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Serious Lower GI Clinical Events with Non-Selective NSAID or Coxib Use: A Double-Blind GI Outcomes Trial of Naproxen vs Rofecoxib

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Epidemiologic studies suggest that NSAIDs increase the chance of lower GI adverse events as well as upper GI events. However, data from prospective studies regarding the rate of clinical lower GI events are not available. Coxibs decrease the rate of upper GI clinical events, but infomation on their effect on lower GI clinical events also is not available. We assessed serious lower GI clinical events in a post hoc analysis of a prospective, double-blind outcomes study of rofecoxib vs. naproxen in 8076 patients followed for a median of 9 months. METHODS: Rheumatoid arthritis patients ≥ 50 years of age (or ≥ 40 years if on steroids) were randomly assigned naproxen 500 mg bid or rofecoxib 50 mg qd. Patients were seen at 6 weeks, 4 months, q 4 months, and prn; phone contact was at 10 weeks and q 4 months. Evaluation and treatment were done at the discretion of investigators based on the clinical situation. Serious lower GI clinical events (assessed blinded to treatment allocation) were defined as bleeding beyond the duodenum with > 2 g/dl hemoglobin drop or hospitalization; and hospitalization for intestinal perforation, obstruction, ulceration, or diverticulitis. RESULTS (SEE TABLE): 35 patients had serious lower GI events: 24 with naproxen and 11 with rofecoxib. 27 patients had bleeding, 2 obstruction, 1 ulceration, 2 diverticular perforation, and 3 diverticulitis without gross perforation. In comparison, rates per 100 patient-years for all clinical upper GI events and complicated upper GI events in this study were 4.5 and 1.4 for naproxen and 2.1 and 0.6 for rofecoxib. CONCLUSIONS: Serious lower GI clinical events occurred at an annualized incidence of 0.9% in patients taking the nonselective NSAID naproxen. This is 64% of the rate for complicated upper GI events with naproxen in the study. Serious lower GI clinical events were 54% lower with the selective COX-2 agent rofecoxib than with naproxen.

Rates of Serious Lower GI Events per 100 Patients-Years in Patients Receiving Rofecoxib vs. Naproxen

Rofecoxib (N=4047)	Naproxen (N=4029)	Relative Risk (95% CI)
0.41	0.89	0.46 (0.22 - 0.93)*

*** Relative risk of rofecoxib to naproxen from COX model; p = 0.03**

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Epstein-Barr Virus-Induced Gene 3 Deficiency Disrupts T Helper 2-Mediated Immune Responses in Experimental Colitis

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The cytokine Epstein Barr virus-induced gene (EBI3) is an interleukin-12 p40 homologue expressed by macrophages and dendritic cells. Its function is unknown, however. To define the functional role of EBI3 we generated knockout mice in which the EBI3 gene was targeted by homologous recombination. EBI-3-deficient mice were phenotypically normal but resistant to the induction of Th2 cytokines and immunopathology associated with oxazolone-induced colitis; a form of colitis thought to be mediated by Th2 type cytokines. In particular, EBI3 deficient mice showed no significant weight loss upon oxazolone sensitization and re-challenge, whereas wild-type mice showed a marked weight loss within 48 hours. Furthermore, there was a significant reduction in histologic signs of colitis in oxazolone-treated knockout mice compared to oxazolone-treated wild-type mice. Finally, we found that the induction of mucosal and systemic IL-4 responses seen in oxazolone-treated wild-type mice were suppressed in oxazolone-treated EBI3 knockout mice. In contrast to the above data on oxazolone-induced colitis, the response to trinitrobenzene sulfonic acid-induced colitis, a T helper 1-mediated model, was unaffected in EBI-3 deficient mice. Although dendritic cells drom EBI-3-deficient mice exhibited normal IL-12 production, EBI3-deficient dendritic cells failed to promote a Th2 response to a model antigen in vivo. These studies imply a critical regulatory role for EBI3 in the induction of a Th2-type immune response and for the development of Th2-mediated chronic intestinal inflammation in vivo.

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Mutations in NOD2 are Associated with Fibrostenosing Disease in Patients with Crohn's Disease (CD)

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Background: The clinical manifestations of CD are diverse ranging from fibrostenosing disease of the small bowel to colon-predominant inflammation. These distinct clinical manifestations may be due to genetic, immunologic and microbial heterogeneity within CD. Mutations in the NOD2 gene have recently been described in CD and may alter innate immune responses in these patients. We hypothesized that mutations in the NOD2 gene may be associated with distinct phenotypic expressions of CD. Methods: Two separate CD patient cohorts of consecutively-identified patients referred to an IBD center (n = 142 collected between 1993-96; n = 50 collected between 1999-2001) were analyzed. An IRB-approved protocol permitted genotype testing and clinical data acquisition. Genotyping was performed for three rare alleles of the NOD2 gene, R675W, G881R, and 980fs, by the Taqman MGB system (Applied Biosystems, Foster City, CA, USA). CD was characterized as fibrostenosing, perianal fistulizing, internal perforating, or ulcerative colitis (UC)-like (Gut 2000:47) by a panel of IBD physicians unaware of genotype information. Results: Univariate analysis revealed that the CD-associated NOD2 variants are significantly associated with fibrostenosing disease in the initial panel ($p = 0.049$) and second independent panel ($p = 0.018$) for the three allelic variants combined. When the two cohorts were analyzed together, the association between NOD2 variants and fibrostenosing disease was more significant ($p = 0.003$). These relationships were observed in both Jews and non-Jews within the cohorts. Approximately 40-50% of CD patients with fibrostenosing disease (Jews 52%, non-Jews 41%) have at least one of these rare alleles as compared to only 25% of CD patients without fibrostenosing disease (OR 2.6 and 95% CI 1.4-4.8). Of the three rare alleles, the frameshift mutation, 980fs, demonstrated the greatest association with fibrostenosing disease (p for cohorts combined = 0.009). Conclusion: This is the first description of a genotype-phenotype correlation in patients with Crohn's disease and the NOD2 variants. These data suggest that certain factors leading to fibrostenosing disease are related to specific impairments in the innate immune response regulated by NOD2. An understanding of this pathway may result in identification of host-microbial factors resulting in fibrostenosis.

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CARD15/NOD2 Mutational Analysis and Genotype-Phenotype Correlation in 612 Patients with Inflammatory Bowel Disease

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Background : CARD15/NOD2 has recently been involved in Crohn's Disease (CD) genetic predisposition. However, a detailed phenotype-genotype correlation has not been investigated. Patients and methods : The entire coding sequence and the exon-intron boundaries of CARD15 was sequenced in 453 CD patients, 159 patients with Ulcerative Colitis (UC) and 103 healthy controls. A detailed phenotype including sex, family history, age at diagnosis, cigarette smoking habits, disease location, granuloma formation, stenosis, transmural involvement, extra-digestive symptoms and therapeutic management was recorded. Phenotype-genotype correlations were tested by ANOVA and Chi Square statistics. Results : 67 sequence variations were identified, of which 9 had an allele frequency higher than 5% in CD patients including 6 polymorphisms and three (R702W, G908R and 1007fs) main disease causing mutations (DCMs) (32%, 18% and 32% of the total CD mutations, respectively). Twenty-seven additional private mutations with a cumulative incidence of 19% were also considered as potential DCMs. No specific mutations were found in UC patients. Patients carrying two mutations in CARD15 were characterized by a younger age at onset (16.9 vs 19.8 years, $P = 0.01$), a more frequent stricturing phenotype (53% vs 28%, $P = 0.00003$, OR = 2.92) and a less frequent colonic involvement (43% vs 62%, $P = 0.003$, OR = 0.44) than those without mutation. The severity of the disease and extra-intestinal manifestations were not influenced by any of the CARD15 genotypes. The proportion of familial and sporadic cases and smoking habits were similar among patients with or without mutation. Conclusion : Patients with a double dose mutation in CARD15/NOD2 are caracterised by a younger age at onset, a structuring phenotype and a less frequent colonic involvement.

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Racial Differences in Nod2 Variation: Characterization of Nod2 in African-Americans with Crohn's Disease

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Background & Aims: Three major coding region polymorphisms within or near the leucine-rich repeat of Nod2 (Arg702Trp, Gly908Arg, Leu1007fsinsC) have been associated with Crohn's disease, each with an allele frequency of less than 5% in control Caucasian populations. We hypothesized that significant racial differences might be observed in African-American Crohn's disease patients and controls. Methods: We examined Nod2 variations in African-Americans with Crohn's disease (n = 66) as well as African-American controls (n = 52), compared to Caucasian Crohn's disease patients and controls. Identification of previously undescribed polymorphisms was performed through sequencing of genomic DNA from 31 unrelated African-Americans with Crohn's disease. Results: No African-American controls carried any of the Crohn's disease-associated risk alleles, with significantly lower carriage observed in African-American CD patients. We identified several variants present solely in African-Americans. Arg790Gln in the second leucine-rich repeat had allele frequencies of 6.9% and 3.3% in African-American Crohn's disease patients compared to controls. Arg702Trp, Gly908Arg, and Leu1007fsinsC all occur on the Pro268Ser variant (allele frequency = 25.8% within Caucasians); among African Americans, the Pro268Ser variant allele frequencies were 5.3% and 2.9% among African-American Crohn's disease and controls, respectively. Conclusions: This study represents the first genetic characterization of CD in African-Americans. The Crohn's disease associated variants are present at significantly lower allele frequencies in African-American compared to Caucasian cohorts. The evolutionary history of Nod2 variants is postulated and marked racial differences are observed for common variants. These findings are consistent with random genetic drift, or alternatively, selective advantage resulting from altered Nod2 expression and/or function.

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The NOD2 Genotype and the Clinical Course of Crohns Disease

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Background: Crohns Disease is a heterogeneous phenotype. Recently, NOD2 was identified as a susceptibility gene for Crohns disease. We investigated the relation between NOD2 genotype and the phenotype characteristics of Crohns disease. Methods: A two-stage design was used. In an exploratory stage, hypotheses about the genotype-phenotype relationship were generated based upon a sample of 447 German patients with Crohns disease at $p<0.05$. Positive findings were verified in an prospectively ascertained cohort of 168 Crohns disease patients. Patients were genotyped for the main coding mutations in the NOD2 gene (SNP8,

SNP12 and SNP13), using Taqman technology. Results: The presence of SNP13 was associated with ileal and fistulizing disease (compound heterozygosity: OR 13.7 (95% CI: 1.9-284) for ileal disease, OR 2.9 (1.5-5.4) for fistulae; heterocygosity: OR 3.5 (1.8-6.9) for ileal disease). The presence of SNP8 was associated with colonic involvement (heterocygosity: OR 2.9 (1.6-5.4) for left colonic disease and OR 2.2 (1.3-3.9) for right colonic disease). Disease severity was not associated with the NOD2 genotype. Conclusions: A distinct relation between NOD genotype and Crohns subphenotypes exists. Test strategies for the NOD2 variations using the prediction of the clinical course of Crohns disease may lead to the development of new therapeutic algorithms.

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Inflammatory Bowel Disease Is Associated with a Functional TNF Polymorphism That Affects an OCT1/NF-KB Transcription Factor Interaction

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Introduction: The tumour necrosis factor-alpha (TNF) gene lies within a replicated inflammatory bowel disease (IBD) genetic susceptibility locus (6p21, IBD3); and TNF is clearly implicated in IBD pathogenesis. Aims: To assess genetic associations of TNF promoter variants in IBD and study the functional biology of associated variants. Methods: Association studies of the common TNF polymorphisms (-1031,-863,-857,-308). Two independent cohorts were used (set A, 457 IBD families: 294 Crohns disease (CD) trios, 252 ulcerative colitis (UC) trios; set B 130 IBD families and 278 healthy controls (HC)). CD analyses were further stratified by possession of NOD2 mutations. Functional studies of -857C/T used: LPS stimulated whole blood TNF ELISA in 46 health y controls; monocyte nuclear extract/ promoter construct electro-mobility shift assays; in vitro GST pull-down assays of transcription factors OCT1, NF-kB, and deletion mutants; in vivo COS cell immuno-precipitation and COS cell luciferase reporter gene analysis. Results: TNF -857C was associated with IBD in both set A and replication set B, using both case control and family based (TDT) association analyses. Proportions homozygous for TNF-857C were IBD = 90.9% (n = 587 unrelated cases, P = 0.001 vs HC 83.1 %), NOD2 negative CD = 92.1% (n = 241, P = 0.002), UC = 92.1% (n = 304, P = 0.001). Higher stimulated TNF production was seen in whole blood from TNF-857C homozygotes (P = 0.03) at 2 hours. The OCT1 transcription factor bound the TNF-857T but not the TNF-857C allele, adjacent to a NF-kB binding site. The DNA binding domains of OCT-1 and NF-kB p65 interacted in vitro and in vivo. OCT1 diminished NF-kB induced reporter gene expression. Conclusion: The TNF-857C allele is associated with IBD, and TNF-857C homozygotes show higher TNF production. We have identified a molecular mode of action, through allele specific binding of OCT1 to the TNF promoter and interaction between OCT1 and NF-kB.

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Role of the 5q31 Cytokine Gene Cluster Haplotype (IBD5) in Japanese and British Inflammatory Bowel Disease: Evidence for Genetic Heterogeneity

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Introduction: Genetic variation in the 5q31 cytokine cluster has recently been linked to and strongly associated with Crohn's disease (CD) in the Canadian population (IBD5 locus, Rioux et al., Nat Gen 2001). Although the disease gene has not been precisely characterised, the cluster contains the immuno-regulatory genes involved in Th1/Th2 responses. The role of the IBD5 risk haplotype in ulcerative colitis (UC) is unknown. Aims: (i) To determine, in UK Caucasian cohorts, whether UC is associated with the 5q31/IBD5 cytokine cluster risk haplotype, and assess its role in CD. (ii) To assess association in Japanese CD and UC. (iii) To assess genetic heterogeneity (as seen in animal models of CD/UC) or epistasis between IBD5 and NOD2 variants. Methods: A genetic variant (IGR2060a1), unique to and of the same frequency as the 250kb CD IBD5 risk haplotype, was genotyped in 457 UK Caucasian IBD families (252 UC, 294 CD trios) and Japanese cohorts (178 CD, 170 UC, 156 healthy controls). Family based and case-control association analyses were performed. CD analyses were sub-stratified by NOD2 status (carriage of Arg702Trp, Gly908Arg, Leu1007fsinsC). To assess genetic heterogeneity or epistasis, we used log-linear analyses in a 'case-control study' based on allele transmissions using the haplotype relative risk model. Results: No association was seen between the 5q31 risk haplotype and UC (TDT Transmitted/Untransmitted: 105/124, P = 0.24). Association was confirmed with CD, specifically in CD patients not carrying NOD2 mutations (110/67, P = 0.003)(CD NOD2 carriers, 52/47, P = 0.3). In the UK population the haplotype plays a significantly lesser role in CD susceptibility than in the Canadian population (P = 0.03, T/U CDall 1.4 versus Canadian CD 2.5). Mean age at diagnosis in UK CD offspring was 22.4 years, similar results obtained for the TDT stratified above and below this age suggest age at diagnosis does not explain the weaker UK association. No interactions were seen between IBD5, Arg702Trp and Leu1007fsinsC. The IBD5 risk haplotype was very rare in the Japanese population (<1% frequency) and no evidence for association was seen. Conclusion: The 5q31/IBD5 cytokine cluster risk haplotype does not influence susceptibility to UC; plays a lesser role in genetic susceptibility to CD in the UK than in the Canadian population; does not influence Japanese UC or CD. CD demonstrates genetic heterogeneity.

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Interleukin-10 Transduced T Lymphocytes Prevent Colitis in the SCID Transfer Model

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Regulatory CD4+ cells secreting the anti-inflammatory cytokine IL-10 play a key role in maintaining the immune balance in the intestinal mucosa. In this study we engineered primary CD4+ cells to express IL-10, and investigated the efficacy of this approach in protecting against experimental colitis. Spleen-derived CD4+ cells were transduced using a retroviral (MMLV) vector to simultaneously express human IL-10 and green fluorescent protein (GFP). The therapeutic benefit of CD4+ cells transduced with IL-10-GFP was studied in colitis, induced by transfer of CD45RBhigh CD4+ cells, and in acute TNBS induced colitis. Transferred engineered GFP fluorescent cells were detected for at least 15 weeks in peripheral blood, spleens, colon and lymph nodes draining the intestine of recipient SCID mice. Human IL-10 was detected in draining lymph nodes and spleen, but not in serum samples of IL-10-GFP CD4+ cell treated mice. Body weights were significantly higher (mean percentage of initial body weight: 112 ± 5%) and colon weights were lower(mean: 174 ± 23 mg) compared to untreated mice (95 ± 3% and 242 ± 31 mg, resp.) and non-transduced CD4+ cell treated mice (103 ± 4% and 287 ± 33 mg, resp.). Histological signs of inflammation were significantly reduced by transfer of IL-10-GFP CD4+ cells mean score: 0.72 ± 0.19) compared to untreated (2.33 ± 0.28) or control CD4+ cell treated mice (2.42 ± 0.29). In addition, IL-10-GFP transduced CD45RBhigh CD4+ cells lost the capacity to induce colitis (mean histological score 1.0 ± 0.21 vs 2.4 ± 0.27 in recipients of non transduced CD45RBhigh CD4+ cells, $p<0.05$). In contrast, no therapeutic benefit was observed in TNBS-induced colitis. Taken together, peripheral CD4+ cells that were engineered to express IL-10 by retroviral transduction act as regulatory cells in CD45RBhigh-induced transfer colitis. This approach may induce long-term maintenance of mucosal immune homeostasis in Crohn's disease.

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Interleukin-10 Knockout Mice: A Model to Study Osteoporosis Associated with Intestinal Inflammation

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Background: Inflammatory bowel disease (IBD) is associated with decreased bone mineral density (BMD) and increased fracture risk. The pathophysiology of bone loss in IBD is poorly understood. RANKL, receptor activator of NF-κB, is a key stimulator of osteoclasts, the bone resorbing cells. Our hypothesis is that intestinal inflammation decreases bone mass via secretion of RANKL by activated T-cells. To test this hypothesis, we compared the bone phenotype with RANKL synthesis by activated T-cells isolated from Interleukin-10 knockout (IL-10$^{-/-}$) mice. Methods: We studied IL-10$^{-/-}$ mice in three different backgrounds (C57BL/6, C57BL/10, and Balb/c) and their appropriate wild type controls. We examined changes in BMD by dual X-ray absorptiometry, bone structure by contact radiography, and trabecular bone mass by quantitative computed tomography (μqCT). Bone RANKL mRNA expression was quantitated by northern blot. T-cells isolated from spleen using positive selection with CD90 (Thy 1.2) microbeads from Miltenyi Biotec (Auburn, CA, USA) were activated by incubation with anti-CD3ε and -CD28 antibodies. An ELISA (R&D Systems, Minneapolis, MN) was used to measure their RANKL production. Results: Contact radiography did not show obvious bone abnormalities or fractures. Starting at 8-weeks of age, there was an age-dependent decrease in whole body BMD and vertebral trabecular bone mass in IL-10$^{-/-}$ mice with symptoms of enterocolitis. These parameters were unchanged in healthy IL-10$^{-/-}$ and in wild type mice, suggesting that the IL-10 deficient state by itself does not influence bone mass. RANKL mRNA expression in calvariae and femur was similar in IL-10$^{-/-}$ and wild type mice. However, RANKL production was significantly higher in activated T-cells derived from IL-10$^{-/-}$ mice compared to wild type. Conclusions: IL-10$^{-/-}$ mice serve as a model to study bone loss associated with intestinal inflammation. In this model, increased RANKL production by activated T-cells may induce bone loss by increasing bone resorption. This will be confirmed by bone histomorphometric analysis. Supported by the Patrick and Catherine Weldon Donaghue Medical Research Foundation, the University of Connecticut Center for Interdisciplinary Research in Women's Health, and NIH grant DK57756 (adl).

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Interleukin 10 Deficient Mice Are Defective in Colonic Muc2 Synthesis Both before and after Induction of Colitis by Commensal Bacteria

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Bacteria are required for the development and perpetuation of colitis in interleukin 10 deficient (IL-10$^{-/-}$) mice. Germ free (GF) IL-10$^{-/-}$ do not show symptoms of intestinal inflammation, whereas IL-10$^{-/-}$ mice with normal enteric bacteria under pathogen free conditions develop chronic colitis. The mucin MUC2 is the major structural component of the mucus layer covering the colonic epithelium. In active ulcerative colitis we showed a decrease in Muc2 synthesis and sulfation. Aims: We sought to assess the effects of deregulation of the immune system in IL-10$^{-/-}$ mice on synthesis of colonic Muc2, prior to and after the induction of colitis by normal enteric bacteria. Methods: GF IL-10$^{-/-}$ and control mice were colonized with specified pathogen free normal murine enteric bacteria (NMEB). Colitis was quantified by histological score and IL-12 secretion. Total amounts of Muc2, Muc2 sulfation and Muc2 precursor synthesis were measured by quantitative assays. Results: GF IL-10$^{-/-}$ mice showed more than 10-fold reduced levels of Muc2 precursor synthesis compared to GF controls. Upon introduction of NMEB, IL-10$^{-/-}$ mice developed severe colitis, whereas control mice remained healthy. Muc2 precursor synthesis was unchanged in control mice. In contrast, IL-10$^{-/-}$ mice showed a peak increase in Muc2 precursor synthesis shortly after introduction of NMEB, returning to GF levels after 2 weeks. In GF controls, total Muc2 levels were 5-fold higher than in GF IL-10$^{-/-}$ mice. After introduction of NMEB, the levels of total Muc2 decreased 2-fold, both in control and IL-10$^{-/-}$ mice. Muc2 sulfation levels were 2-fold lower in GF IL-10$^{-/-}$ mice compared to GF control mice. Upon introduction of NMEB, Muc2 sulfation increased 2-fold in control mice, whereas in IL-10$^{-/-}$ mice Muc2 sulfation levels decreased about 10-fold, compared with GF IL-10$^{-/-}$ levels. Conclusions: The absence of IL-10 in GF non-inflamed IL-10$^{-/-}$ mice resulted in significant reductions in Muc2 synthesis, total Muc2, and Muc2 sulfation. This indicates that, due to a cytokine imbalance, the mucosal protection by Muc2 shows a primary defect. NMEB induced colitis in IL-10$^{-/-}$ mice, followed by a aggravated secondary reduction in total Muc2 and Muc2 sulfation, compared to control mice. Both the primary and secondary reduction in Muc2 levels and Muc2 sulfation in IL-10$^{-/-}$ mice likely reduce the capability of the epithelium to cope with non-pathogenic micro-organisms, and contribute to the development as well as perpetuation of colitis.