

**THE ROLE OF GENETIC (ATG16L1, IL23R AND NFKB) AND
CLINICAL FACTORS ON PATHOGENESIS AND
APPEARANCE OF INFLAMMATORY BOWEL DISEASES**

PhD thesis

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Introduction

The chronic inflammatory conditions in the human intestines with undetermined etiology are called “inflammatory bowel diseases” (IBD). IBD includes two well-defined medical conditions, Crohn’s disease (CD) and ulcerative colitis (UC).

According to our current knowledge both diseases are multifactorial, explained by the dysregulation of the mucosal immune system in the presence of enteral antigens, which generates an aberrant immune response in the genetically susceptible host, resulting in self-sustained inflammation and tissue damage. Disturbed immune tolerance may be presumed as another etiological factor. At the present time there is no one known single cause explaining the development of IBD.

Identifying the genetic variants associated with the disease and understanding their role in the development and course of IBD can be instrumental in the comprehension of the etiopathogenesis of the disease and can improve therapeutic possibilities.

The incidence of IBD is rapidly increasing which draws attention on the contribution of environmental factors to the pathogenesis and the clinical course of the disease and their possible therapeutic consequences.

Intestinal flora and smoking are the two most well-established environmental factors involved in the pathogenesis of IBD but even in their cases the exact mechanism of action, and their possible relationship with the phenotype of the disease or their effect on the treatment (need for surgical treatment, efficacy of conservative treatment) still unknown.

The cause of IBD is unknown, therefore causal therapy is unattainable, available treatments rest on empirical bases. On the other hand extensive knowledge was gained on the mechanism of action of the conventional therapies through the achievements of clinical investigations,

and in the last years “biologic” treatments became available as targeted therapies against known elements of the pathogenesis. In both forms of IBD usually life-long medical, partly symptomatic, often surgical treatment is needed. Improvement in the state of health can be achieved in 80-85% of the patients in UC and 60-70% in CD respectively. By analysing the relationship between changes of clinical signs and symptoms of the disease, results of the treatments and the molecular genetics of IBD the efficacy of the available treatments could be improved.

Aims

1) To investigate the effect of genetic, environmental and clinical factors on the clinical course of Hungarian IBD patients, particularly with regard to specific molecular genetic factors and smoking.

2) To search for relationship between either genotype or smoking or clinical phenotype and treatments (necessity of surgical resection, efficacy of conservative treatment).

The dissertation investigates the possible relationships of individual genetic polymorphisms, so

- the relevance of NFKB1 -94ins/delATTG and NFKBIA 3'UTR mutations,
- and the relevance of ATG16L1 and IL23R mutations

with the risk of IBD, the clinical phenotype, the outcome of pharmaceutical therapy and the necessity of bowel resection.

In respect of environmental factors we investigated the complex associations between smoking and the clinical phenotype of the disease, the pharmaceutical therapy and the risk of surgical treatment, with particular attention devoted to the role of smoking and aggressive medical treatment

in connection with the presence of complicated disease course and the need for surgery.

Patients and methods

- **Study population and definitions**

Genetic investigations: in total, 415 unrelated IBD patients (CD: 266 patients, mean age 35.2 ± 12.1 years, male/female 130/136, duration of disease 8.7 ± 7.6 years; UC: 149 patients, mean age 44.5 ± 15.4 years, male/female 73/76, duration of disease 10.7 ± 8.9 years) were included in our investigations. The control group for mutation analysis consisted of 149 age- and gender-matched healthy blood donors (male/female: 72/77, age: 37.9 ± 10.9 years). Control subjects did not have any gastrointestinal and/or liver diseases and were selected from consecutive blood donors in Szeged and Budapest. Only patients with a confirmed diagnosis for more than 1 year were enrolled. The diagnosis was based on the Lennard-Jones criteria. Factors such as presence of extraintestinal manifestations (ocular: uveitis, iridocyclitis; skin lesions: erythema nodosum, pyoderma gangrenosum; peripheral and axial arthritis; primary sclerosing cholangitis) were investigated by reviewing the medical charts and evaluating a questionnaire completed by both patients and controls. The disease phenotype (age at onset, duration, location, and behavior) was determined according to the Vienna classification. Upper gastrointestinal involvement, perianal disease, and age at onset were also analyzed, according to the Montreal classification. We investigated the presence of familial IBD and smoking habits. IBD associated medical therapy and therapeutical response were registered and investigated by reviewing the medical charts and completing a questionnaire. Response and resistance to steroids was classified

according to the accepted criteria published by the European Crohn's and Colitis Organisation. Patients were regarded as AZA users if they took a dose of ≥ 1.5 mg/kg body weight for at least 6 months. Response to infliximab induction therapy (dose 5 mg/kg at weeks 0, 2, and 6) was measured at week 8. We classified partial response as a CDAI decreased by ≥ 70 points and/or $\geq 50\%$ decrease in the number of draining fistulas, while remission was a CDAI score < 150 or closure of all fistulas.

In the second study, the complex associations between smoking as an environmental factor and the clinical phenotype of the disease, the pharmaceutical therapy and the risk of surgical treatment 681 IBD patients (CD 340, men/women 155/185, duration 9.4 ± 7.5 yr; UC 341, men/women 174/164, duration 11.5 ± 9.7 yr) were investigated. The definitions used during the processing of the clinical data were the same as the ones used in the genetic investigations. Early use was considered if the use of immunomodulatory therapy preceded the behavior change by at least 6 months. According to the Center's policy, if AZA was started, its use was not halted even in patients with long term clinical remission. The definition of smoking consisted of smoking ≥ 7 cigarettes/week for at least 6 months at the time of diagnosis and/or during follow-up, within 1 year of diagnosis or behavior change. Patients were interviewed on their smoking habits, changes in smoking habits, clinical activity of the disease, phenotype of the disease, pharmaceutical therapy and its changes and time and type of surgical resection at the time of referral and thereafter during the regular follow-up visits. Smoking cessation was defined as complete abstinence of at least 1 year's duration. Only 16 CD and 3 UC patients stopped smoking during the course of the disease, while 2 additional CD patients started smoking after the diagnosis. In patients with change in disease behavior, all

CD patients stopped smoking following the change. Since macroscopic lesions on the ileal side of the anastomosis observed 1 year following surgery were not different between smokers and non-smokers and there has no significant difference reported between ex-smokers and nonsmokers in reoperation rates in a recent meta-analysis, ex-smokers at the time of diagnosis were included in the non-smoker group.

- **Genotyping**
- **DNA isolation**

Genomic DNA was isolated from whole blood using High Pure PCR Template Preparation kit according to the manufacturers' instructions (Roche, Budaors, Hungary).

- **Detection of NFKB polymorphisms**

Polymorphisms in the NFKB1 promoter (rs28362491) and in the 3'UTR region of NFKBIA (rs696) were genotyped by restriction fragment length polymorphism (RFLP). In terms of the -94ins/delATTG polymorphism in the promoter region of the NFKB1 gene, a 289-bp PCR fragment was amplified and digested by Van91I, resulting in two fragments in the presence of -94delATTG (254 and 35 bp) and three fragments in the presence of -94insATTG (206, 48, and 35 bp). In the case of the single nucleotide polymorphism (SNP) in the 3'UTR (G/A) of the NFKBIA gene, a 426-bp fragment was amplified and digested by HaeIII, which cleaves only the G-containing allele, resulting in two fragments (308 and 118 bp). For both sequence variants, the resulting fragments were separated on 2.5% agarose gels and visualized with ethidium bromide staining.

- **Detection of IL23R and ATG16L1 polymorphisms**

Genotyping of IL23R (rs11209026, R381Q, c.1227G>A) and ATG16L1 (rs2241880, T300A, c.1338A>G) mutations were performed using the LightCycler (Roche Diagnostics) allelic discrimination system. Amplification primers and hybridisation probes were designed by the LightCycler Probe Design software (Roche Diagnostics) or Primer3 primer design software. All oligonucleotides were synthesised by Integrated DNA Technologies (Coralville, USA). PCR was performed by rapid cycling in glass capillaries in a reaction volume of 10µl with 50 ng genomic DNA, 5_1 2× PCR Master Mix (Promega), supplemented by 0.7U *Taq* DNA polymerase (Finnzyme, Espoo, Finland), 1.5mMMgCl₂, and 2.5 pmol of the respective labelled oligonucleotide (sensor and anchor). Asymmetric PCR was applied in the composition of the unlabelled amplification oligonucleotides (1.5:5 pmol forward:reverse amplification oligonucleotide ratio in case of *ATG16L1* and 5:1.5 pmol F:R ratio in case of *IL23R*). Cycling conditions were the following: initial denaturation at 94 °C for 2 min, followed by 70 cycles of denaturation at 94 °C, annealing at 55 °C for 10 s for the *ATG16L1* and 50 °C for 10 s for the *IL23R* variants, and extension at 72 °C for 15 s, with a ramping rate of 20 °C/s. After amplification, a melting curve analysis was performed by cooling the samples to 45 °C, followed by gradual heating to 85 °C with a ramp rate of 0.1 °C/s. The decline of fluorescence was continuously monitored. Melting curves were converted to melting peaks with wild type and variant alleles showing distinct melting points. Results were evaluated by two independent investigators.

Statistical methods

Variables were tested for normality using Shapiro Wilk's W test. The genotype frequencies for each polymorphism were tested for deviation from the Hardy–Weinberg equilibrium by means of the χ^2 test, with 1 df used. The t test with separate variance estimates, the χ^2 test, and the χ^2 test with Yates correction were used to evaluate differences between IBD patients and controls as well as within subgroups of IBD patients. The results are expressed as odds ratios (OR) with 95% confidence intervals (95%CI). For analysis of variance for continuous factors ANOVA with *post hoc* Scheffe test was used, in case of scale type variables Pearson- or Spearman-correlation was calculated. The Bonferroni correction was used to correct for multiple testing. Logistic regression was used for multi-variance analysis to compare genetic and clinical data. A P value of 0.05 was considered to be significant, all results were corrected to the duration of the illnesses. Kaplan-Meier survival curves were plotted for analysis with the LogRank and Breslow tests. Additionally, forward stepwise Cox regression analysis was used to assess the association between categorical clinical variables and surgical requirements. Factors resulted $p < 0.1$ in univariate analysis were included in multi-variance analysis, with some factors chosen by us beforehand. If there were no other criteria $p < 0.05$ values were described as significant differences. Data were described in mean \pm standard deviation, median (quartile) and n (%) forms. For the statistical analysis, SPSS version 13.0 (SPSS, Chicago, IL) was used with the assistance of a statistician (Dr. Peter Vargha).

Results

- **Genetic polymorphisms and IBD**
- **NFKB1-94ins/delATTG and NFKBIA 3'UTR polymorphisms**

All investigated polymorphisms were in Hardy–Weinberg equilibrium (HWE $P = 0.63–0.96$). The genotype and allele frequencies of either NFKB1-94ins/delATTG or NFKBIA 3'UTR variants were not different among CD, UC, and controls, furthermore, there was no interaction between NFKB1-94ins/delATTG and NFKBIA 3'UTR variant SNPs in Hungarian patients. *The carriage of the variant NFKBIA 30UTR GG was associated with extensive colitis in patients with UC ($P = 0,003$, $OR_{GG \text{ vs } AA/AG} 2,97$, $CI_{95\%} 1,45- 6,08$) that remained significant after Bonferroni correction.*

In CD patients the carriage of the NFKB1-94ATTGdel allele was associated with frequent relapses (54,3 a 31,3%-kal szemben $P < 0,0001$, OR 2,61, $CI_{95\%} 1,57- 4,33$) and increased risk for arthritis, however, this association became insignificant after correction for multiple testing. We also investigated the association between the NFKB1-94ins/delATTG or NFKBIA 30UTR variants and the response to steroids, infliximab, or need for surgery in patients with CD. 47 unrelated CD patients (male/female: 24/23, mean age 33.2 ± 11.6 years, duration 7.6 ± 4.7 years) received infliximab therapy. The location of the disease was ileal in 2 patients, colonic in 19 patients, and ileocolonic in 25 patients. In addition, three patients had upper gastrointestinal (GI) involvement. Disease behavior was inflammatory in 17 patients and penetrating in 30 patients. Perianal complications were observed in 27 (57.4%) patients. Almost all patients received immunosuppressant treatment (steroids 93.6%, AZA 93.6%). Overall, no association was found between either of the polymorphisms

investigated and short-term response (assessed at week 8) to infliximab induction therapy, steroid resistance, need for surgery or reoperation rate.

- **The ATG16L1 and IL23 receptor (IL23R) gene polymorphisms**

All investigated polymorphisms were in Hardy–Weinberg equilibrium (HWE $p = 0.38–0.95$). *The genotype frequencies of both IL23R Arg381Gln ($p = 0.018$) ATG16L1 Thr300Ala ($p = 0.027$) were significantly different between CD patients and controls. The carriage of the ATG16L1 300Ala/Ala ($p = 0.037$) genotype or the Ala allele (58.1% vs. controls:50.0%, OR: 1.39, 95% CI: 1.05–1.85) was associated significantly with the risk for CD. In addition, the heterozygous carriage of the IL23R variant ($p = 0.018$) was inversely associated with the risk for CD compared to the controls. No homozygous IL23R carriers were identified. In contrast, although the tendency was similar, no significant associations were found in UC compared to controls or CD ($p = \text{NS}$). Furthermore, there was no interaction between ATG16L1 Thr300Ala and IL23R Arg381Gln variant SNPs in Hungarian patients.*

The carriage of the variant IL23R 381Gln allele was significantly associated with inflammatory disease phenotype in CD ($p = 0.037$, ORhet: 0.22, 95% CI: 0.06–0.87). *The homozygous carriage of the ATG16L1 300Ala was associated with disease restricted to the colon ($p = 0.036$, OR_{Ala/Ala} colonic: 1.83, 95% CI:1.04–3.25).* No other significant associations were found in either CD or UC patients.

The association between the IL23RArg381Gln or ATG16L1 Thr300Ala variants and the response to steroids, infliximab or need for surgery in patients with CD were also investigated. The clinical data of patients treated with infliximab didn't differ from the group described in

connection with the NFKB1 -94ins/delATTG, and NFKBIA 3'UTR polymorphisms. Overall, there was a tendency for *ATG16LI* to be associated with short-term response to infliximab induction therapy, but we could not identify any association with the steroid resistance or need for surgery.

In UC, no association was found between *IL23R* Arg381Gln and *ATG16LI* Thr300Ala variant alleles and steroid use/resistance, azathioprine use or need for surgery

- **The complex examination of environmental factors with the clinical phenotype, the pharmaceutical therapy and the risk of surgery**
- **The frequency of smoking in IBD and the association with the clinical phenotype**

At the time of diagnosis, 45.5% of CD patients and 15.8% of UC patients were smokers. In CD, smoking at diagnosis was associated with change of behavior from B1 to B2/B3 during follow-up (smokers: 38.6% vs. nonsmokers: 25.2%, OR: 1.86, 95% CI: 1.02–3.46) and increased need for resection (smokers: 52.9% vs. nonsmokers: 41.2%, OR: 1.61, 95% CI: 1.04–2.48), but current smoking was not associated with risk for re-operation. The mean disease duration (smokers: $9,2 \pm SD7,2$ years vs. nonsmokers: $9,5 \pm SD 7,7$ years) was identical in both groups.

In UC, smoking at diagnosis was associated with lower risk for colectomy (0 vs. 6.6%, $P=0.05$). No other associations (including age at onset) were found.

- **Frequency of aggressive therapy in IBD patients and association with the clinical phenotype**

In CD, AZA was used at least 6 months long before the first operation in 46.2% of patients and 63.5% after the first operation. The rate of AZA intolerance was altogether 14.3% (additional 36 patients), and these patients were classified as AZA-nonusers. Biological (infliximab or adalimumab) exposure was 10.9% before the first operation and 20.9% altogether for the entire cohort. There were only seven patients before the first operation and 13 altogether treated only with biological therapy, without concomitant AZA.

In UC, AZA was used in 21.2% of the patients, there wasn't any patient on biologics.

In CD, early AZA and AZA/biological use was more common in patients with ileocolonic location (54.8 and 55.9% vs. 41 and 40.8%, $P=0.001$ and $P<0.001$). *Behavior change from B1 to B2/3 (23,3% és 23,1% vs. 38,9% és 40,0%, OR_{AZA} : 0,48, 95%CI: 0,29-0,88 és $OR_{AZA/biológiai}$: 0,45, 95%CI: 0,24-0,84) was less common in patients with either early AZA or AZA/biological therapy.* If smoking and early AZA/biological use was combined, patients receiving immunomodulator therapy were more likely to have extensive location (L3, nonsmokers, no-AZA/biological use: 43.6%, vs. nonsmoker and AZA/biological use: 51.6% vs. smoker and no AZA/biological use: 37% and smoker and AZA/biological use: 58.3%, $P<0.001$

- **Association between early AZA/biologic treatment and disease behavior change in CD**

In a univariate analysis, behavior change from B1 to B2/B3 during follow-up was associated with disease duration, location, presence of perianal disease, smoking at diagnosis, frequency of relapses, steroid use,

early AZA use, AZA/biological therapy use and need for resective surgery. Although ocular manifestations were also associated with behavior change (3.6% vs 11.5%, $P = 0.033$), this became non-significant after Bonferroni correction. Patients with a change in disease behavior had significantly longer disease duration (12.3 ± 7.6 years vs 7.4 ± 6.5 years, $P < 0.001$).

In a logistic regression model, disease duration, presence of perianal disease, smoking, steroid use, and early AZA use prior to behavior change were independent predictors for change in disease behavior. If early AZA use was changed to early AZA and/or biological therapy use (Coefficient: -1.221, $P = 0.002$, OR: 0.29, 95% CI: 1.34-0.64) in the same logistic regression model, the associations remained unchanged.

Disease location, perianal disease, early AZA or AZA/biological therapy, steroid use (LogRank $P = 0.004$ and Breslow $P = 0.005$) and smoking were significant determinants for time to behavior change surgery in a Kaplan-Meier analysis using LogRank and Breslow tests.

After performing a forward stepwise proportional Cox regression analysis each of the above variables was independently associated with the probability of disease behavior change. The result was the same if early AZA/biological therapy use ($P = 0.002$, HR: 0.43, 95% CI: 0.25-0.73) was incorporated in the same analysis.

- **Association between early AZA or AZA/biologic treatment and time to surgery/reoperation**

In univariate analysis localization, disease behavior, smoking habits and early AZA or AZA/biologic treatment showed association with surgical resection. *Early AZA or AZA/biologic treatment showed to be protective against the risk of surgical resection.* Reoperation was associated with frequent relapses (OR: 4,83, 95%CI: 2,17-11,8), perianal disease (OR: 3,20,

95%CI: 1,59-6,43) and disease behavior (without reoperation vs. reoperated B1: 15,9% vs 7,7% és B3: 55,1% vs 76,9%, $p = 0,02$), but AZA and AZA/biologic treatment or starting immunosuppressive/biologic therapy after the first operation were not associated.

Both smoking (LogRank $p = 0,025$ és Breslow $p = 0,047$) and early AZA use ($P < 0.001$ for both) were significant determinants of time to first surgery in a Kaplan–Meier analysis. Similarly, the combination of early [AZA only]/smoking ($P < 0.001$ and $P = 0.001$) and [AZA/biological use before first surgery]/smoking ($P < 0.001$ for both) were significantly associated with time to first surgery using the same analysis. Disease location ($P < 0.001$ and $P = 0.002$), behavior ($P < 0.001$ for both), change of disease behavior from B1 to B2/B3 ($P < 0.001$ for both) but not sex were significantly associated with the need for first surgery in the same Kaplan–Meier analysis using log-rank and Breslow tests for comparison. In case of a change in disease behavior the protective role of early AZA or AZA/biologic therapy against the first surgical resection could be shown in either in the inflammatory (B1) group or the whole study population, but if change in behavior occurred, the positive tendency could be seen only later during the follow up period.

Early AZA/biological use, disease location, and behavior were independently associated with need and time to first surgery in a forward stepwise proportional Cox-regression analysis, but sex was not associated with the same measure. The result was the same if early AZA only/smoking ($P = 0.001$) was analyzed in the same analysis. In contrast, frequent relapses ($P = 0.001$).

Frequent relapses ($p = 0,001$ both in the case of LogRank and Breslow test) and perianal disease ($p = 0,007$ and $p = 0,048$) were associated with time to re-operation in a Kaplan–Meier analysis, similarly to

the results of the former univariate analysis, but early AZA or AZA/biologic treatment were not associated.

In UC, smoking was preventive against surgery in a Kaplan–Meier survival analysis using log-rank ($P=0.042$) but not Breslow tests ($P=0.08$) for comparison (Fig. 3). Similarly, extensive disease location was associated with an increased need for colectomy ($P<0.001$ for both log-rank and Breslow). In a Cox regression analysis, both smoking ($P=0.02$) and location ($P=0.008$) were independently associated with the need for colectomy.

Conclusions

The following new observations were made in the dissertation:

1. An association was demonstrated between both the *ATG16L1* and the *IL23R* gene polymorphisms and Crohn's disease in Hungarian patients.
2. None of the *NFKB1* -94ins/delATTG, the *NFKBIA* 3'UTR, the *ATG16L1* and the *IL23R* gene polymorphisms could be associated with response to the pharmaceutical (steroid-azathioprin-infliximab) treatment or necessity for surgical resection in Hungarian IBD patients.
3. The carriage of the variant *NFKBIA* 3'UTR GG was associated with extensive colitis in patients with ulcerative colitis in Hungarian IBD patients.
4. The homozygous carriage of the *ATG16L1* 300Ala variant was associated with colonic localization in Hungarian patients suffering from Crohn's disease.
5. The presence of *IL23R* 381Q allele was associated with the inflammatory phenotype (B1) of Crohn's disease in our study population.
6. In a long-term follow up study we demonstrated, that the inceptive clinical characteristics of the disease (location-behavior), the pharmaceutical therapy and the environmental factors, such as smoking independently influence the long-term surgical risk in Crohn's disease and in ulcerative colitis, and the progressive behavior of the disease in Crohn's disease.
7. We were the first to demonstrate, that early immunosuppressive (azathioprin based), aggressive medication independently can influence the risk of surgical resection in Crohn's disease, and may play a protective role against the negative effect of smoking in point of the surgical risk in Crohn's disease.
8. In accordance to the literature in Crohn's disease the more progressive behavior is associated with the ileal and perianal localization in Hungarian patients, too, and both localization accompanied by a higher risk of surgical resection similarly to the stenotizing/fistulizing forms of the disease.
9. In ulcerative colitis we confirmed in multi-variance analysis that smoking and the extent of the disease are independent predictive factors of colectomy.

List of publications

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Publications directly related to this thesis:

1. **Szamosi T**, Banai J, Lakatos L, Czegledi Z, David G, Schafer E, Pandur T, Erdelyi Z, Gemela O, Papp M, Papp J, Lakatos PL. Early azathioprine/biological therapy is associated with decreased risk for first surgery and delays time to surgery but not reoperation in both smokers and non-smokers with Crohn's disease, while smoking prevents colectomy in UC. **Eur J Gastroenterol Hepatol** 2010;22:872-9.

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