SHORT REPORT

Pre-clinical Crohn's disease: Diagnosis, treatment and six year follow-up

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Abstract

Diagnosis of Crohn's disease is usually made at a symptomatic stage. However diagnosis at a pre-clinical stage might provide valuable information on etiology/pathogenesis and allow early intervention to alter its natural history. We describe here the case of a 27 year old woman who was diagnosed with Crohn's disease at a completely asymptomatic stage and followed up for more than six years. She was part of an ongoing screening study in first degree relatives of Crohn's disease patients. At diagnosis, colonoscopy showed modest inflammation and few superficial ulcerations and erosions in the ileo-cecal valve and the terminal ileum. Fecal calprotectin was only modestly elevated. Intestinal permeability was also increased. During follow-up and while still asymptomatic the patient was sequentially treated with therapeutic doses of 5-ASA, budesonide, azathioprine and infliximab in an attempt to stop disease progression. Only infliximab appeared capable of inducing profound mucosal healing—however the disease recurred several months after the medication was ceased. Over time, quantification by immunohistochemistry of a number of cell types and cytokines revealed a positive correlation between CD4-CD25-FOXP3 (Treg) cell number and inflammation, a finding potentially consistent with tissue resistance to Tregs' activity.

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1. Introduction

Diagnosis of Crohn's disease (CD) is invariably made at an advanced, symptomatic stage.1 Yet diagnosis of preclinical CD may shed light on the early pathogenetic features of CD. In addition, disease course prediction may impact on treatment/disease prevention.2 Screening programs for CD in the general population are essentially considered unfeasible.3 However, first degree relatives (FDRs) of CD patients could be tested for the purpose with a relatively high yield.4 Since symptomatic CD is localized in the colon, ileocolon or terminal ileum in ≥ 90% of patients5 by inference the disease must originate in sites which can be reached by the...
ileocolonoscopy in the vast majority of cases. Based on these premises, we have initiated such screening study several years ago. Among the screened individuals, we identified and diagnosed with early CD a 27 year old woman who was then carefully followed for several years with endoscopy, blood and fecal tests. In addition, intestinal tissue samples were analyzed over time for a number of cell types and cytokines believed to play a role in CD pathogenesis.

2. Material and methods

2.1. Endoscopy

Endoscopy and biopsies were performed under light sedation with standard Olympus (Segrate—Italy) or Pentax (Milano—Italy) equipment. During each colonoscopy, the ileum was intubated and explored for at least 25 cm.

Endoscopic damage was scored using a simplified Crohn’s Disease Endoscopic Index of Severity (CDEIS)6: a score of 1 was given for each ulceration; for ulcers larger than 2 mm such scores were multiplied by 2; for deep (as opposed to superficial) ulcers, the score was further multiplied by 2; for any number of erosions or in the presence of edema or hyperemia, 0.5 points were added to the total score. Endoscopic lesions were scored in the ileocecal valve and terminal ileum by review of the film of the colonoscopy that day after the examination by an independent investigator who was unaware of the patient's treatment. Each time, pictures were taken at the same anatomical location.

2.2. Histology and immunohistochemistry

Histological scoring was based on methods previously described7,8, which include epithelial damage, architectural changes, infiltration of mononuclear and polymorphonuclear cells in lamina propria, polymorphonuclear cells in surface epithelium, presence of ulcer and granuloma and proportion of abnormal biopsies.

Tissue was taken in the immediate vicinity of lesions (erosions and ulcerations) in diseased areas and preserved in formalin for H&E staining and immunohistochemistry. An antibody panel was used to measure the following cytokines, cell surface markers and chemokine receptors: IL1β, IL4, IL6, IL10, IL12, IL17, IL22, IL23N, IL23p19, TNF-α, TGF-β, IFN-γ, CD4, FOXP3, CCR4, CCR6 (Abcam, Prodotti Gianni, Milan-Italy), IL21 (Acris Antibodies, Space Imports Milan-Italy) and CD25 (Imgenex, San Diego, CA, USA), at time 0, 18, 30, 38, 50, 57, and 73 months. CD4, CD25 and FOXP3 are markers of Treg cells9 and CD4-CCR4-CCR6 are expressed in Th17 cells.10

From formalin fixed and paraffin embedded biopsies, appropriate 4 μm thick sections were immunostained using antigen retrieval.11 Immunostaining was accomplished in triplicate on a Dako Autostainer Link 48, using Envision + Detection (Dako, Carpinteria, CA, USA). The slides were examined by a single pathologist (C.A.). After low power scanning to identify the distribution of the inflammatory cells, the whole specimen in each slide was examined at high power view (40×), on a minimum of 8 fields, evaluating the immunohistochemical reactivity as absolute number of reactive cells per mm². Two different biopsy specimens (ileal and colonic mucosa) from patients with normal ileocolonoscopy were used as negative controls for each patient's specimen. The slides were treated as described above.

2.3. Fecal calprotectin

Fecal calprotectin (FC) was measured by a commercially available ELISA test (Calprest, Eurospital, Trieste—Italy) after protein extraction on a weighted stool sample a few days before bowel preparation for colonoscopy.

2.4. Permeability test

Lactulose and mannitol (10 and 5 g respectively) were dissolved in 50 mL of water and administered after an overnight fast. Over the following 6 h, two stools were collected in plastic containers with chlorhexidine (1 mL of a 2% solution) added as preservative. The urine volume was measured and an aliquot stored at −20 °C until analysis. Sugars were measured in the urine after sample preparation using standard sugar solutions by HPLC as described by Marsilio et al.12 The results are given as the percentage of orally administered quantity and expressed as lactulose/ mannitol (L/M) ratio. The normal threshold was set at 0.025, mean value of the control population + 2 SD.

2.5. Statistical analysis

Two correlation analyses were performed using the endoscopic score, the histologic score, FC and the above cell types and cytokines. Firstly, a functional principal component analysis (fPCA)13 was performed in order to reduce the dimensionality of the data to a smaller number of factors (principal components) while including the temporal dimension. Hence, both cross-correlation and temporal autocorrelation are accounted for by fPCA. A smoothing spline (B-spline of order 4) was used for modeling the nonparametric relationship $y = f(t)$, where $y$ is the variable of interest, $t$ denotes time ($t = 0, 18, 30, 38, 50, 57, 73$ months) and $f$ is a nonparametric function. For comparison purposes, data were standardized on a scale from 0 (min) to 1 (max).

Secondly, the pairwise Pearson’s correlation coefficient and its p-value were calculated.

3. Case report

D.M. is the 27 year old sister of a CD patient. As a part of a larger study – approved by the University of Udine Ethical Committee – we proposed to screen her for CD with ileocolonoscopy and other tests as needed. She was asymptomatic and denied alcohol, NSAID’s or other medication use, recent traveling or infections, pregnancy and exposure to tuberculosis. Neoplasia, cellular, rheumatologic, endocrine, cardiovascular, lung, liver, renal, ocular, skin and genital diseases and food allergies/intolerance were also excluded.

Ileocecal valve and terminal ileum images (taken at the same location) as well as valve histology are shown in Fig. 1. The initial exam (Fig. 1A) showed a hyperemic valve with 2 small superficial ulcerations. The terminal ileum also showed hyperemia and few erosions. Histology showed a focal...
pleomorphic inflammatory component in lamina propria, small and superficial microaphtae and hystoid subepithelial aggregates—aspects consistent with, although not diagnostic for, CD. FC was 64 mg/kg (normal 0–50 mg/kg). Proximal disease localization was excluded by magnetic resonance enterography and upper endoscopy. Stool cultures and ANCA/ASCA antibodies were negative. Intestinal permeability was increased at 0.076.

Initial management was limited to observation. After 18 months endoscopy showed a significant worsening of inflammation both at the valve – with the appearance of new ulcerations – and at the terminal ileum where the erosions became aphthous ulcers. Histology also worsened and showed the presence of microgranulomas (Fig. 1B). FC was 148 mg/Kg. After discussion with the patient, a step-up therapeutic approach (beginning with mesalamine) was initiated to arrest disease progression. After 12 months of mesalamine 2.4 g/day colonoscopy showed wider and deeper valve and ileal ulcerations while new ones appeared in the ileum, extending the disease proximally. Histology also worsened (Fig. 1C). FC was now 194 mg/kg. Mesalamine was stopped and budesonide (9 mg/day) was initiated and given for 8 months after which time the tests were repeated. Colonoscopy and histology (Fig. 1D) showed further disease progression with new and wider ulcerations on the valve and new, more proximal ulcerations in the ileum. FC increased further to 270 mg/kg. A third line therapy (azathioprine) was discussed and shortly initiated at a dose of 2.5 mg/kg body weight. No side effects were recorded during a full year of therapy with azathioprine after which time another repeat colonoscopy showed, for the first time, a modest improvement of the valve and ileal ulcerations—which had become confluent and more superficial. However new ulcerations appeared in the ileum, more proximally (Fig. 1E). FC levels
approached normal values (65 mg/kg) but histology only showed a modest reduction of the inflammatory component (Fig. 1F).

At this point the patient – extremely concerned about disease progression – decided to stop azathioprine and try (after extensive discussions) one single infusion of infliximab. The latter (5 mg/kg body weight) was given 6 months after the last azathioprine dose after screening for latent tuberculosis (skin test and chest x-rays) yielded negative results. One month later ileocolonoscopy showed complete disappearance of ulcerations while histology showed a minimal degree of residual inflammation (Fig. 1F). FC was reduced to 40 mg/kg. At this point we reiterated the experimental nature of this approach for the patient condition and the possibility of long term side effects with maintenance infliximab. After extensive discussions, the patient discontinued infliximab but she also refused to take other medications. The following colonoscopy was performed 16 months later (6 years after the initial diagnosis) with the patient still completely asymptomatic. It showed disease recurrence with deep valve and ileal ulcerations, typical of full blown CD (Fig. 1G). Histology confirmed disease progression with frequent ulcerations, lymphoid follicular hyperplasia and focal fibrosis in the lamina propria and submucosa. FC had increased again to 270 mg/kg. All along, all the routine blood tests including CRP have remained within limits. The patient has since refused further instrumental follow-up. As of today, she has reported 2 episodes of self-limited diarrhea (2–3 bowel movements/day unclear if related) but no pain or other symptoms. All along, given the goal of arresting the progression of the disease (rather than inducing symptom remission) traditional steroid therapy was not considered.

Tissue cytokines and cell types from diseased areas were quantified by immunohistochemistry after each colonoscopy and correlation analyses were performed (see Section 2). The score map using the first (PC1) and second (PC2) principal components (Fig. 2) shows that almost all variation in the data is accounted for by these two independent factors.

The most important component (82.3%) was associated with inflammation. PC1 scores were positively associated with endoscopy/histology scores, FC, TNF-α, IL-18, IL-21, IL-23N, IL-23p19, IFN-γ but also with the antiinflammatory cytokine IL-10 and with CD4/CD25/FOX3+ (Treg cell) number (group I). There was a strong correlation (≥0.9) between endoscopic scores and TNF-α, IFN-γ and Treg’s (all p ≤ 0.005); between histologic score and Treg’s (p = 0.002); between FC and TNF-α, IL-18, IFN-γ, and Treg’s (all p ≤ 0.002); and between IL-21 and IL-18, IL-23N, and IFN-γ (all p ≤ 0.004). IL-23p19 had a moderate correlation (≥0.55) with IL-21, IL-18, and IL-23N (all p > 0.1).

PC1 scores were negatively associated with the antiinflammatory cytokine TGF-β but also with the proinflammatory cytokines IL-4, IL-6, IL-22, IL-17 and CD4/CCR4 + CCR6+ cell numbers (group II). There was a strong correlation (≥0.9) between IL-4 and TGF-β (p = 0.005); between IL-6 and CD4/CCR4 + CCR6+ cell numbers (p < 0.001); between CD4/CCR4 + CCR6+ cell numbers and TGF-β (p = 0.003). IL-22 had a small correlation with all the other variables in group I. The pairwise correlation coefficients between variables in group I and II were all negative, except for IL-22 vs histologic score (0.16) and IL-22 vs Treg’s (0.09). IL-12 was unmeasurable at most time points and therefore excluded from the analyses.

The second component (15.8%) was strongly associated with IL-22 and IL-23p19 levels.

In Fig. 3, standardized values are plotted against time. The behavior of all group I variables (panel A) differed little over time except for IL-23p19 which peaked earlier. All group II variables (panel B) followed a mirror-like pattern compared to those of group I except for IL-22 which departed from the overall pattern and reached its lowest level earlier in time.

4. Discussion

We have described here – to our knowledge – the first detailed diagnosis and follow-up of preclinical CD. Care was taken from the initial colonoscopy to exclude all the other known causes of ileitis. The disease first appeared at the valve-ileal transition and within several years became more severe and progressed more proximally. It is unclear whether, 6 years after the initial diagnosis, the patient has developed CD related symptoms or not. Overall, the time course from diagnosis to the full blown endoscopic picture of CD in our patient appears similar to the time interval from diagnosis to symptoms recently reported by Esch and colleagues in patients diagnosed by chance with asymptomatic ileitis. This is a most interesting finding because it suggests that CD diagnosis could be made much earlier than it is customary today in clinical practice and because such a long latency period might offer great opportunities for timely intervention—well before complications have taken place. Indeed, prospective follow-up of this patient allowed us – after an initial period of observation of 18 months – to start a step-up therapeutic approach to arrest disease progression. Mesalazine, budesonide and to a large extent azathioprine were incapable of healing the mucosa. By contrast, one single infusion of infliximab quickly restored the mucosal integrity. However, the disease recurred several months after the infusion.

Over time, a number of typical inflammatory cytokines (TNF-α, IL-18, and IFN-γ) correlated with the endoscopy/histology scores and FC. However, Treg’s and IL-10 also correlated with endoscopic/histologic inflammation and FC. By contrast, CD4/CCR4 + CCR6+ (Th17) cell numbers and the cytokines IL-4, IL-6, IL-22 and IL-17 were negatively correlated with inflammation. The cytokines IL-23p19 and IL-22 appeared to reach their highest and lowest tissue values, respectively, at an earlier time point during follow-up compared to the other cytokines.

Increased Treg numbers – possibly TNF-α-induced – may reflect partial tissue resistance to their antiinflammatory activity. However, Treg’s excess may also inhibit Th17 response which may in turn be ineffective in blocking Th1 response, a potential major driver of inflammation. However it should be kept in mind that FOX3 is also transiently expressed in activated human effector T cells, a potentially confounding factor when using CD4, CD25 and FOX3 as markers of Treg’s in humans. Furthermore, it should be noticed that immunohistochemistry is a technique heavily dependent on the choice of antibodies and laboratory methods and false negative results or nonspecific staining are not
uncommon. Although each test was run at least in triplicate and with proper controls (see Section 2) we do acknowledge that these results should be replicated on a larger scale and with a variety of approaches.

In conclusion, we have shown here that a colonoscopy-based strategy might allow early diagnosis of CD in asymptomatic FDRs. In our patient the disease evolved over the course of many years, thus offering the opportunity of therapeutic intervention to prevent symptoms onset and complications. In this individual patient only infliximab appeared capable of reversing the endoscopic lesions—which recurred after stopping the medication. Immunohistochemistry studies suggest that in these early years relative tissue resistance to Treg's might drive the disease.

Figure 2  Score map for the first two functional principal components. The first (PC1) and second (PC2) components accounted for, respectively, 82.3 and 15.8% of the variability in the data. PC1 scores are positively associated with variables on the positive horizontal semi-axis (group I) but negatively associated with variables on the negative horizontal semi-axis (group II). Each circle is color-coded according to the value of the pairwise correlation with endoscopic score (filled triangle) representing positive correlation with inflammation (red) or negative correlation with inflammation (blue).

Figure 3  Smoothed temporal trajectories for group I (A) and group II (B) variables as defined in Fig. 2. All values are standardized to the unit interval. The curves for the cytokines IL-23p19 and IL-22 are marked by a black bold line. Curves within each group differ little—mainly by amplitude except for IL-23p19 (A) which shows an earlier temporal peak compared to the other variables and IL-22 (B) which appears to reach its lowest tissue value at an earlier time point. Each time point represents the time at which the biopsy was taken after diagnosis, as in Fig. 1.
Ethical, safety and cost effectiveness issues are always at stake in managing asymptomatic individuals\textsuperscript{14,25} and great care should be taken when proposing a screening procedure to young, apparently healthy FDRs of CD patients. In addition, the psychological issues raised by such a diagnosis could be overwhelming and should also be properly taken into account from the outset. Nevertheless, we believe that a large but similarly designed study may offer important clues to effective ways of halting disease evolution and help us unravel the initial steps of CD pathogenesis.

**Conflict of Interest**

D Sorrentino has been a consultant for Janssen, Centocor, AbbVie, MSD, Hoffmann-LaRoche, Giuliani, Schering-Plough, and Ferring. The other authors have no conflicts of interest to disclose. No writing assistance was utilized in the production of this manuscript.

**References**


