Early-onset Crohn Disease Is Associated With Male Sex and a Polymorphism in the IL-6 Promoter

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ABSTRACT

Aims: Pediatric onset of Crohn disease (CD) is characterized by male sex predominance while adult-onset disease demonstrates female sex predominance. It has been postulated that this phenomenon may be genetically determined or due to an effect of estrogen on age of onset. Interleukin (IL)-6 modulates the T<sub>H</sub>17 pathway, and the IL-6 promoter is modulated by estrogen, possibly linking genetically determined inflammation and the presence of estrogen. The aim of our study was to investigate whether differences in IL-6 promoter genotype could explain male sex in earlier disease onset.

Patients and Methods: We genotyped 333 patients with CD and 100 controls. 162 pediatric-onset patients (age of onset 18 years and younger) for the IL-6-174 polymorphic site. Genotype, sex, and age of onset were compared.

Results: Males with IL-6-174GG genotype (the wild-type allele) had an increased risk for a younger age of onset compared to males with IL-6-174GC or CC genotype (G → C genotype), hazard ratio (HR) 1.49, P = 0.02, 95% confidence interval (CI) 1.07–2.09. Females with GG genotype were not found to have an increased risk for a younger age of onset compared with females with G → C genotype, HR 1.01, P = 0.96, 95% CI 0.72–1.41.

Conclusions: Males with IL-6-174GG genotype are prone to develop CD at a younger age than males with the IL-6-174G → C genotype. Our study suggests that age of onset may be modified by the IL-6-174GG genotype and this modification is sex dependent. This may be due to increased transcription of IL-6, an effect that may be repressed by estrogen in females.

Key Words: age of onset, Crohn disease, child, genes, interleukin-6, inflammatory bowel disease, phenotype

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C rohn disease (CD) is a complex chronic inflammatory disorder of the gastrointestinal tract that can affect both children and adults. The pathogenesis of the disease remains enigmatic, although there are clear genetic and environmental components. The disease is characterized by a T helper 1 (T<sub>H1</sub>) and T helper 17 (T<sub>H17</sub>) response leading to gut inflammation (1).

Evidence for the genetic susceptibility of the disease was provided initially by epidemiological studies and concordance rates in twins (2–4), and the breakthrough in our understanding of the genetic basis for CD occurred in 2001, with identification of the Nucleotide-binding Oligomerization Domain2/Caspase Recruitment Domain15 (NOD2/CARD15) gene in the pericentromeric region of chromosome 16 (5,6). Subsequently mutations in the interleukin-23 receptor (IL-23R) (7–13) and an autophagy gene (ATG 16L1 gene) were identified as important susceptibility genes (14,15). Multiple other genes identified by genomewide association screens may also play a role in inflammatory bowel disease susceptibility (16–18).

Early age of onset (pediatric onset) could be a random event, it could be environmentally determined, or it could be determined, by genetic factors. Identification of factors that determine age of onset could lead to a better understanding of the pathogenesis of the disease, especially if it is genetically determined. Two consistent differences between early-onset CD and adult-onset CD are the site of the disease (more colonic involvement in younger children) and the predominance of male sex in early-onset disease (19–21).

Several lines of evidence suggest that early age of onset may be a genetically determined phenotype (20,22). Epidemiological studies in adult patients with CD have demonstrated a 20% to 30% higher incidence of females in CD than in males. In the pediatric CD population there is a male predominance (about 62% males, depending on the population). The male predominance is reversed after adolescence (23–27).

Three plausible theories have been put forward to explain the difference between the sexes in age of onset: One possibility is that certain disease susceptibility genes may increase the risk for male sex and early onset, or conversely, that these same genes may have a protective effect in females (22,28). A second possibility is that a sex-specific – disease-modifying gene (independent of any disease susceptibility gene) may affect age of onset in 1 of the sexes. Lastly, sex hormones could affect the age of onset through a regulatory effect on inflammatory pathways.

Interleukin-6 (IL-6) is a crucial cytokine in the inflammatory reaction in CD. It plays an important role in differentiation of T helper 17 (T<sub>H17</sub>) cells and induces upregulation of the IL-23R (11). The IL-6 promoter is regulated by estrogen and estrogen receptors, which act to reduce expression of IL-6 (12,13).

In addition, the C allele from a single nucleotide polymorphism (SNP) at position -174 of the promoter of IL-6 (-174G → C) is associated with decreased transcription and secretion of IL-6.

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These 2 characteristics of IL-6 promoter make it an ideal candidate as a disease-modifying gene responsible for the unexplained phenomena of pediatric male predominance. We hypothesized that a higher level of IL-6, which could activate the T1/17 pathway, would predispose to earlier onset of the disease. In accordance with this hypothesis, we surmised that patients under the influence of estrogen or the -174G → C SNP would produce less IL-6, which would be less prone to a T1/17 inflammatory response, and therefore develop CD at a later age of onset. Conversely, patients with no exposure to estrogen (males) and absence of the modifying effect of the 174G → C SNP on IL-6 transcription would be prone to early-onset CD. To evaluate this premise, we investigated the role of -174G → C SNP stratified by sex and age of onset of patients with CD.

Patients and Methods

Patients were recruited from pediatric and adult gastroenterology programs distributed throughout central and northern Israel and were declared eligible if CD was confirmed by established criteria based on clinical, radiological, endoscopic, and histopathological findings (31). A diagnosis of CD for this study required at least 2 of the following: history of abdominal pain, weight loss, short stature, malaise, rectal bleeding, or diarrhea; characteristic endoscopic findings of discontinuous ulcerations, cobblestoning, fistula, or severe perianal disease; radiologic features of stricture, fistula or evidence of cobblestoning, or ulceration of the mucosa; macroscopic appearance at laparotomy of typical bowel induration, mesenteric lymphadenopathy, or serosal involvement showing creeping fat or other inflammatory changes; or histopathology showing transmural inflammatory cell infiltrate or epithelial granulomas and absence of identifiable infectious agents (32). Recruitment took place from March 2003 to March 2005.

The Wolfson Medical Center review board and the Israel Ministry of Health genetic research review board approved the recruitment protocol. Informed consent was obtained from all of the parents or patients. Data regarding the age of onset and sex were obtained from the patients and the referring gastroenterologist and registered earlier to genotyping. Site of disease was recorded using the Vienna classification. We did not analyze disease behavior due to the significant variability in duration of disease among the patients, nor was this the objective of the study.

All of the control donors were adult Israeli citizens (older than 18 years) and gave written informed consent for the study of their genetic material (DNA) for biomedical research. All of them reported that they were in general good health. Their DNA was purchased from the National Laboratory for the Genetics of Israeli Populations, Tel-Aviv University, Israel.

Genotyping for the IL-6-174G → C Polymorphism

Genomic DNA was extracted from whole peripheral venous blood, using a commercially available kit (Genta, Minneapolis, MN) in accordance with the manufacturer’s instructions.

The -174G → C polymorphism in the IL-6 promoter was genotyped by amplifying a 234-bp fragment with the primer pair: P 5’-AAA GGA AGA GTG GTT CTT-3’ and R: 5’-TCC TAT ATT TAT TGG GGG TCG AGA-3’. The 50-μL reaction mixture contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 200 μM dNTPs, 5 μM/L of each primer, 100 ng of genomic DNA, and 1.25 U of AmpliTaq Gold DNA polymerase (Perkin Elmer Applied Biosystems, Roche Diagnostic Systems, Indianapolis, IN) with an initial denaturing step of 7 minutes at 95 °C to activate the polymerase followed by 35 cycles of 94 °C; 15 seconds, 60 °C; 45 seconds, 72 °C; 45 seconds and a final extension of 10 minutes at 72°C. Predicted sizes were confirmed by agarose gel electrophoresis. Polymerase chain reaction product was digested overnight with NlaIII (New England Biolabs, Beverly, MA). Digests were run on a 3% agarose to determine the G or C alleles: 45- and 188-bp fragments for the G allele, 45-, 75-, and 111-bp fragments for the C allele.

Data Analysis

Data analysis was carried out using SPSS version 9.0 statistical analysis software (SPSS Inc, Chicago, IL). Continuous variables, such as age of onset, are described by mean ± standard deviation (SD). Categorical variables such as sex and presence or absence of a polymorphism are described by frequency tables. The t test for independent samples was used to compare means of continuous variables by polymorphism. The Pearson χ² test for independence was used to assess associations between categorical variables by polymorphism. Additionally, allelic frequency was compared between males and females using the Pearson χ² test. Age at disease onset was modeled using a Cox proportional hazards regression model with sex and presence of polymorphism as covariates, and the cumulative hazard function was estimated. All of the tests are 2-sided and considered significant at P < 0.05 unless specified otherwise.

Results

Demographic Data

We collected 433 DNA samples, 333 patients with CD, and 100 controls. Among the patients with CD were 168 males and 165 females and among 100 controls were 54 males and 46 females. Twenty-three samples (6.9%) of the patients with CD were insufficient for genotyping because of previous genetic studies. Sex prevalence for the patients with the missing genotype data, 13 males (56.5%) and 10 females (43.5%), was compared with patients of the genotyped group, 155 (50%) males and 155 (50%) females, and was not found to be significantly different (P = 0.6). Approximately 51% of patients were of Ashkenazi origin, 18% mixed Ashkenazi + Sephardic, and the rest of Sephardic origin. Clinical and demographic characteristics of our patients are presented in Table 1.

The mean ± SD age of onset for male patients was 18.15 ± 9.88 years, significantly younger than female patients, 23.4 ± 12.7 years (P = 0.0001).

Data on the prevalence of SNP IL-6-174G → C within cases and controls is presented in Table 2. No difference in the prevalence

<table>
<thead>
<tr>
<th>Parameter</th>
<th>% (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>50.5% (168)</td>
</tr>
<tr>
<td>Age of onset ± SD, y</td>
<td>18.15 ± 9.88</td>
</tr>
<tr>
<td>Females</td>
<td>49.5% (165)</td>
</tr>
<tr>
<td>Age of onset ± SD, y</td>
<td>23.4 ± 12.7</td>
</tr>
<tr>
<td>Ileitis</td>
<td>38.4% (128)</td>
</tr>
<tr>
<td>Colitis</td>
<td>15.9% (53)</td>
</tr>
<tr>
<td>Ileocolitis</td>
<td>38.7% (129)</td>
</tr>
<tr>
<td>Isolated upper GI tract</td>
<td>5.1% (17)</td>
</tr>
<tr>
<td>Missing data</td>
<td>1.8% (6)</td>
</tr>
</tbody>
</table>

GI = gastrointestinal; SD = standard deviation.

Table 1. Clinical and demographic characteristics of patients with CD

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of the polymorphism was detected between the cases and the controls ($P = 0.9$).

### Age of Onset by Genotype and Sex

The mean age of onset for males and females within the different genotypes is presented in Table 3. Males with GG genotype (absence of SNP) had a significantly younger age of onset than males with IL-6G→C (presence of SNP at 1 or both alleles; ie, GC or CC), 16.7 ± 7.3 years and 20.37 ± 12.02 years, respectively ($P = 0.017$). In females, no difference in age of onset was detected by genotype, such that the mean age of onset in females with the GG genotype was 23.6 ± 12.6 years compared with 23.1 ± 13.1 years among G→C females, $P = 0.8$.

The cumulative incidence of disease onset by age stratified by sex and by IL-6-174 genotype is presented in Figure 1. This figure plots the fact that males with CD with the GG genotype in our study had a higher cumulative incidence of disease onset at an earlier age compared to males with G→C genotype and compared to females with both genotypes. Remarkably, 80% of males with the GG genotype developed CD before the age of 20 years.

A Cox proportional hazards regression model was applied to age of onset of the 310 patients, with polymorphism status and sex as covariates. Figure 2 depicts the 4 hazard rate functions according to sex and polymorphism status. In the polymorphism status, we compare patients with GG genotype versus patients with G→C genotype.

Male sex significantly increased the risk of early disease onset compared to females (hazard ratio (HR) 1.7, $P < 0.0001$, 95% confidence interval [CI] 1.4–2.2). The increase risk of GG genotype for early age of onset was significant compared to G→C genotype (but close to significant) (HR = 1.3, $P = 0.06$, 95% CI 0.99–1.6).

The interaction effect of sex and polymorphism was observed to add an additional risk rather than sex or genotype alone (HR 1.6, $P = 0.04$, 95% CI 1.02–2.6).

Males with GG genotype had an increased risk for a younger age of onset compared to males with G→C genotype (HR 1.49, $P = 0.02$, 95% CI 1.07–2.09).

### DISCUSSION

Determinants for early age of onset in CD remain enigmatic. Increased prevalence of male sex in pediatric CD may provide a clue to factors determining age of onset or pathogenesis. We decided to explore the role of the IL-6 promoter as a candidate disease-modifying gene because IL-6 plays a critical role in the inflammatory response characteristic of CD (T lymphocyte pathway) (10,11) and its transcription is modulated by estrogen (12,13), therefore providing a possible link between sex and the T lymphocyte pathway. Four previous studies involving 384 patients with CD and 1292 controls did not find an association between SNP

#### Figures

**Figure 1.** Incidence of CD by age of onset stratified for sex and polymorphism. CD = Crohn disease.

Females with GG genotype were not found to have an increased risk for a younger age of onset compared with females with G→C genotype (HR 1.009, $P = 0.96$, 95% CI 0.72–1.4). Among patients with the GG genotype, male sex significantly increased risk (HR 2.14, 95% CI 1.59–2.88, $P < 0.0001$). Among the subjects with the GC genotype, male sex did not significantly alter risk (HR 1.2, $P = 0.3$, 95% CI 0.85–1.79).

It is evident that males with GG genotype (no polymorphism) have a higher hazard risk for childhood-onset CD than the other 3 groups.

#### Table 3. Mean and standard deviation of age of onset by sex and IL-6-174 genotype

<table>
<thead>
<tr>
<th>Age of onset mean ± SD, y</th>
<th>GG genotype</th>
<th>G→C (GC + CC) polymorphism</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>16.17 ± 7.3</td>
<td>20.37 ± 12.02</td>
<td>0.017</td>
</tr>
<tr>
<td>Female</td>
<td>23.6 ± 12.6</td>
<td>23.1 ± 13.1</td>
<td>0.83</td>
</tr>
</tbody>
</table>

C = polymorphic allele; G = wild-type allele; SD = standard deviation.
IL-6-174G → C and disease susceptibility (33–36). Two studies found an association between the SNP and phenotype. The first found the GG genotype to be associated with ileocolonic disease, whereas isolated colonic disease was related to CC genotype (34). The second study found that patients having CD with GG genotype are associated with significant growth retardation (35). Furthermore, this SNP has not been identified by genomewide association screen studies as a candidate susceptibility gene (16–18).

As anticipated, according to previous reports, our pediatric-onset cohort was characterized by male sex predominance (62.8%). The age of onset was significantly affected by the IL-6 genotype. Within the male population, patients carrying the IL-6-174GG genotype had a statistically significant younger age of onset. The genotype had no effect on females’ age of onset (Table 3).

Furthermore, we observed that 80% of male patients with CD who carried the GG genotype developed the disease during the first 2 decades of life. The hazard risk for developing CD at a younger age was 49% higher in the males with the GG genotype as compared with males with the G → C genotype. A Cox regression model found an interaction effect between sex and genotype with an HR of 1.6, demonstrating that the 2 risk factors for early age of onset create an additional risk for pediatric onset when both are present. This dataset supports our basic premise that IL-6 promoter plays an important role as a disease-modifying gene. The major question is why the effect of the SNP is observed significantly in males. A plausible explanation is that a state of high IL-6 transcription will favor early onset. Thus, patients with IL-6-174GG genotype should be at a higher risk for early-onset disease. The lack of affect of GG genotype on women could be that any modulation that dampens IL-6 transcription will therefore be protective from early onset. Circulating estrogen, which rises in the second decade of life in females, mitigates the effect of IL-6-174GG genotype by decreasing the transcription, resulting in males being preferentially affected and females preferentially protected from the GG genotype. It is evident (Table 3) that males tend to have a younger age of onset in comparison with females within each polymorphism status. Furthermore, Cox modeling confirmed an interaction between sex and the polymorphism that suggests an additive risk when both male and GG genotype risk factors are present. This hypothesis is investigated for the first time in a pediatric cohort and needs to be corroborated in other cohorts. The estrogen receptor is not the only polymorphism that influences the transcription of IL-6. Four polymorphic sites are known to participate in the transcription control in a synergistic fashion (37); however, the exact mechanism by which the IL-6-174 SNP may exert its effect remains unknown, and may be because of an association with other polymorphisms at binding sites for transcription factors on the promoter.

We did not perform an assay of IL-6 to try and verify our results for several reasons. Studies that have evaluated the effect of this polymorphism on IL-6 transcription have already been performed and published (30,31). Although measurements of IL-6 levels by polymorphism would have been better, this would have required us to take serum samples at disease onset in all of the patients and this was not a part of our ethical mandate. The time point of collecting cytokine samples is critical, unlike the timing of DNA collection. IL-6 levels will fluctuate with inflammation and treatment. To judge the effect of the polymorphism, all of the samples would have to be collected at a uniform time point. Duration of disease in our cohort varied: some patients had active disease, others were in remission, and treatments varied widely. IL-6 transcription is increased with disease activity (38), and use of agents that repress tumor necrosis factor-α (eg, infliximab) would also be likely to alter circulating IL-6, because tumor necrosis factor-α is a potent inducer of IL-6 (39). Soluble IL-6 receptors are also associated with active disease (40). These factors place a limitation on interpretation of circulating IL-6 under any circumstances other than the treatment of naive patients with active disease.

Our study is not the only study to find an impact of genotype on sex-dependent risk. Two other studies have linked a variant in the disc large homolog gene (DLG5) and sex-related susceptibility. Bianke et al (22) found that the DLG5 variant R30Q had a protective effect on susceptibility among females with CD. Another study that collected data from 12 studies found that the R30Q allele is associated with a small decreased risk of CD in females but not in males and that there is no difference in the allele frequencies between control males and females (28). DLG5 has been identified as a progesterone target gene (41).

Our study, as well as recent studies performed by other groups, join a growing body of evidence that suggests that pediatric-onset CD may differ from adult-onset CD, and that age of onset is not a totally random event (7–9,20–22,24,42,43). Taken together, these studies may illustrate that the differences in observed phenotype (male sex and more frequent colitis) may have their origins in different disease-susceptibility genes or in genes that modify the phenotype.

Results of the previously mentioned studies also indicate that previous methods for disease association with susceptibility genes may be inadequate. Because early-onset disease may differ from adult-onset disease, detection of determinants for early-onset disease may require cohorts composed of both pediatric-onset and adult-onset disease to detect associations, as well as analysis by sex.

In conclusion, we have shown preliminary evidence that early onset of CD may not be a totally random event but is due to a complex interaction between genotype and sex. Our data suggest that factors that increase inflammation and possibly factors involved via the T17,17 pathway may play a role in early onset of CD. Furthermore, this may suggest that genetic factors unrelated to susceptibility genes (disease-modifying genes) may affect disease phenotype. Given the importance of replication in multiple cohorts to establish causality in genetics studies, we believe that additional studies need to be performed to confirm our findings.

REFERENCES

24. Durno C. Mode of Inheritance and Demographics of Pediatric-onset Inflammatory Bowel Disease. [MSc Clin Epi thesis]. University of Toronto; 1999.