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Genetic and environmental factors as predictors of disease severity and extent at time of diagnosis in an inception cohort of inflammatory bowel disease, Copenhagen County and City 2003–2005

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Abstract

Background and aims: The etiology of the inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), remains unknown. We aimed to investigate the influence of genetic, serological, and environmental factors on phenotypic presentation of IBD at diagnosis in a population-based Danish inception cohort from 2003–2005.

Methods: Three-hundred-forty-seven (62%) of 562 cohort patients were genotyped. ASCA and p/c-ANCA were determined and patients answered a questionnaire concerning environmental factors with possible influence on IBD.

Results: Fourteen percent of CD patients vs. 11% of controls were positive for common CARD15 mutation (ns), whereas more CD patients than healthy controls were homozygous for the OCTN-TC haplotype ($p=0.03$). ASCA was more common in CD (22%) than UC (14%) ($p=0.045$) and was related to age and localization of CD. p-ANCA was more frequent in UC ($p=0.00001$) but was related to pure colonic CD ($p=0.0001$). Sugar consumption was significantly higher in CD patients than in UC patients ($p=0.0001$) and more CD patients than UC patients had undergone appendectomy prior to IBD diagnosis ($p=0.03$). A possible relation between tonsillectomy and disease severity in CD, and a relation between use of oral contraception and disease localization of UC to rectum/left-sided colon were found.

Conclusions: In this cohort of unselected IBD patients we found a very low frequency of mutations in IBD susceptibility genes and observed a greater impact of ASCA and ANCA than of genetic factors on disease phenotypes. In addition, several environmental factors seemed to influence disease occurrence and disease presentation in both UC and especially CD.

1. Introduction

The incidence and prevalence of both Crohn's disease (CD) and ulcerative colitis (UC) have increased in recent years, but there is still a variation across Europe.[1–8] The causes of the rise in incidences have not been identified.

Despite excessive research, the etiology of inflammatory bowel diseases (IBD) remains unknown. CD and UC are proposed to be multifactorial entities caused by a combination of genetic and environmental factors.

Epidemiological studies[9,3] and linkage analyses[10–13] have provided firm evidence that genetic determinants of susceptibility to IBD exist, and that further knowledge of genetics could lead to a better understanding of the etiology of IBD and thereby improvement of treatment.

Genetic factors represent some pieces of the etiological puzzle, but there is no doubt that several pieces remain to be identified, and there is, therefore, a need to focus more on the impact of environmental factors.[3]

The aim of the present study was to assess a Danish inception cohort of IBD patients, in which a significant increase in incidence of both CD ($8.6/10^5$/year) and UC ($13.4/10^5$/year) has recently been demonstrated,[14] in order to (i) analyze the frequencies of specific genetic and serological markers and their possible influence on the initial disease presentation, and (ii) identify possible environmental factors that may influence the initial disease presentation.

2. Patients and methods

All patients residing in a well-defined geographical area and fulfilling the strict international diagnostic criteria for IBD[15,16] were included during a two-year period from January 1, 2003 to December 31, 2004. Patients with colonic affection and acute and chronic histopathological colitis but without classical histological signs of CD (granulomas) or consistent signs of UC that could make differentiation possible, and where the condition demanded medical treatment, were classified as indeterminate colitis (IC).

A total of 209 CD patients, 326 UC patients and 27 patients with IC were initially registered and followed up as previously described.[14] Patients with IC were excluded in this paper. Within the short observation time in this study none of the patients changed diagnosis.

The CD and UC patients were carefully reviewed regarding ethnicity to match 755 healthy Caucasian controls with age above 50 years and no IBD diagnosis, obtained from a colorectal cancer case-control study (manuscript in preparation). Fifteen CD patients and 32 UC patients were excluded from the IBD cohort because of non-Caucasian ethnicity. Also one of two CD twin patients was excluded due to the family relation.

The remaining patients were invited to blood sampling, which were collected from 156 (81%) of the 193 CD patients, and from 191 (65%) of 294 UC patients. The UC patients were more reluctant to respond initially and since mainly CD is found in previous studies to have a possible genetic cause no second contact was established to enroll further UC patients. The CD patients received a reminding letter.

The patients received a questionnaire reviewed by the IOIBD (International Organization of Inflammatory Bowel Diseases) covering 25 different topics proposed to be environmental risk factors for IBD.[17] In order to analyze possible associations between environmental factors and initial disease localization and initial disease severity, we chose to group the topics to the following 14 parameters: smoking, appendectomy, tonsillectomy, use of oral contraceptives (defined as never used, former user (>12 months prior to symptom onset), and current user (<12 month

before onset of symptoms) according to Corrao et al.,[18] breastfeeding during infancy, childhood infections, measles vaccination, BCG vaccination, sugar consumption (defined as daily use of either two or more teaspoons in each cup of tea or coffee, additional sugar on cereals or intake of several deciliters of soft drinks daily), fiber intake, fast food consumption, intake of caffeine (three or more cups of coffee or tea per day), hygiene and stressful events (e.g. death of close relative, financial problems, divorce).

Seventy-eight percent (121/156) of the genotyped CD patients and 75% (144/191) of UC patients answered the questionnaire.

2.1. Data collection

Information on date of onset of symptoms and IBD diagnosis, extent of disease, familial occurrence of IBD, extra intestinal manifestations, number of births, and occupational status was collected for all patients at the time of diagnosis.

2.1.1. Definition of extent and severity

The extent of disease at the time of diagnosis was for UC defined as proctitis (proximal extent to the sigmoid colon), left sided (to the splenic flexure), or extensive disease (beyond the splenic flexure), whereas for CD, the Vienna Classification[19] was used; L1: disease limited to the lower third of the small bowel with or without spill over into caecum, L2: any colonic location between caecum and rectum with no small bowel or upper gastrointestinal involvement, L3: disease of the terminal ileum and any location between ascending colon and rectum and L4: any disease location proximal to the terminal ileum regardless of any additional involvement of the terminal ileum or colon.

Both UC and CD patients were scored as having either severe disease or not at the time of diagnosis.

In UC, severe disease was defined as any extent and need of high dose steroid (0.5−1 mg/kg), and/or immunomodulator therapy (azathioprine, methotrexate, infliximab or other biological modifiers), and/or surgery.

The severity of CD was more complex to define. The general principles were to consider the site (ileal, ileocolonic, colonic, other), the pattern (inflammatory, stricturing, fistulizing) and the activity of the disease before therapeutic decisions were made.

Severe CD was defined as necessity for immunomodulator therapy (that was used in case of (1) $\geq$2 relapses/year or (2) steroid dependency/refractory disease, or (3) penetrating disease) and/or surgery within the first year after diagnosis.[20]

2.2. Blood samples

EDTA stabilized blood samples were drawn from patients. A plasma sample was collected for anti-*Saccharomyces cerevisiae* antibodies (ASCA) and antineutrophil cytoplasmatic antibodies (p/c-ANCA) typing, and the sediment and plasma were remixed. The DNA was purified from the peripheral leukocytes using QIAamp blood mini kit (Qiagen, Hilden, Germany), or as previously described.[21]

2.3. CARD15, OCTN and DLG5

CARD15 was genotyped for three genetic variants, 2104 C→T (SNP8), 2722 G→C (SNP12), and 3020insC (SNP13) using multiplex capillary electrophoresis single strand conformation polymorphism and DNA sequencing essentially as described previously[22] except that a multiplex PCR reaction and subsequent CE-SSCP analysis were performed rather than typing each variant separately. Three genetic variants in the IBD5 genetic region (5q31)[23] were investigated, OCTN (SLCA22A4, SLC22A5) and RS4705950. They were analyzed by Taqman analysis (Applied Biosystems, Foster City) (SLC22A4 1672C→T), by restriction enzyme cleavage (NciI; New England Biolabs, UK) (SLC22A5 -209 G→C) and by CE-SSCP (RS4705950 (IGR2230)). A genetic variant of DLG5 on chromosome 10q23 (113 G→A)[24] was genotyped by Taqman analysis (Applied Biosystems).

2.4. Antineutrophil cytoplasmatic antibodies (p/c-ANCA)

Freshly isolated human leukocytes were ethanol-fixed on microscope slides and used for p/c-ANCA (IgG) detection by indirect immunofluorescence (IIF) (for details see [25]). Slides were read on a fluorescence microscope and results given as negative, weakly, medium, or strongly positive for p/c-ANCA.

2.5. Anti-*S. cerevisiae* antibodies (ASCA)

Enzyme linked immunosorbent assay (ELISA) for antibodies (IgG, IgA, IgM) to *S. cerevisiae* was performed essentially as previously described.[26] Results were calculated in arbitrary units from a standard curve and cut-off (>10 units/ml) defined as the mean + 3 SD of 100 donors.

2.6. Statistical methods

All statistical analyses were performed using SPSS and the S-PLUS statistical software packages. Chi square tests were used to test for independence. A p-value of <0.05 was considered to be statistically significant. Logistic regressions were performed with disease severity and disease extent as dependent variables in order to elucidate the influence of various genetic, serological, and environmental factors.

3. Results

Demographic characteristics are outlined in Table 1.

3.1. Genetics

Table 2 shows allele frequencies (OCTN) and prevalence of at least one mutation for CARD15 and DLG5. The prevalence of having at least one CARD15 mutation was 14% in CD patients compared with 11% in controls (ns), and 11% in UC patients (ns). Among CD patients, no association between CARD15 frequency and age, gender, disease extent or severity at time of diagnosis was observed.

Both polymorphisms of the OCTN gene were significantly associated with occurrence of CD, as 47% of CD patients carried the SLC22A4 vs. 39% in controls (OR 1.36 [1.06–1.74], p=0.014)

Table 1 Demographic characteristics of inflammatory bowel disease patients from the Copenhagen inception cohort, 2003–2005

	Crohn's disease $n=156$	Ulcerative colitis $n=191$
Age median (range)	33 (10–85)	38 (8–90)
Observation time in months median (range)	11 (0–24)	12 (0–24)
Gender female:male ratio	1.3	1.2
Familial IBD (%)	32 (21)	24 (13)
Extraintestinal manifestations (%)	23 (15)	5 (3)
Localization at time of diagnosis (%)	Terminal ileum (L1) 48 (31) Colon (L2) 60 (38) Ileocolonic (L3) 37 (24) Upper GI (L4) 11 (7)	Proctitis 54 (28) Left sided 74 (39) Extensive 63 (33)
Fistulas (%)	25 (16)	–
Severe disease (%) (see definition in "Patients and methods")	63 (40)	66 (35)

variant and 52% carried the SLC22A5 variant vs. 44% in controls (OR 1.36 [95% CI: 1.07–1.74], $p=0.013$). In addition, the two point haplotype, 1672T and -207C (OCTN-TC), was associated with increased risk of CD in individuals who were homozygous for the mutation (24% of CD patients vs. 17% of controls; OR 1.61 [95% CI: 1.06–2.43], $p=0.02$). The significant difference persisted when frequencies of homozygous of the risk associated allele, RS4705950 (IGR2230), were combined with the TC-haplotype: 23% in CD patients vs. 16% in controls (OR 1.64 [95% CI: 1.07–2.49], $p=0.02$). Among CD patients no associations between the OCTN-TC haplotype and age, gender, disease extent or severity at diagnosis were observed. No significant differences were found between UC patients and controls or between CD patients and UC patients regarding the aforementioned polymorphisms.

Finally, mutations in the DLG5 gene occurred with similar frequency in CD patients, UC patients and controls, as 23% of CD patients vs. 18% of UC patients and 19% of controls carried at least one mutation.

3.2. ASCA and p/c-ANCA

Thirty-five of 156 CD patients (22%) were ASCA positive compared with 26 of 191 (14%) of UC patients ($p=0.045$). Among CD patients, no association was found between ASCA and gender, but a significant association was noted between positive ASCA and age <40 years at diagnosis (OR 5.34 [95%CI: 1.94–14.7], $p=0.0004$) and between ASCA and any small bowel involvement, as 31% of CD patients were ASCA positive compared with 8% with pure colonic CD (OR 2.58 [95% CI: 1.82–13.76], $p=0.001$). Regarding initial disease severity, the prevalence of positive ASCA seemed higher, although not significant, among CD patients with severe disease as compared to patients with no severe disease (30% vs.17%, OR 2.08 [95% CI: 0.97–4.45], $p=0.057$). No associations were found between ASCA status and CARD15, DLG5 or OCTN-TC mutations.

Positive p/c-ANCA was significantly more frequent among UC patients (104/191, 54.5%) than among CD patients (19/156, 12.2%) (OR 8.62 [95% CI: 4.93–15.06], $p=0.00001$). In UC patients, p/c-ANCA was not correlated to age, gender, disease severity or disease extent, whereas in CD, 15 of the 19 patients (79%) with positive p/c-ANCA had pure colonic disease ($p=0.0001$). Of the 15 patients with positive p/c-ANCA, three were ASCA positive (20%). In comparison 13.3% of UC patients were p-ANCA+/ASCA+.

Table 2 Allele frequencies of OCTN and the prevalence of at least one mutation of CARD15 and DLG5 (*N* and percentages) in Crohn's disease (CD) and ulcerative colitis (UC) patients and controls

	Crohn's disease	Ulcerative colitis	Controls
CARD15			
At least one mutation *n* (%)	22 (14)	20 (11)	86 (11)
Arg702Trp (SNP8) *n* (%)			
– Wild type	141 (90)	186 (97)	707 (94)
– Heterozygote	14 (9)	5 (3)	48 (6)
– Homozygote	1 (0.5)	–	–
Gly908Arg (SNP12) *n* (%)			
– Wild type	151 (97)	188 (98)	742 (98)
– Heterozygote	5 (3)	3 (2)	13 (2)
– Homozygote	–	–	–
Leu1007insC (SNP13) *n* (%)			
– Wild type	151 (97)	179 (94)	729 (97)
– Heterozygote	4 (2.5) [a]	12 (6)	26 (3) [a]
– Homozygote	1 (0.5)	–	–
OCTN			
SLC22A4 *n* (%)			
– Wild type	48 (31)	61 (32)	288 (38)
– Heterozygote	70 (45)	91 (48)	341 (45)
– Homozygote	38 (24)	39 (29)	126 (17)
– Allele frequencies	146 (47) [b]	169 (44)	593 (39)
SLC22A5 *n* (%)			
– Wild type	39 (25)	52 (27)	249 (33)
– Heterozygote	73 (47)	92 (48)	349 (46)
– Homozygote	44 (28)	47 (25)	157 (21)
– Allele frequencies	161 (52) [b]	186 (49)	663 (44)
RS4705950 *n* (%)			
– Wild type	49 (31)	65 (34)	290 (38)
– Heterozygote	70 (45)	90 (47)	340 (45)
– Homozygote	37 (24)	36 (19)	125 (17)
– Allele frequencies	144 (46) [b]	162 (42)	590 (39)
DLG5			
At least one mutation *n* (%)	36 (23)	34 (18)	142 (19)

[a] Three of four SNP 13 heterozygote were compound with SNP8 in CD patients, and one of controls was compound SNP13 and SNP8.
[b] $p=0.01$ Crohn's disease vs. controls.

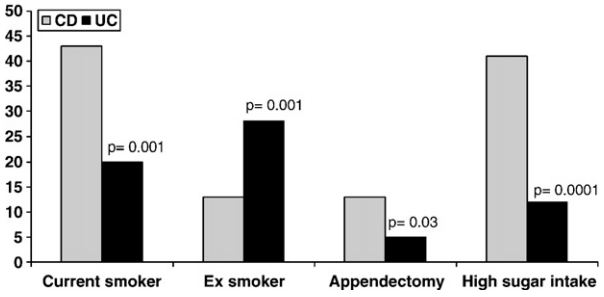

Figure 1 Relation between selected environmental factors and inflammatory bowel disease.

3.3. Environmental factors

Relations between selected environmental factors and IBD are outlined in Fig. 1.

No interplay between CARD15, OCTN, DLG5 and environmental factors was observed.

When comparing CD and UC patients, significant differences regarding smoking habits, sugar consumption, and frequency of appendectomy were observed. More CD patients (43%) than UC patients (20%) were current smokers at diagnosis ($p=0.001$) and more UC patients (28 %) than CD patients (13%) were ex-smokers ($p=0.001$). Sugar consumption was significantly higher in CD patients than in UC patients ($p=0.0001$) and more CD patients than UC patients had undergone appendectomy prior to IBD diagnosis ($p=0.03$). A possible relation between tonsillectomy and disease severity in CD, and a possible relation between use of oral contraception and disease localization of UC to rectum/left-sided colon were found (data not shown).

Besides the already mentioned environmental factors, other risk factors investigated such as vaccinations, childhood exposure to virus, i.e. measles, breastfeeding, other dietary factors, hygiene, and stress were not found to be associated with disease severity or extent.

3.4. Logistic regression

Logistic regression showed that age below 40 years at diagnosis (OR 2.53 [1.04–6.15], $p=0.04$) was independently associated with disease severity. None of the genetic or serological factors investigated seemed to influence the severity or extent of CD or UC.

4. Discussion

In the present study of genetic, serologic and environmental factors and their influence on the initial disease presentation in a cohort of unselected IBD patients, we found that CD susceptibility was, although weakly, related to the OCTN-TC haplotype, but not to CARD15 or DLG5 mutations. No gene polymorphisms were related to specific disease phenotypes, whereas ASCA and p/c-ANCA were related to localization of CD, and ASCA was related to severity of CD. The influence on IBD of the well described environmental factor, smoking, could be confirmed. Sugar consumption was significantly higher in CD patients compared with UC patients and the rate of appendectomy was higher in CD than in UC patients.

IBD are thought to be diseases with a more complex relation between genotypes and phenotypes than in simple Mendelian disorders. Only a minority of IBD patients report a positive family history of IBD. Sporadic cases are therefore of great interest in solving the etiological riddle of IBD. One may even ask whether the genetic polymorphisms identified initially in familial cases of IBD are of the same importance in the pathogenesis of sporadic IBD.[27] This study presents relatively high familial IBD rates and low occurrence of genetic polymorphisms with possible impact on IBD etiology. This was not to be expected, and the explanation must be that the definition of familial IBD is somewhat broader including third and fourth degree relatives.

To our knowledge, the present study is one of few studies investigating the frequency of IBD susceptibility genes in an unselected cohort of sporadic IBD patients.[28] A low frequency of the CARD15 mutation in both CD patients, UC patients, and controls was observed, in accordance with the findings reported in a recent study of unselected patients from the same area[29]; whereas previous studies of highly selected patients showed higher frequencies of CARD15 in CD.[22,30] On the other hand, we found that carriers of OCTN variants were slightly more susceptible to CD but not to UC, as recently shown by Waller et al.[31] We could, however, not confirm the previously reported interaction between OCTN-TC haplotypes and CARD15 genotype in the etiology of CD,[32] perhaps due to the low frequency of CARD15 variants in the Danish population. No associations between OCTN-TC haplotypes or disease severity or extent of CD as is recently reported[33] were observed. Lastly, no associations between DLG5 variants and CD or UC were found, as reported previously by others.[34,35]

Thus, overall low frequencies of genetic variants were observed in the present study, and the use of susceptibility gene frequencies as diagnostic markers, therefore, does not seem to be of great value in unselected patient groups. Whether genetics can be of any value as prognostic markers is yet too early to say, therefore follow-up studies of unselected cohorts are needed.

The prevalence of especially ASCA was low in this cohort of unselected patients compared to observations made by other groups over the years,[36–38] but in spite of this finding significantly more CD patients than UC patients had positive ASCA serology, and ASCA was found to be related to age below 40 years at CD diagnosis and to small bowel CD. Furthermore, a trend towards an association between positive ASCA and severity of CD was observed. p/c-ANCA was more common in UC patients, and with a prevalence similar to other studies.[36–38] p/c-ANCA was not associated with either disease severity or extent of UC, but was related to pure colonic CD.

The low prevalence of these markers and almost identical frequencies in ulcerative colitis and Crohn's colitis could indicate that an application as markers for clinical use is of limited value. These markers may, however, be useful in predicting disease course.

Over the past 25 years several studies have been accomplished investigating the role of several and seemingly unrelated environmental factors which may play a role in the etiology of IBD.[3] The drawback of these types of studies, typically based on retrospective questionnaires, is the obvious risk of recall bias. In the present inception cohort study, this problem was to some extent avoided, since the

majority of patients were diagnosed at a young age[14] and because the questionnaire was handed to the patients within a short time after diagnosis. A control group was, however, not included when investigating these environmental factors, which is a drawback of this study. The questionnaire is, however, recently validated in a control group of patients with orthopedic diseases recruited from the same geographical area.[39]

Smoking is undoubtedly the most investigated environmental factor in relation to IBD and is known to have a strong negative impact on the course[40] and severity of CD[41] but a protective effect in UC. These observations were confirmed in the present study. It has been hypothesized that nicotine in cigarettes has an anti-inflammatory effect by affecting the antigen-receptor-mediated signals.[42]

In our study, appendectomy performed before diagnosis of IBD was more frequent in CD patients than in UC patients. This is in accordance with a Danish registry cohort study[43] and with recent Swedish studies, reporting an increased risk of CD following appendectomy[44] and an inverse relation in UC patients who had undergone appendectomy before the age of 20.[45] The mechanism by which appendectomy protects against UC but enhances the risk of CD is unknown. It is suggested, but still not clarified, that appendicitis is mediated by the same inflammatory response (Th-1 response) as observed in CD, rather than by the Th-2 response related to UC.[45]

Dietary factors have been another suspect on the list of potential environmental factors playing a role in development of IBD, and CD patients have previously been shown to consume more sucrose and less fiber than controls.[46,47] Accordingly, we observed that the two patient groups also differed when it came to reported sugar consumption CD patients consumed more (refined) sugar than UC patients. It remains, however, to be elucidated why CD patients are reported to have higher sugar consumption than UC patients and controls. Our question regarded sugar consumption prior to diagnosis of CD and does therefore not support the hypothesis, that the inflammatory response, which in CD is far more disseminated than in UC, may lead to fatigue and weight loss and thereby to increased sugar consumption.[48] The highly significant difference in sugar consumption, which has previously been shown[49] could be a real finding. There is, however, a great need of focused and quantitatively well-planned studies on a few dietary components to validate these results.

In UC patients, an association between current usage of oral contraceptives and localization of UC to rectum/left-sided colon was observed. Previous studies have suggested a relation between oral contraceptive use and risk of IBD, although there has been some controversy over the years as to whether usage increases the risk of both UC and CD[50] or does not increase the risk of CD.[51]

The observed relation between oral contraceptive use and proctitis/left-sided localization of UC in the present study actually suggests a somewhat protective role of these medications, however, in this study it was not possible to asses the all over risk of using oral contraceptives on IBD because no controls were available.

In addition to smoking, appendectomy, sugar intake, and usage of oral contraceptives, other risk factors such as vaccinations, childhood exposure to virus, breastfeeding, hygiene, and stress have been suggested in the etiology of CD and UC. However, some of these factors may actually not be factors in themselves but rather markers for other unidentified influences. In the present study, we found associations between severity of CD and absence of tonsillectomy — in contrast to what has been reported by some[52] but questioned by others.[53]

As it appears, our findings concerning environmental factors both confirmed and differed from what previously has been hypothesized. The rise in IBD incidence is undoubtedly linked to some of these factors.

In conclusion, this population-based study of patients with IBD showed that in spite of a worldwide evidence of heritable factors in the genesis of both CD and UC, both serologic and environmental factors play important roles as well. We found a very low frequency of mutations in IBD susceptibility genes and observed a greater impact of ASCA and ANCA than of genetic factors on disease phenotypes. In addition, several environmental factors seemed to influence disease occurrence and disease presentation in both UC and especially CD.

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