), Hepatocyte growth factor activator (HGFA), Thrombospondin-1 (TSP1), 72 kDa type IV collagenase (MMP2), and Fibulin-1 (FBLN1) were identified. Considering that PRM assays are not readily available in clinical laboratories, we also examined proteins of interest using traditional ELISA assays (Figure 2). ELISA and PRM quantitation results for ECM1, COMP, MMP9, and FINC were similar, suggesting that conventional methods may be sufficient for clinically relevant measurement of these proteins of interest (Table 2).

The magnitude of the candidate biomarkers at diagnosis was not associated with how quickly conversion to the B2 fibrostenotic phenotype occurred. Comparing protein levels in those developing early stenosis (within 1 year of diagnosis) to late stenosis (1–3 years

following diagnosis), no significant differences in the mean level of ECM1 (3540.3ng/mL vs. 3323.7ng/mL, $p=0.352$), COMP (200.2 vs. 198.6, $p=0.850$), MMP9 (600.5ng/mL vs. 508.0ng/mL, $p=0.600$), or FINC (128.2ug/mL vs. 100.3 ug/mL, $p=0.223$) were detected. However, among those who went on to develop stricturing complications, higher ECM1 levels at diagnosis were associated with needing surgical bowel resection during the 3 year follow up (3555.2 ng/mL vs. 3005.8 ng/mL, $p=0.034$). COMP, MMP9, and FINC levels at diagnosis were not associated with future surgical bowel resection.

CRP levels and candidate biomarkers of fibrosis collected at the time of diagnosis were assessed for co-linearity, as serum proteins associated with future conversion to the B2 phenotype could be surrogates of inflammatory burden. No linear relationship or correlation was found between CRP and ECM1 ($r=0.127$, $p=0.293$), COMP ($r=-0.072$, $p=0.489$), or MMP9 ($r=-0.154$, $p=0.139$); a trend of weak, non-significant, positive correlation was found between CRP and FINC ($r=0.1806$, $p=0.082$).

Adjusted Models of Conversion from Inflammatory to Stricturing Phenotypes

A multivariable model of conversion to a B2 stricturing phenotype was constructed incorporating all candidate serum proteins of fibrosis and clinical features, yielding an AUROC of 0.797 (Table 3). ECM1 levels in the upper quartile at diagnosis (>3900 ng/mL) were associated with an increased risk of future stricturing disease on adjusted analysis (HR 3.43, 95% CL 1.33, 8.42). Further, upper quartile ECM1 values at diagnosis resulted in a stronger association with surgical bowel resection due to obstruction (HR 11.80, 95% CL 3.35, 31.64). While MMP9 level was not significantly associated with conversion to stricturing disease, it was retained because of contribution to model fit. When candidate biomarkers were examined independently, low MMP9 and COMP levels were associated with B2 conversion, while high FINC levels demonstrated a non-significant trend toward B2 conversion, though these effects were overshadowed by ECM1 in the multivariable model (Supplemental Table 2). Increasing age (HR 1.3, 95% CL 1.14, 1.49 per year) was also associated with converting to stricturing disease. Models using PRM in place of ELISA protein quantitation produced similar results in terms of effects, with a comparable AUROC of 0.766.

Neither therapeutic choice or CRP at diagnosis were significant factors in models of future conversion to a fibrostenotic phenotype. While therapy choice and timing were uncontrolled, the initiation of anti-TNF therapy within 3 months of diagnosis (25.8% of all patients) was not protective against the development of stricturing disease (HR 1.46, 95%CL 0.67, 3.18). No correlations or interactions were observed between early anti-TNF use and ECM1, FINC, COMP, MMP9 or standardized anthropomorphic values, including height velocity. As both an independent variable (HR=1.01, 95%CL 0.98, 1.02) and in interaction with ECM1 (HR=0.86, 95%CL 0.78, 1.29), CRP was not significantly associated with conversion to B2 phenotype on adjusted multivariable analysis.

To assess whether candidate serum markers of fibrosis were additive, synergistic or superceeded by established prognostic serum markers, models incorporating both candidate proteins and IBD serologies were generated (Table 4). CBir (HR 5.19, 95%CL 1.83, 14.74) and ASCA IgA (HR 4.99, 95%CL 1.50, 16.68) both exhibited strong positive effects for

stricture development, while ASCA IgG, pANCA, and OMPc were non-significant. High values of ECM1 (above 3900ng/mL) remained strongly associated with future stricture development (HR 5.33, 95% CL 1.83, 14.74) even with adjustment for baseline IBD serologies. The final model of future stricturing disease using ECM1, ASCA IgA, and CBir had a time dependant AUROC of 0.833 (95% CL 0.756, 0.911). MMP9, COMP, and FINC, as well as non-listed clinical variables did not exert influence and were not forced into the model to prevent over-fitting.

Finally, despite its association with conversion to stricturing disease, the diagnostic performance of ECM1 alone is fair. As an independent and unadjusted biomarker for predicting future conversion from a B1 to B2 phenotype, ECM1 levels >3900ng/mL had PPV and NPV of 75.9% and 60.7%, a sensitivity and specificity of 38.3% and 89.7%, and an accuracy of 63.6% respectively. Adjusting for age, disease location, and serologies, the final multivariate model including ECM1 level had a PPV and NPV 79.4% and 73.9%, a sensitivity and specificity of 80.0% and 70.7%, and an accuracy of 75.4% for predicting future conversion to structuring disease.

DISCUSSION

In this study we identified ECM1 as a potential biomarker to improve our ability to risk stratify the propensity for future fibrostenotic complications in CD patients. These findings are in agreement with mucosal tissue data from this cohort demonstrating ECM1 expression was upregulated 1.50 fold in ileal biopsies collected at diagnosis in those converting from B1 to B2 disease ($p=0.000392$), along with other pro-fibrotic expression signatures involving ECM organization, fibronectin binding, and collagen binding²¹. Further, while CBir, ASCA IgA, and the total number of positive serologies have previously been shown to prognostic for predicting future complex disease, the addition of ECM1 levels improved model performance³. Importantly, the association of ECM1 and future stricturing disease, independent of IBD serologies and CRP at diagnosis, suggests ECM1 could be additive to risk-stratification panels in development, though prospective validation studies are needed. We unexpectedly found that despite low abundance, the examined biomarkers did not require highly sensitive techniques such as PRM and were adequately detected using conventional ELISA methods. While further work is needed before clinical implementation, ECM1 may be an important biomarker of future fibrostenotic disease considering its known biologic functions and elevated levels in serum and tissue prior to macroscopic stricture development.

MMP9, COMP, and FINC remain biomarkers of interest, though their associations with conversion from B1 to B2 stricturing phenotypes were overshadowed by the stronger effect of ECM1. Differences in MMP9 levels between groups did not achieve significance, but it was an influential factor in our model of future stricture development. The behavior of COMP in this pediatric population was unexpected, as prior work demonstrated elevated serum levels in adult CD patients with established severe intestinal stricturing disease⁸. Though COMP has a direct relationship with several key elements of tissue fibrosis, it also is a strong morphogenic growth factor²⁷. We hypothesize that patient growth characteristics were a major confounder limiting the use of COMP in pediatric CD, as COMP is associated

with normal connective tissue expansion and would be expected to be elevated during childhood growth periods. Despite adjustment for growth velocity and Tanner stage, COMP remained a non-significant risk factor of future B2 stricturing disease within this study population.

Several limitations are relevant when interpreting the results. The primary limitation, as a function of the observational cohort study design, was that objective reassessments needed to immediately detect interval stricturing disease were not protocolized or scheduled. Decision for the timing of follow up endoscopy and imaging assessments were at the discretion of the treating provider. While we expect the majority of patients with clinically significant stricturing disease would prompt objective reassessment, there is nonetheless potential for delay or even omission of detecting conversion to a B2 phenotype. Additionally, the non-interventional aspect of the study prevented control of therapeutic decisions which may have influenced the results. Importantly, when anti-TNF agents were used, dose and frequency decisions were not protocolled, nor was the use of therapeutic drug monitoring. Potentially, subtherapeutic drug levels may have limited anti-TNF impact on disease progression from B1 to B2 phenotypes. While the data were unable to demonstrate anti-TNF effect on stricture development, variability in the timing of drug initiation and method for dose optimization used remain key limitations for assessing whether medications modify intestinal fibrosis development.

A major barrier in clinical CD-fibrosis research to date is that stricturing disease is often described as a singular event or occurrence. While consensus statements and definitions of features constituting stricturing disease have been proposed by expert multidisciplinary teams, fibrosis is not a binary phenomenon^{28,29}. Time series data from animal models of IBD-like chronic injury indicate intestinal fibrosis is incremental with variability in the rate of progression based on local environmental factors³⁰. Further, clinical, genetic and serologic markers have been associated with the rate of change from inflammatory to fibrostenotic phenotypes in human observational studies^{3,4,31}. Ideally, descriptions of fibrostenotic features would use quantitative measures of deep bowel damage as compared to the binary classifications of stricturing disease offered by the Montreal and other clinical phenotyping systems. The quantitation of intestinal fibrosis would dramatically improve our ability to detect precursors of severe stenosis and modify treatment, as well as measuring the efficacy of anti-fibrotic therapies in development. In this study, ECM1 levels did not differ by the rate of transition from non-stricturing to stricturing disease (B2). Understanding the interval existence and rate of developing fibrosis before frank macroscopic intestinal stenosis occurs will require better quantitative measurement tools, most likely relying on imaging modalities.

Finally, fundamental interactions between inflammation and wound healing pathways present additional challenges in developing clinical biomarkers for fibrosis. Without full thickness histology it is difficult to immediately conclude these candidate biomarkers are reflective of fibrosis as compared to predictors of future phenotype or disease severity. Though there was no correlation between CRP and ECM1 or other studied biomarkers, excluding the possibility these proteins are actually surrogates of inflammatory activity requires further study. At present, we are inferring a role in fibrogenesis based on their

known functions and the presented observations in CD. Future prospective validation studies will require protocolled image acquisition at scheduled intervals with central reading of radiographs, ideally quantifying deep bowel damage rather than simply reporting the presence of stenosis. Repeated blood sampling and protein measurements will aid in understanding the precision and reproducibility of the candidate biomarkers. Ongoing work will detail the longitudinal kinetics of ECM1, MMP9, FINC, and COMP as predictors of fibrostenotic phenotype and examine protein modifications associated with disease complications.

In conclusion, ECM1 and other serum-based proteins are associated with the future development of stricturing complications in a pediatric Crohn's disease cohort. Fibrostenotic complications drive a substantial degree of the morbidity in CD. Improving our ability to predict the likelihood of future complications at the time of diagnosis would better personalize the choice and intensity of therapy. Other methods to gauge the propensity for future fibrostenotic complications are showing promise, including early serologic and genetic markers of disease progression^{3,32}. New technologies to distinguish and quantify existing inflammatory and fibrotic burden are progressing, including ultrasound elastography, magnetization transfer MRI, and photoacoustic imaging^{33,34}. Recent data demonstrating measurements of fibrostenotic burden can stratify the likelihood of anti-TNF treatment success highlights the potential utility of fibrosis measurement tools in treatment decision making³⁵. We envision a future state where both inflammation and fibrosis will be independently assessed to better describe phenotype and assess the response to therapy. In conjunction with clinical, genetic, serologic and imaging assessments, continued research in blood-based biomarkers may further improve risk stratification in patients with Crohn's disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES:

1. Peyrin-Biroulet L, Loftus EV, Colombel JF, Sandborn WJ. The Natural History of Adult Crohn's Disease in Population-Based Cohorts. *Am J Gastroenterol*. 2009;105(2):289–297. doi:10.1038/ajg.2009.579. [PubMed: 19861953]
2. Cosnes J, Cattan S, Blain A, et al. Long-term evolution of disease behavior of Crohn's disease. *Inflamm Bowel Dis*. 2002;8(4):244–250. [PubMed: 12131607]
3. Dubinsky MC, Kugathasan S, Mei L, et al. Increased immune reactivity predicts aggressive complicating Crohn's disease in children. *Clin Gastroenterol Hepatol*. 2008;6(10):1105–1111. doi:10.1016/j.cgh.2008.04.032. [PubMed: 18619921]

4. Sands BE, Arsenault JE, Rosen MJ, et al. Risk of early surgery for Crohn's disease: implications for early treatment strategies. *Am J Gastroenterol.* 2003;98(12):2712–2718. doi:10.1111/j.1572-0241.2003.08674.x. [PubMed: 14687822]
5. Lichtenstein GR, Hanauer SB, Sandborn WJ, Practice Parameters Committee of American College of Gastroenterology. Management of Crohn's disease in adults. *Am J Gastroenterol.* 2009;104(2):465–483; quiz 464, 484. doi:10.1038/ajg.2008.168. [PubMed: 19174807]
6. Rieder F, Zimmermann EM, Remzi FH, Sandborn WJ. Crohn's disease complicated by strictures: a systematic review. *Gut.* 2013;62(7):1072–1084. doi:10.1136/gutjnl-2012-304353. [PubMed: 23626373]
7. Adler J, Punglia DR, Dillman JR, et al. Computed tomography enterography findings correlate with tissue inflammation, not fibrosis in resected small bowel Crohn's disease. *Inflamm Bowel Dis.* 2012;18(5):849–856. doi:10.1002/ibd.21801. [PubMed: 21710535]
8. Stidham RW, Wu J, Shi J, Lubman DM, Higgins PDR. Serum Glycoproteome Profiles for Distinguishing Intestinal Fibrosis from Inflammation in Crohn's Disease. Green J, ed. *PLoS ONE.* 2017;12(1):1–15. doi:10.1371/journal.pone.0170506.
9. Fujimoto N, Terlizzi J, Aho S, et al. Extracellular matrix protein 1 inhibits the activity of matrix metalloproteinase 9 through high-affinity protein/protein interactions. *Exp Dermatol.* 2006;15(4):300–307. doi:10.1111/j.0906-6705.2006.00409.x. [PubMed: 16512877]
10. Sercu S, Zhang M, Oyama N, et al. Interaction of extracellular matrix protein 1 with extracellular matrix components: ECM1 is a basement membrane protein of the skin. *J Invest Dermatol.* 2008;128(6):1397–1408. doi:10.1038/sj.jid.5701231. [PubMed: 18200062]
11. Chan I, South AP, McGrath JA, et al. Rapid diagnosis of lipoid proteinosis using an anti-extracellular matrix protein 1 (ECM1) antibody. *J Dermatol Sci.* 2004;35(2):151–153. doi:10.1016/j.jdermsci.2004.03.010. [PubMed: 15265527]
12. Yabluchanskiy A, Ma Y, Iyer RP, Hall ME, Lindsey ML. Matrix metalloproteinase-9: Many shades of function in cardiovascular disease. *Physiology (Bethesda).* 2013;28(6):391–403. doi:10.1152/physiol.00029.2013. [PubMed: 24186934]
13. Ram M, Sherer Y, Shoenfeld Y. Matrix metalloproteinase-9 and autoimmune diseases. *J Clin Immunol.* 2006;26(4):299–307. doi:10.1007/s10875-006-9022-6. [PubMed: 16652230]
14. Tan K, Duquette M, Joachimiak A, Lawler J. The crystal structure of the signature domain of cartilage oligomeric matrix protein: implications for collagen, glycosaminoglycan and integrin binding. *FASEB J.* 2009;23(8):2490–2501. doi:10.1096/fj.08-128090. [PubMed: 19276170]
15. Acharya C, Yik JHN, Kishore A, Van Dinh V, Di Cesare PE, Haudenschild DR. Cartilage oligomeric matrix protein and its binding partners in the cartilage extracellular matrix: interaction, regulation and role in chondrogenesis. *Matrix Biol.* 2014;37:102–111. doi:10.1016/j.matbio.2014.06.001. [PubMed: 24997222]
16. Agarwal P, Schulz J-N, Blumbach K, et al. Enhanced deposition of cartilage oligomeric matrix protein is a common feature in fibrotic skin pathologies. *Matrix Biol.* 2013;32(6):325–331. doi:10.1016/j.matbio.2013.02.010. [PubMed: 23507196]
17. Schulz J-N, Nüchel J, Niehoff A, et al. COMP-assisted collagen secretion - a novel intracellular function required for fibrosis. *J Cell Sci.* 2016;129(4):706–716. doi:10.1242/jcs.180216. [PubMed: 26746240]
18. Farina G, Lemaire R, Pancari P, Bayle J, Widom RL, Lafyatis R. Cartilage oligomeric matrix protein expression in systemic sclerosis reveals heterogeneity of dermal fibroblast responses to transforming growth factor beta. *Annals of the Rheumatic Diseases.* 2009;68(3):435–441. doi:10.1136/ard.2007.086850. [PubMed: 18408251]
19. Happonen KE, Saxne T, Geborek P, et al. Serum COMP-C3b complexes in rheumatic diseases and relation to anti-TNF- α treatment. *Arthritis Res Ther.* 2012;14(1):R15. doi:10.1186/ar3694. [PubMed: 22264230]
20. Norman GL, Gatselis NK, Shums Z, et al. Cartilage oligomeric matrix protein: A novel non-invasive marker for assessing cirrhosis and risk of hepatocellular carcinoma. *World J Hepatol.* 2015;7(14):1875–1883. doi:10.4254/wjh.v7.i14.1875. [PubMed: 26207169]

21. Kugathasan S, Denson LA, Walters TD, et al. Prediction of complicated disease course for children newly diagnosed with Crohn's disease: a multicentre inception cohort study. *Lancet*. 2017;389(10080):1710–1718. doi:10.1016/S0140-6736(17)30317-3. [PubMed: 28259484]
22. Satsangi J, Silverberg MS, Vermeire S, Colombel J-F. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. In: Vol 55 BMJ Publishing Group; 2006:749–753. doi:10.1136/gut.2005.082909.
23. Breslow NE, Day NE. Statistical methods in cancer research. Volume II--The design and analysis of cohort studies. IARC Sci Publ. 1987;(82):1–406.
24. Peterson AC, Russell JD, Bailey DJ, Westphall MS, Coon JJ. Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics. *Mol Cell Proteomics*. 2012;11(11):1475–1488. doi:10.1074/mcp.O112.020131. [PubMed: 22865924]
25. Hoofnagle AN, Whiteaker JR, Carr SA, et al. Recommendations for the Generation, Quantification, Storage, and Handling of Peptides Used for Mass Spectrometry-Based Assays. *Clin Chem*. 2016;62(1):48–69. doi:10.1373/clinchem.2015.250563. [PubMed: 26719571]
26. MacLean B, Tomazela DM, Shulman N, et al. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics*. 2010;26(7):966–968. doi:10.1093/bioinformatics/btq054. [PubMed: 20147306]
27. Ishida K, Acharya C, Christiansen BA, Yik JHN, DiCesare PE, Haudenschild DR. Cartilage oligomeric matrix protein enhances osteogenesis by directly binding and activating bone morphogenetic protein-2. *Bone*. 2013;55(1):23–35. doi:10.1016/j.bone.2013.03.007. [PubMed: 23528838]
28. Bruining DH, Zimmermann EM, Loftus EV, et al. Consensus Recommendations for Evaluation, Interpretation, and Utilization of Computed Tomography and Magnetic Resonance Enterography in Patients With Small Bowel Crohn's Disease. *Gastroenterology*. 2018;154(4):1172–1194. doi:10.1053/j.gastro.2017.11.274. [PubMed: 29329905]
29. Rieder F, Bettenworth D, Ma C, et al. An expert consensus to standardise definitions, diagnosis and treatment targets for anti-fibrotic stricture therapies in Crohn's disease. *Aliment Pharmacol Ther*. 2018;48(3):347–357. doi:10.1111/apt.14853. [PubMed: 29920726]
30. Johnson LA, Rodansky ES, Sauder KL, et al. Matrix stiffness corresponding to strictured bowel induces a fibrogenic response in human colonic fibroblasts. *Inflamm Bowel Dis*. 2013;19(5):891–903. doi:10.1097/MIB.0b013e3182813297. [PubMed: 23502354]
31. Colombel JF, Narula N, Peyrin-Biroulet L. Management Strategies to Improve Outcomes of Patients With Inflammatory Bowel Diseases. *Gastroenterology*. 2016;152(2):351–361.e355. doi:10.1053/j.gastro.2016.09.046. [PubMed: 27720840]
32. Marigorta UM, Denson LA, Hyams JS, et al. Transcriptional risk scores link GWAS to eQTLs and predict complications in Crohn's disease. *Nat Genet*. 2017;49(10):1517–1521. doi:10.1038/ng.3936. [PubMed: 28805827]
33. Dillman JR, Stidham RW, Higgins PDR, et al. Ultrasound shear wave elastography helps discriminate low-grade from high-grade bowel wall fibrosis in ex vivo human intestinal specimens. *J Ultrasound Med*. 2014;33(12):2115–2123. doi:10.7863/ultra.33.12.2115. [PubMed: 25425367]
34. Stidham RW, Higgins PD. Imaging of intestinal fibrosis: current challenges and future methods. *United European Gastroenterology Journal*. 2016;4(4):515–522. doi:10.1177/2050640616636620. [PubMed: 27536361]
35. Bouhnik Y, Carbonnel F, Laharie D, et al. Efficacy of adalimumab in patients with Crohn's disease and symptomatic small bowel stricture: a multicentre, prospective, observational cohort (CREOLE) study. *Gut*. January 2017:gutjnl-2016-312581. doi:10.1136/gutjnl-2016-312581.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- Preventing fibrostenotic disease is essential for best outcomes in Crohn's disease.
- Risk stratification for the development of future complications of Crohn's disease remains difficult.
- Biomarkers reflecting existing intestinal fibrosis or the potential for fibrogenesis are needed.

WHAT IS NEW HERE

- High levels of extracellular matrix protein 1 (ECM1) are associated with conversion from inflammatory to fibrostenotic phenotypes of Crohn's disease.
- ECM1 association with future stenosis is independent of information reflected by traditional IBD serologies.
- Serum biomarkers of fibrosis, in combination with imaging and serologies, may further personalize treatment selection in Crohn's disease.

Figure 1.

Comparison of candidate biomarker protein quantitation by PRM between subjects with CD remaining B1 vs those converting to B2 after 3-year follow-up. Peptide abundance plots from PRM analysis using 4 candidate biomarkers demonstrating a significant difference between those remaining B1 and those converting to B2 stricturing disease. Peptide fragments were standardized using light/heavy (L/H) ratios (*P < 0.05; **P < 0.01). CD, Crohn's disease; COMP, cartilage oligomeric matrix protein; ECM1, extracellular matrix protein 1; FINC, fibronectin; MMP9, matrix metalloproteinase 9; PRM, parallel reaction monitoring.

Figure 2.

ELISA quantitation of candidate proteins associated with future conversion to stricturing phenotype. Elevated concentrations of ECM1 and FINC and reduced concentrations of COMP and MMP9 were associated with future intestinal stricture development on unadjusted analysis (* $P < 0.05$, ** $P < 0.01$). Results on ELISA quantitation were similar to PRM biomarker trends. COMP, cartilage oligomeric matrix protein; ECM1, extracellular matrix protein 1; ELISA, enzyme-linked immunosorbent assay; FINC, fibronectin; MMP9, matrix metalloproteinase 9; PRM, parallel reaction monitoring.

Table 1.**Patient Characteristics at Diagnosis**

	Remain B1 (n=58)		Convert to B2 (n=58)		<i>p</i>
	mean, n	95% CL	mean, n	95% CL	
Age at Diagnosis	11.3	10.6, 11.9	12.8	12.1, 13.6	0.003
Gender, F (%)	20 (34.4)		25 (41.7)		0.317
Height, cm	143.5	139.1, 147.8	149.2	144.7, 153.8	0.071
Height, percentile	0.41	0.33, 0.48	0.36	0.28, 0.44	0.347
Weight, kg	35.4	31.9, 38.8	38.9	35.4, 42.4	0.150
Weight, percentile	0.31	0.24, 0.38	0.26	0.19, 0.33	0.341
pCDAI	28.7	24.9, 32.5	28.3	24.8, 31.7	0.859
<u>Disease Location</u>					
Ileal, n (%)	9 (15.5)		19 (32.8)		0.039
Colonic, n (%)	1 (1.7)		2 (3.4)		0.579
Ileocolonic, n (%)	46 (79.3)		36 (62.1)		0.023
Upper Disease, n (%)	34 (58.6)		27 (46.6)		0.114
<u>Laboratory Data</u>					
WBC, kcell/mL	10.9	9.4, 12.4	9.2	8.2, 10.1	0.048
Hematocrit, %	35.4	34.5, 36.3	34.8	33.8, 35.8	0.349
Hemoglobin, g/dL	11.4	11.1, 11.71	11.1	10.7, 11.5	0.242
Platelets, 10 ⁹ cells/L	508.6	467.7, 549.6	507.3	464.6, 550	0.963
Albumin, g/L	3.56	3.41, 3.73	3.48	3.32, 3.64	0.443
CRP, mg/L	14.3	1.7, 27.0	14.2	6.6, 21.9	0.994
ESR, mm/hr	36.6	30.4, 42.7	34.8	29.4, 40.1	0.656

Abbreviations:

CL: confidence limit, pCDAI: pediatric Crohn's disease activity index, WBC: White Blood Cell, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate.

Table 2.

Comparison of PRM and ELISA-based protein quantitation methods by B2 structuring conversion status.

Protein	PRM Quantitation			ELISA Quantitation		
	Remain B1	Convert B2	<i>p</i>	Remain B1	Convert B2	<i>p</i>
ECM1	0.00141 (0.0017)	0.00271 (0.0026)	0.0026	2995.2 ng/mL (880.1)	3415.5ng/mL (875.4)	0.0109
FINC	0.6648 (0.9177)	0.9847 (1.27)	0.0061	75.8 µg/mL (87.5)	116.6 µg/mL (86.5)	0.0123
COMP	0.0211 (0.0065)	0.0178 (0.0056)	0.0038	225.1 ng/mL (61.1)	199.5 ng/mL (30.9)	0.0047
MMP9	0.00604 (0.0055)	0.00454 (0.0054)	0.0400	860.8 ng/mL (648.2)	561.9 ng/mL (665.3)	0.0150

Means and standard deviations are displayed for each protein by PRM (parallel reaction monitoring) compared to ELISA analysis. PRM results are relative abundance to referenced reporter protein; the most discriminatory peptide is shown for comparison to ELISA results.

Table 3.

Multivariable Cox regression model of conversion to B2 stricturing phenotype.

Variable	Hazard Ratio	95% CL	p value
<i>Age (yrs) (per year)</i>	1.30	1.14, 1.49	0.0001
Height Percentile (per 0.10)	0.64	0.23, 1.73	0.3777
Albumin (per 0.5g/dL)	0.67	0.41, 1.07	0.0924
ECM1 Quartiles			
1st (0–2529ng/mL)		reference	
2nd (2530–3250ng/mL)	1.84	0.74, 4.58	0.1906
3rd (3251–3899ng/mL)	1.19	0.45, 3.21	0.7241
4th (3900–5177ng/mL)	3.43	1.33, 8.42	0.0087
MMP9 Quartiles			
1st (0–231ng/mL)	1.80	0.75, 4.62	0.1802
2nd (232–549ng/mL)	1.64	0.68, 3.99	0.2716
3rd (550–1009ng/mL)	0.67	0.28, 1.64	0.3838
4th (1010–1612ng/mL)		reference	

Table 4.

Multivariable Cox regression model of conversion to B2 stricturing phenotype incorporating both candidate biomarkers of fibrosis and IBD serologies.

Variable	Hazard Ratio	95% CL	<i>p</i> value
<i>Age (yrs) (per year)</i>	1.31	1.08, 1.60	0.0073
Disease Distribution (Ileocecal vs. colonic)	0.60	0.12, 3.06	0.2439
<u>ECM1 Quartiles</u>			
2nd (2530–3250ng/mL)	1.33	0.37, 4.75	0.7861
3rd (3251–3899ng/mL)	0.67	0.16, 2.78	0.6837
4th (3900–5177ng/mL)	5.33	1.29, 22.13	0.0040
<u>Serologies</u>			
CBir	5.19	1.83, 14.74	0.0020
ASCA IgA	4.99	1.50, 16.68	0.0089
ASCA IgG	0.83	0.22, 3.06	0.4309