3

Genotyping of *CARD15/NOD2*, *ATG16L1* and *IL23R* Genes in Polish Crohn's Disease (CD) Patients – Are They Related to the Localization of the Disease and Extra-Intestinal Symptoms?

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1. Introduction

Crohn's Disease (CD), together with ulcerative colitis (UC) belongs to inflammatory bowel diseases (IBD). Among its typical symptoms are inflammation, abdominal pain, melaena, diarrhea, ulcers and even fistulas. So far the etiology of CD remains unclear, but what is certain, is that it is multifactorial. Causing chronic inflammation and starting an immunological response requires both genetic factors, as well as environmental ones. What is more, CD is multigenetic – various genes could be a potential cause of the disease; among them are e.g. CARD15/NOD2, *ATG16L1 and IL23R (*Barrett et al., 2008; Cukovic-Cavka 2006, Cho 2008, Lesage 2002).

2. Aim of the study

The aim of this study was to investigate the polymorphisms in the *CARD15/NOD2* rs2066845 (p.Gly908Arg), *ATG16L1* rs2241879, *ATG16L1* rs2241880, *IL23R* rs7517847 and *IL23R* rs1004819 gene. Firstly, this research project focuses on determining whether there are any differences in the frequency of these polymorphisms among CD (Crohn's disease) patients and in the population group. Secondly, the aim of the study is to determine any

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correlations, or associations between the genotypes and alleles, and the localization of gastrointestinal and extra-intestinal symptoms of the disease.

3. Materials and methods

A total of 289 subjects took part in this study: 139 CD patients (60 women, 79 men) and 150 individuals in a population based control group (73 women, 77 men). All the patients were patients of Poznan University of Medical Sciences, Poland, Department of Gastroenterology, Human Nutrition and Internal Diseases. Moreover, all the subjects participating in this research project were adults, who had agreed to take part in genetic testing. The population group came from the DNA bank of the Institute for Human Genetics at the Polish Academy of Science. Genotyping was performed for all the investigated individuals. If obtaining reliable result was impossible, a given probe was eliminated from further analysis. That is why, the number of individuals in particular analyses may differ. In some cases, obtaining the data pertaining to the localization of the symptoms, as well as other co-existing symptoms was also impossible. Similarly, probes without an complete disease history were not included in further research.

Peripheral blood samples were collected on EDTA in the amount of 5 ml. Next, DNA was isolated from leukocytes, using the GTC (guanidine thiocyanate) and phenol-chlorophorm extraction method (Slomski et al, 2008). The isolates were dissolved in 1xTE buffer to obtain 500ng/µl concentration.

In the investigation of single nucleotide polymorphism (SNP) pyrosequencing was performed. A polymerase chain reaction (PCR) was performed for each probe with a set of primers: two for PCR, including a biothynylated one, and primer for sequencing. The sequences of the primers were designed with the use of computer software software dedicated to pyrosequencing (Table 1).

Primer	Sequence 5'→3'	Amplicon length in bp
ATGrs2241879_Fbiotin	gcttagctgcaggcctagaaa	152
ATGrs2241879_R	tggatactcatcctggttctgg	
ATGrs2241879-SEQ	ccacgcttgatatgg	
ATGrs2241880_F	ttacgaagacacacaaggcagtag	98
ATGrs2241880_Rbiotin	tgtctcttccttcccagtcc	
ATGrs2241880_SEQ	tttaccagaaccaggat	
IL23Rrs7517847_F	cctcttggttttcccatttca	292
IL23Rrs7517847_Rbiotin	tgaatttgaggggcctagga	
IL23Rrs7517847_SEQ	tttcacctattcccaag	
IL23Rrs1004819_F	tagcagcacaagcattctagga	125
IL23Rrs1004819_Rbiotin	actgacctgctttatgctgtga	
IL23Rrs1004819_SEQ	cttatgagaaatgcagatag	
CARD15_908_Fbiotin	gactcttttggccttttcagatt	243
CARD15_908_R	ccaatggtctttttttccttactcc	
CARD15_908_SEQ	tcgtcacccactctgt	

Table 1. Primers used for analysis.

SNP	Analyzed sequence
ATGrs2241879	TA/GACAGAGCCTGGCAATTAAAGGGTCC
ATGrs2241880	GAGC/TATCCACATTGTCCTGGGGGACTGGG
IL23Rrs7517847	GCCG/TCAGCTACACCTGTATGTAGGCTAGA
IL23Rrs1004819	CAC/TAGTAAGAATCACAGCATAAAGCAGG
CARD15_908	TGCC/GCCAGAATCTGAAAAGGCCAAAAGAG

Table 2. Analyzed sequences. SNPs are marked.

SNP	Population group		CD patients		p
CARD15_G908R	Amount	%	Amount	%	
CC	139	93,9	136	91,9	ns
CG	9	6,1	11	7,4	ns
GG	0	0,0	1	0,7	ns
ATGrs2241879	Amount	%	Amount	%	
GA	39	55,7	75	48,7	ns
AA	19	27,1	48	31,2	ns
GG	12	17,1	31	20,1	ns
ATG16L1rs2241880	Amount	%	Amount	%	
TC	46	47,9	74	47,4	ns
CC	22	22,9	48	30,8	ns
TT	28	29,2	34	21,8	ns
IL23Rrs 7517847	Amount	%	Amount	%	
GT	51	53,7	75	48,7	ns
TT	33	34,7	62	40,3	ns
GG	11	11,6	17	11,0	ns
IL23Rrs 1004819	Amount	%	Amount	%	
TT	4	4,3	18	15,9	ns
TC	48	51,1	40	35,4	ns
CC	42	44,7	55	48,7	ns

Table 3. Genotype frequencies and statistical significance.

Statistical analysis was performed using the exact Fisher test and the test for differences between two frequencies (odds ratio – OR). A p-value of less than 0.05 was accepted as statistically significant.

Symptoms of CD can be localized in the area of the entire gastrointestinal tract (GIT), starting at the oral cavity and ending with the anus; however, extra-intestinal symptoms are also characteristic of this disease. The GIT symptoms were divided into: symptoms occurring in the oral cavity, esophagus, stomach and duodenum, small intestine, colon, and the presence of perianal fistulas. Extra-intestinal symptoms were divided into: arthralgia,

uveitis, iritis, stomatitis aphtosa, erythema nodosum, fatty liver and elevated body temperature.

4. Results

4.1 Genotyping

The first aim of the project was to determine the differences in the genotype and allele frequencies, taking into consideration the two investigated groups of individuals: CD patients and the population group.

A statistical analysis of the genotypes did not reveal any significant differences in genotype frequencies (Table 3), but the allele analysis revealed that the frequency of alleles in ATG_rs2241880 and IL23Rrs7517847 differs significantly between the CD subjects and the population group (Table 4).

SNP	CD patients		Population group		p
CARD15_G908R	Amount	%	Amount	%	
G	12	7,55	9	5,66	ns
C	147	92,45	148	93,08	ns
ATGrs2241879	Amount	%	Amount	%	
G	106	46,29	51	46,29	ns
A	123	53,71	57	53,71	ns
ATG_rs2241880	Amount	%	Amount	%	
T	108	46,96	78	33,91	p=0,0775
C	122	53,04	68	29,57	p=0,0017
IL23Rrs7517847	Amount	%	Amount	%	
T	137	59,83	84	36,68	p=0,0011
G	92	40,17	62	27,07	p=0,0987
IL23Rrs1004819	Amount	%	Amount	%	
T	58	37,91	52	33,99	ns
C	95	62,09	90	58,82	ns

Table 4. Allele frequencies and statistical significance.

4.2 The correlation between genotypes and the localization of GIT symptoms

Symptoms were divided into: symptoms occurring in the oral cavity, esophagus, stomach and duodenum, symptoms displayed by the small intestine, colon, and the presence of fistulas. Genotype analysis and its relation to the localization of GIT symptoms revealed that the only polymorphism that is connected with localization of the disease symptoms in the Polish CD population is *IL23R* rs7517847, and which is related to the symptoms localized in the small intestine.

CARD G908R	oral cavity, esophagus, stomach or duodenum				small intestine				colon				perianal fistulas			
Geno-type	CC	CG	GG	∑	CC	CG	GG	∑	CC	CG	GG	∑	CC	CG	GG	∑
yes	4	1	0	5	40	3	0	43	50	6	0	56	22	0	0	22
no	73	6	0	79	36	4	0	40	26	1	0	27	55	7	0	62
Total	77	7	0	84	76	7	0	83	76	7	0	83	77	7	0	84
p	ns				ns				ns				ns			
Genotype compa-rison	ns				CC vs GG p<0.0001 CC vs CG p< 0.001				ns				ns			

Table 5. *CARD15/NOD2* G908R genotype analysis and its relation with the localization of GIT symptoms.

Statistical analysis did not indicate any significant discrepancies, but some tendencies were observed. A comparison of the genotypes proved that the CC genotype is statistically more frequent than the GG and CG ones, among patients suffering from enteritis.

ATG rs2241 879	Oral cavity, esophagus, stomach or duodenum				small intestine				colon				perianal fistulas			
Geno-type	GA	AA	GG	∑	GA	AA	GG	∑	GA	AA	GG	∑	GA	AA	GG	∑
yes	1	1	2	4	22	16	8	46	33	16	10	59	16	3	5	24
no	43	25	16	84	22	9	10	41	11	9	8	28	29	22	13	64
Total	44	26	18	88	44	25	18	87	44	25	18	87	45	25	18	88
p	ns				ns				ns				ns			
Genotype compa-rison	ns				GA vs GG p=0.005				ns				ns			

Table 6. *ATG16L1 rs2241879* genotype analysis and its relation to the localization GIT symptoms.

Genotype comparison revealed certain tendencies, i.e. that in the case of the *ATG* rs2241879 polymorphism, among CD patients with symptoms localized in the small intestine, the GA genotype is significantly more frequent than the GG one.

ATG rs2241 880	Oral cavity, esophagus, stomach or duodenum				small intestine				colon				perianal fistulas			
Geno-type	TC	CC	TT	∑	TC	CC	TT	∑	TC	CC	TT	∑	TC	CC	TT	∑
yes	2	2	1	5	23	15	9	47	35	15	11	61	14	4	6	24
no	44	24	18	86	23	10	10	43	11	10	8	29	33	21	13	67
Total	46	26	19	91	46	25	19	90	46	25	19	90	47	25	19	91
p	ns				ns				ns				ns			
Genotype compa-rison	ns				TCvsTTp=0.005				ns				ns			

Table 7. *ATG16L1 rs2241880* genotype analysis and its relation to the localization of GIT symptoms.

The comparison of genotypes suggests, that among CD patients with symptoms localized in small intestine, as far as the ATG16L1 rs2241880 polymorphism is concerned, the TC genotype is significantly more frequent than the TT one.

IL23R rs1004 819	Oral cavity, esophagus, stomach or duodenum				small intestine				colon				perianal fistulas			
Geno-types	TT	TC	CC	∑	TT	TC	CC	∑	TT	TC	CC	∑	TT	TC	CC	∑
yes	1	1	1	3	3	17	20	40	8	22	25	55	2	9	11	22
no	10	32	35	77	8	15	16	39	3	10	11	24	9	23	25	57
Total	11	33	36	80	11	32	36	79	11	32	36	79	11	32	36	79
p	ns				ns				ns				ns			
Genotype compa-rison	ns				TTvsCCp=0.005				ns				ns			

Table 8. *IL23R rs1004819* genotype analysis and its relation to the localization of GIT symptoms.

Genotype comparison showed that, among CD patients, in the IL23R rs1004819 polymorphism, the TT genotype is significantly more related to the localization of the symptoms in the small intestine than the CC genotype.

IL23Rrs 7517847	Oral cavity, esophagus, stomach or duodenum				small intestine				colon				perianal fistulas			
Geno-types	TT	GT	GG	∑	TT	GT	GG	∑	TT	GT	GG	∑	TT	GT	GG	∑
yes	1	2	0	3	23	15	7	45	24	28	7	59	11	9	3	23
no	36	37	11	84	14	23	4	41	13	10	4	27	26	30	8	64
Total	37	39	11	87	37	38	11	86	37	38	11	86	37	39	11	87
p	ns				p=0.0364				ns				ns			
Geno-type compa-rison	ns				TTvsGGp<0.0001 GTvsGGp=0.005 GTvsTTp=0.0161				ns				ns			

Table 9. *IL23R rs7517847* genotype analysis and its relation with the localization of GIT symptoms.

Statistical analysis demonstrates that the only polymorphism that can statistically be connected with intestinal localization of CD is rs7517847 in the *IL23R gene (p=0.0364).*
What is more, genotype comparison revealed that the TT genotype is significantly more frequent than the GG one in the case of CD patients, whose symptoms are localized in the small intestine; additionally, the GT genotype prevails over the TT.

	oral cavity, esophagus, stomach or duodenum			small intestine			colon			perianal fistulas		
CARD15 _908	G	C	Σ	G	C	Σ	G	C	Σ	G	C	Σ
yes	1	5	6	3	43	46	6	56	62	0	22	22
no	6	79	85	4	40	44	1	27	28	7	62	69
Total	7	84	91	7	83	90	7	83	90	7	84	91
p	ns			ns			ns			ns		
Genotype compari-son	ns			C vs G p<0.001			C vs G p<0.001			C vs G p<0.001		

Table 10. *CARD15/NOD2 G908R* allele analysis and its relation to the localization of symptoms in the GI track.

4.3 Correlation between alleles frequency and GIT symptoms localization

For each of the investigated polymorphisms, allele distribution analysis was performed, as well as an analysis investigating the correlations between particular alleles and the

localization of the symptoms in the GI track. The results are presented in tables 10 – 14. None of the alleles seem to be significantly related with the localization of the GI track symptoms. However, genotype comparison revealed certain tendencies, which are presented in the tables below.

In *CARD15/NOD2 G908R* polymorphism, allele C was more frequent among patients with fistulas and symptoms in the area of the small intestine.

	oral cavity, esophagus, stomach or duodenum			small intestine			colon			perianal fistulas		
ATGrs 2241879	G	A	Σ	G	A	Σ	G	A	Σ	G	A	Σ
yes	3	2	5	30	38	68	43	49	92	21	19	40
no	59	68	127	32	31	63	19	20	39	42	51	93
Total	62	70	132	62	69	131	62	69	131	63	70	133
p	ns			ns			ns			ns		
Genotype comparison	ns			ns			ns			ns		

Table 11. ATG16L1 *rs2241879* alleles analysis and its relation to the localization of symptoms in the GI track.

	oral cavity, esophagus, stomach or duodenum			small intestine			colon			perianal fistulas		
ATGrs 2241880	T	C	Σ	T	C	Σ	T	C	Σ	T	C	Σ
Yes	3	4	7	32	38	70	46	50	96	20	18	38
No	62	68	130	33	33	66	19	21	40	46	54	100
Total	65	72	137	65	71	136	65	71	136	66	72	138
p	ns			ns			ns			ns		
Genotypes comparison	ns			ns			ns			ns		

Table 12. *ATG16L1 rs2241880* allele analysis and its relation to the localization of symptoms in the GI track.

	oral cavity, esophagus, stomach or duodenum			small intestine			colon			perianal fistulas		
IL23Rrs 7517847	T	G	Σ	T	G	Σ	T	G	Σ	T	G	Σ
yes	3	2	5	38	22	60	52	35	87	20	12	32
no	73	48	121	37	27	64	23	14	37	56	38	94
Total	76	50	126	75	49	124	75	49	124	76	50	126
p	ns			ns			ns			ns		
Genotypes compari-son	ns			ns			T vs G p<0.02			ns		

Table 13. *IL23R rs7517847* allele analysis and it's relation to the localization of symptoms in GI track.

In *IL23R* rs7517847 what was observed, was the tendency for the predominant presence of allele T in patients with a colonic manifestation of symptoms.

	oral cavity, esophagus, stomach or duodenum			small intestine			colon			perianal fistulas		
IL23Rrs 1004819	T	C	Σ	T	C	Σ	T	C	Σ	T	C	Σ
yes	2	2	4	20	37	57	30	47	77	11	20	31
no	42	67	109	23	31	54	13	21	34	32	48	80
Total	44	69	113	43	68	111	43	68	111	43	68	111
p	ns			ns			ns			ns		
Genotype compa-rison	ns			ns			C vs T p<0.04			ns		

Table 14. *IL23R rs1004819* alleles analysis and its relation to the localization of symptoms in GI track.

In *IL23R* rs1004819, what was observed, was the tendency for allele C, rather than T to be present more often in patients with a manifestation of the symptoms in the colon.

4.4 The correlation between the frequency of genotypes and the localization of non-GIT symptoms

The second part of this research project was devoted to the analysis of the relations between the genotypes and extra-intestinal symptoms. Extra-intestinal symptoms were divided into: arthralgia, uveitis, iritis, *stomatitis aphtosa*, erythema nodosum, fatty liver or elevated body temperature. The results are presented in tables 15-24.

	Arthralgia				uveitis				iritis				stomatitis aphtosa				erythema nodosum			
CARD G908R	CC	CG	GG	∑	CC	CG	GG	∑	CC	CG	GG	∑	CC	CG	GG	∑	CC	CG	GG	∑
Yes	45	5	0	50	10	2	0	12	8	1	0	9	22	2	0	24	15	4	0	19
no	31	2	0	33	65	5	0	70	67	6	0	73	53	5	0	58	60	3	0	63
Total	76	7	0	83	75	7	0	82	75	7	0	82	75	7	0	82	75	7	0	82
p	ns				ns				ns				ns				ns			
Genotype compa-rison	CCvsCG p<0.001 CCvsGG p<0.001				CCvsCG p<0.03				ns				CCvsGG p<0.01 CCvsCGp<p <0.01				CCvsGG p<0.03			

Table 15. Analysis of genotypes and their correlation with the extra-intestinal symptoms.

A comparison of the genotypes revealed the tendency for the CC genotype to be the statistically predominant one in all the investigated cases.

	fatty liver				elevated body temperature			
CARD15 G908R	CC	CG	GG	∑	CC	CG	GG	∑
yes	4	1	0	5	35	6	0	41
no	71	6	0	77	40	1	0	41
Total	75	7	0	82	75	7	0	82
p	ns				ns			
Genotype compa-rison	ns				CCvsCG p<0.001 CCvsGG p<0.0001			

Table 16. Analysis of genotypes and their correlation with extra-intestinal symptoms.

Genotype comparison revealed the tendency for the CC genotype to be statistically more frequent than GG and CG ones among patients declaring elevated body temperature. Although, in our opinion, elevated body temperature cannot be treated as a symptom specific to CD (rather as a symptom related to the chronic inflammation), the decision was made to include it into the group of extra-intestinal symptoms, since it was mentioned as a symptom by the patients.

	arthralgia				uveitis				iritis				stomatitis aphtosa				erythema nodosum			
ATG rs2241879	GA	AA	GG	∑	G A	AA	GG	∑	GA	AA	GG	∑	G A	AA	GG	∑	GA	AA	GG	∑
Yes	25	15	12	52	9	1	2	12	6	1	3	10	13	5	5	23	11	5	3	19
no	20	10	5	35	35	24	15	74	38	24	14	76	31	20	12	63	33	20	14	67
Total	45	25	17	87	44	25	17	86	44	25	17	86	44	25	17	86	44	25	17	86
p	ns				ns				ns				ns				ns			
Genotype comparison	GAvsAAp <0.01 GAvsGGp <0.01				ns				ns				ns				GAvsGG p<0.02			

Table 17. Analysis of the genotypes, and their correlation with extra-intestinal symptoms.

Genotype comparison showed the tendency for the GA genotype to be the statistically prevailing one among patients declaring joint pain and erythema nodosum.

	fatty liver				elevated body temperature			
ATG rs2241879	GA	AA	GG	∑	GA	AA	GG	∑
Yes	3	0	2	5	22	14	6	42
no	41	25	15	81	22	11	11	44
Total	44	25	17	86	44	25	17	86
p	ns				ns			
Genotype comparison					GAvsGG p<0.001			

Table 18. Analysis of the genotypes, and their correlation with extra-intestinal symptoms.

Genotype comparison showed a tendency for the GA genotype to be statistically more frequent than GG genotype in patients declaring an elevated body temperature.

ATG rs2241880	arthralgia				uveitis				iritis				stomatitis aphtosa				erythema nodosum			
	TC	CC	TT	∑	TC	CC	TT	∑	TC	CC	TT	∑	TC	CC	TT	∑	TC	CC	TT	∑
yes	27	15	12	54	8	1	3	12	5	1	4	10	13	6	6	25	12	5	4	21
no	20	10	6	36	38	24	15	77	41	24	14	79	33	19	12	64	34	20	14	68
Total	47	25	18	90	46	25	18	89	46	25	18	89	46	25	18	89	46	25	18	89
p	ns				ns				ns				ns				ns			
Genotype comparison	TCvsTT p<0.01 TCvsCC p<0.01				ns				ns				ns				TCvsTT p<0.04			

Table 19. Analysis of the genotypes, and their correlation with extra-intestinal symptoms.

Genotype comparison showed that in patients complaining of joint pain and erythema nodosum, the TC genotype was statistically the most common one.

ATG rs2241880	fatty liver				elevated body temperature			
	TC	CC	TT	∑	TC	CC	TT	∑
Yes	3	0	2	5	22	14	8	44
no	43	25	16	84	24	11	10	45
Total	46	25	18	89	46	25	18	89
p	ns				ns			
Genotype comparison	ns				TCvsTT p<0.01			

Table 20. Analysis of the genotypes, and their correlation with extra-intestinal symptoms.

The TC genotype was more frequent than the TT one among patients with an elevated body temperature.

	arthralgia				uveitis				iritis				stomatitis aphtosa				erythema nodosum			
IL23R rs7517 847	TT	GT	GG	∑	TT	GT	GG	∑	TT	GT	GG	∑	TT	GT	GG	∑	TT	GT	GG	∑
yes	24	21	8	53	7	3	2	12	4	5	1	10	10	10	4	24	6	8	5	19
no	12	18	3	33	28	36	9	73	31	34	10	75	25	29	7	61	29	31	6	66
Total	36	39	11	86	35	39	11	85	35	39	11	85	35	39	11	85	35	39	11	85
p	ns				ns				ns				ns				ns			
Genotype compa-rison	TTvsGG p<0.01 GTvs GGp<0.01				ns				ns				ns				ns			

Table 21. Analysis of genotypes, and their correlation with extra-intestinal symptoms.

Genotype comparison showed two tendencies: in patients complaining of joint pain, the TT genotype was statistically more frequent than the GG one, but GT was more frequent than GG.

	fatty liver				elevated body temperature			
IL23R rs7517847	TT	GT	GG	∑	TT	GT	GG	∑
yes	2	3	0	5	22	14	5	41
no	33	36	11	80	13	25	6	44
Total	35	39	11	85	35	39	11	85
p	ns				ns			
Genotype comparison	ns				TTvsGG p<0.03 GTvsGG p<0.01			

Table 22. Analysis of the genotypes, and their correlation with extra-intestinal symptoms.

Again, an elevated body temperature was related to the presence of the TT but not the GG genotype, as well as the GT but not GG genotype. However, as it was mentioned above, we do not believe it should be associated with CD specifically.

	arthralgia				uveitis				iritis				stomatitis aphtosa				erythema nodosum			
IL23R rs1004 819	TT	TC	CC	∑	TT	TC	CC	∑	TT	TC	CC	∑	TT	TC	CC	∑	TT	TC	CC	∑
Yes	8	19	19	46	3	3	6	12	2	4	3	9	5	7	11	23	2	4	13	19
no	3	13	17	33	8	29	30	67	9	28	33	70	6	25	25	56	9	28	23	60
Total	11	32	36	79	11	32	36	79	11	32	36	79	11	32	36	79	11	32	36	79
p	ns				ns				ns				ns				ns			
Genotype compari-son	ns				ns				ns				ns				TTvsCC p<0.03 TCvsCC p<0.03			

Table 23. Analysis of the genotypes, and their correlation with extra-intestinal symptoms.

Patients with erythema nodosum showed the tendency for statistically prevalent TT and TC genotypes.

	fatty liver				elevated body temperature			
IL23R rs1004819	TT	TC	CC	∑	TT	TC	CC	∑
Yes	2	2	1	5	8	11	18	37
no	9	30	35	74	3	21	18	42
Total	11	32	36	79	11	32	36	79
p	ns				P=0.078			
Genotype comparison	ns				CCvsTT p<0.03			

Table 24. Analysis of the genotypes and their correlation with extra-intestinal symptoms.

A comparison of the genotypes revealed the tendency for the CC genotype to be statistically more frequent than the TT one in patients declaring elevated body temperature.

4.5 The correlation between allele frequency and the localization of non-GIT symptoms

The analysis of allele frequency in exact polymorphisms among CD patients was correlated with extra-intestinal symptoms. Although no significant results were obtained, some interesting tendencies have been observed. The results are presented in table 25.

	arthralgia		Uveitis		iritis		stomatitis aphtosa		Erythema nodosum		Fatty liver		Elevated body temperature	
CARD15 G908R	**C**	**G**	**C**	**G**	**C**	**G**	**C**	**G**	**C**	**G**	**C**	**G**	**C**	**G**
no	33	2	70	5	73	6	58	5	63	3	77	6	58	5
yes	50	5	12	0	9	1	24	2	19	4	5	1	24	2
p	ns		ns		ns		ns		ns		ns		ns	
Alleles comparison	C vs G p<0.001		ns		ns		ns		ns		ns		C vs G p<0.01	
ATG rs2241879	**A**	**G**	**A**	**G**	**A**	**G**	**A**	**G**	**A**	**G**	**A**	**G**	**A**	**G**
yes	40	37	59	40	62	52	51	43	53	47	66	56	33	33
no	30	25	10	11	7	9	18	18	16	14	3	5	36	28
p	ns		ns		ns		ns		ns		ns		ns	
Alleles comparison	ns		ns		ns		ns		ns		ns		ns	
ATG rs2241880	**T**	**C**	**T**	**C**	**T**	**C**	**T**	**C**	**T**	**C**	**T**	**C**	**T**	**C**
yes	39	42	53	62	55	65	55	52	48	54	59	68	34	35
no	26	30	11	9	9	6	19	19	16	17	5	3	30	36
p	ns		ns		ns		ns		ns		ns		ns	
Alleles comparison	ns		ns		ns		ns		ns		ns		ns	
IL23R rs7517847	**T**	**G**	**T**	**G**	**T**	**G**	**T**	**G**	**T**	**G**	**T**	**G**	**T**	**G**
yes	45	29	64	45	65	44	54	36	60	37	69	47	38	31
no	40	21	10	5	9	6	20	14	14	13	5	3	36	19
p	ns		ns		ns		ns		ns		ns		ns	
Alleles comparison	T vs G p< 0.04		ns		ns		ns		ns		ns		ns	
IL23R rs1004819	**T**	**C**	**T**	**C**	**T**	**C**	**T**	**C**	**T**	**C**	**T**	**C**	**T**	**C**
yes	27	38	37	59	37	61	31	50	37	51	39	65	24	39

no	16	30	6	9	6	7	12	18	6	17	4	3	19	29
p	ns		ns		ns		ns		ns		ns		ns	
Alleles compa-rison	ns		ns		ns		ns		ns		ns		ns	

Table 25. Correlation between allele frequency and the localization of non-GIT symptoms

5. Discussion

Crohn's Disease (CD) has been known to physicians for many decades. In 1904 Antoni Leśniowski, already tried to characterize the disease, and in later years his efforts were undertaken by many other researchers. Unfortunately, up to this day we know very little about CD. There are numerous descriptions of clinical cases presenting different localization of disease symptoms, descriptions of symptoms localized solely in the digestive tract, as well as symptoms accompanying the disease, typical for CD, but not necessarily associated with the digestive tract. Since the second half of XX century it has also been known that CD is conditioned by genetic factors. However, these do not act independently, but instead need to overlap with immunological and environmental factors, resulting in an onset of the disease. All this information sheds much light on the etiology of inflammatory bowel disease (IBD), including CD, but does not entirely solve the puzzle constituted by this complex condition. Genetic research initiated by the discovery of the IBD1 region, and later the CARD15/NOD2 gene lying in its vicinity, appears to have provided answers to the numerous questions and doubts of researchers: What is the cause of CD? What increases the risk of the disease developing and what is the protective factor? Why is its clinical picture so diverse and complex, and what is it determined by? Is it hereditary and if so how? It quickly became clear that what seemed to have provided the answer and cleared all doubts, only opened new perspectives for ascertaining the possible causes of CD, but still did not solve the problem. Today CD is thought to be polygenic, which means that complicated dependencies related to numerous genes lie at its basis. Until recently, it was assumed that a group of 40 genes are involved as possible determinants of the disease, but the most recent findings point to a number twice as large.

While planning this research project, the idea which emerged, was to research the genotype distribution within the human genome, amongst Polish patients diagnosed with Crohn's disease. Although the clinical course might be similar everywhere, research as to the genetic factors underlying CD differs depending on the population which patient stems from. Consequently, we decided to define the span of genotypes and alleles for the Polish population. What is more, having the information about the clinical course of the disease, it seemed interesting to study whether any dependencies exist between a particular genome and the clinical course, as well as localization of the disease symptoms.

Recently, some information surfaced related o the dependency between the localization of the disease symptoms and the particular genotypes. The latest of these studies, (Jurgens et al., 2010), carried out by German researchers on patients with CD, showed that there is a clear dependency between the presence of homozygotic mutations in the CARD15/NOD2 gene and the occurrence of fistulas and simultaneously post-inflammatory intestinal strictures. As part of the research work span of this project, a single change of the SNP type

(G908R) in CARD15/NOD2 gene was studied. However, it did not show a statistically significant change in the frequency of occurrence of particular genotypes and alleles in the patient group, as compared to the healthy population. None the less, certain tendencies were observed, which pointed to a relation between the presence of the CC genotype iand an intestinal localization of the disease (both the small intestine, as well as the colon). Our research also confirmed the association of perianal fistulas with the presence of the CC genotype, in this respect, confirmed the results of the above-mentioned German research project. What is more, our studies also indicated certain tendencies with respect to the occurrence of specific disease symptoms not related to the gastrointestinal tract, such as joint pain, iritis, erythema nodosum, or aphtous ulcerations in the mouth (these symptoms were predominant in patients with the CC genotype). This aspect of the research presented here seems to supplement our current knowledge in an interesting way, as it has not been taken into account in the German study and therefore is in itself innovative and unique.

In this research project two polymorphic changes, located in the IL23R gene (IL23Rrs7517847, as well as IL23Rrs1004819) were analyzed. In the case of the former, statistically significant changes were observed in the frequency of occurrence of the alleles, as compared with the population group (allele T p=0,0011, allele G p=0,0987). This points to the fact that polymorphism IL23Rrs7517847 can be associated with an increased risk of morbidity in significant way. Studies conducted as part of this research project demonstrated the tendency for the development of the disease related changes in the small intestine and colon. Similarly, for IL23Rrs7517847, the TT genotype was significantly more frequent in patients with intestinal changes, while the TG genotype was most frequent in the case of changes within the colon. Although our research does not show any significant association of the IL23R gene with the occurrence of fistulas, it does bear out the results of the German publication cited above.

Another study on the relation between the CARD15/NOD2 gene with the course of the disease is the one conducted on a Croatian group of patients from 2006 (Cukovic-Cavka et al., 2006). They reported that mutations in the CARD15/NOD2 gene were associated with an increased risk of developing CD, and were also responsible for an earlier diagnosis on the disease (the patients were diagnosed at an earlier age) as well as the need for surgical intervention (a more severe disease course). As part of the research conducted on Croatian patients three most common mutations/polymorphisms (Arg702Trp, Gly908Arg, Leu1007fsinsC) occurring in CARD15/NOD2 gene were studied. The research published by Annese i et al. (2005) corroborates the results of the work published above and proves that the existence of even one of the key mutations/polymorphisms is responsible for an aggressive course of the disease. Possibly, because only one analysis, of the mentioned polymorphisms - Gly908Arg –was planned in the project no similar dependence was shown. According to the literature data, the largest impact as far as increasing the risk of developing CD is concerned, as well as a more severe course of the disease is caused by insertional mutation. Although it was not studied as part of this research project as of yet, we do plan to carry out research focussing on this aspect in the future.

Another publication, which confirms the association of the CARD15/NOD2 gene with the clinical symptoms of the disease, is a study by Mendoza et al. from 2003 who scrutinized this dependence in the Spanish population. One aspect makes it particularly interesting; not only does this research point to the influence of the three main SNP changes in the CARD15/NOD2 gene on the occurrence of the intestinal disease symptoms, but also proves

that carriers of the G908R mutation show a disease type characterized by intestinal strictures, which additionally requires more frequent surgical interventions, including appendectomy. The results of the study presented here do confirm the Spanish data pertaining to the association of G908R mutation with the intestinal localization of the disease; however, the remaining aspects discussed by Mendoza et. al. are not analyzed in this project, and, therefore, cannot be compared. The need for more frequent surgical intervention associated with the presence of a CARD15/NOD2 gene mutation, was also confirmed in 2010, when pediatric cases of CD were analyzed (Lacher et al., 2010). It was discovered that in the case of children under the age of 17, surgical interventions were more common in those who displayed the presence of the mutation.

A very interesting scientific publications surfaced in 2008, when Barrett et al., performed a GWAS analysis (genome-wide association studies) on a group of 3230 CD patients, as well as a control group of 4829 subjects (GWAP studies include the analysis of a huge number of polymorphisms in the research (patient) group and a subsequent comparison of the same types of changes in the control/ population group; the differences in the occurrence of changes in both of these groups (presented mainly through a very precise and thorough statistical analysis) point to a relation between the potential polymorphisms associations with the disease studied. These studies showed that within the human genome there are at least 32 genes associated with predisposition for CD (11 loci mentioned in earlier publications were confirmed, and 21 new ones were identified). This lends support to the currently held belief that CD may be conditioned by 30-35 different genes.

The reports presented above clearly point to the fact that for a number of years worldwide research has been resorting to multilocus studies. These are not aimed at determining one particular mutation or polymorphism conditioning a given disease, but rather at discovering complex inter-genetic dependencies. Similarly, this research project strives to follow these global trends, at least to an extent. Of course the authors realize that the relatively small patient group (compared to large-scale research projects) is not a match for the reports presented above; nevertheless, we believe it is still an interesting attempt at investigating the described dependencies in the Polish patient population.

Not all of the polymorphisms studied in this doctoral thesis have been analyzed previously in reference to the clinical course of the disease. In Poland this kind of research is quite innovative, although studies of this type have surfaced on the international stage. Most frequently it is the CARD15/NOD2 gene that is subject to analysis as so far, it has seemed to be most closely associated CD. However, recent studies have pointed to other susceptibility genes, for example ATG16L1 or IL23R, which may shed more light on the results obtained so far

In this research project the authors decided to scrutinize those genes which were most frequently linked to CD in publications appearing over the course of last several years. In the planning process of the study presented here we decided to concentrate on the analysis of three chosen genes, as well as the polymorphisms present within them, in order to eventually uncover certain dependencies. We were fortunate to observe certain tendencies in the occurrence of disease symptoms related to a particular genotype; however, we found no relation which might be crucial in explaining the mystery of CD. It is possible that the reason for this is either the absence of such a dependency, or the insufficient size of the research group. Although 160 patients seem to constitute a rather substantial sample, worldwide, these analyses are conducted on groups with numbers going into thousands.

Unfortunately, conducting such large-scale study in Poland is extremely problematic; therefore, a project based on a group of even 160 patients, yet featuring an analysis of 10 polymorphic loci, might be considered a success.
When this research project was still in its infancy, the topic of multilocus analyses of both clinical symptoms and the course of the disease was entirely innovative in Poland; what is more, foreign studies of this type were also extremely scarce. In recent years, the topic has attracted much attention worldwide; nevertheless, in Poland, a genetic multilocus study complemented by an analysis of the clinical symptoms and demonstrating such a wide scope remains a rarity. It is also worth mentioning that despite the fact the relevant genetic dependencies underlying CD remain undiscovered, such a possibility is not unattainable in the future. Global research aimed at unearthing its causes is being carried out as we speak, and it is the authors' firm belief that Polish researchers will also participate in this trend. After all, CD is a complex condition, and the research project presented here does not exhaust all the possible hypotheses pertaining to its etiology.

6. Conclusions

In this research project, 139 patients with Crohn's Disease were analyzed taking into consideration 5 single nucleotide polymorphisms (SNPs):

- CARD15/NOD2 G908R,
- *ATG16L1* rs2241879,
- *ATG16L1* rs2241880,
- *IL23R* rs7517847,
- *IL23R* rs1004819,

The obtained results were compared to an adequate population group.
All SNPs were polymorphic in the Polish population, which has been described in detail in the previous section of this publication. Unfortunately, no statistically significant differences in the distribution of particular genotypes were observed. However, the analysis of alleles showed, that:

- in the *ATG16L1* gene (rs2241880), allele C was statistically significantly more frequent among CD patients than in the population group (p=0.0017, for allele T p=0.0775).
- in the *IL23R* gene (rs7517847), allele T was statistically significantly more frequent among CD patients than in the population group (p=0.0011, for allele G p=0.0987).

Due to these results, it is possible that allele C for *ATG16L1* Thr300Ala (rs2241880) and T for *IL23R* (rs7517847) are connected with a higher risk of developing CD.
The second part of this research project focused on alleles and genotypes; in particular, on their relation to the localization of symptoms. It revealed that there are certain tendencies that can predict some predispositions to exact disease symptoms:

- among the CD patients, in the *CARD15/NOD2* gene G908R, genotype CC and allele C were the most frequent ones, which is connected with the localization of the symptoms in the area of intestines and the presence of perianal fistulas.
- the analysis of the extra-intestinal symptoms also revealed some interesting dependences: patients with joint pain, uveitis, stomatitis aphtosa, erythema nodosum, or elevated body temperature have statistically more frequent alleles C and the CC genotype.

- in the *ATG16L1* gene (rs2241879), patients with symptoms localized in the small intestine more frequently displayed the GA genotype; what is more, the same genotype was observed more often among patients with joint pain and erythema nodosum. Unfortunately, the allele analysis did not reveal statistically significant dependences.
- in the *ATG16L1* gene (rs2241880), the TC genotype was observed more frequently for patients with symptoms localized in the small intestine, arthralgia and erythema nodosum. Allele analysis did not reveal any statistically significant dependences.
- in the *IL23R* gene (rs1004819), the TT genotype was statistically the predominant one in CD patients with the disease symptoms manifested in the small intestine; however, allele analysis revealed that the presence of allele T alone may be crucially connected with the colonic symptoms as well. What is more, the TT and TC genotypes were statistically more frequent among patients declaring the presence of erythema nodosum.
- in the *IL23R* gene (rs7517847) the TT genotype was statistically prevalent among CD patients with symptoms localized in the small intestine. Together with the GT genotype, the TT genotype was statistically more frequent among individuals complaining of joint pain.

Although this research project did not reveal statistically significant differences in the distribution of particular genotypes in CD patients and the population group, some tendencies have been observed. They can be helpful when predicting the development of the disease, but they cannot be treated as a diagnostic parameter; rather, they indicate certain predispositions to the development of the disease. As a result, currently patient history, physical examinations, colonoscopy, histopathology and radiological studies (e.g. USG examination) still remain the main sources of the diagnosis.

It needs to be noted that although spectacular genetic dependencies underlying the causes of CD have not been discovered as of yet, it is still feasible in the future. Indeed, with every next research project – including this one – new advances are made in the field; therefore, it is possible that the genetic background of CD will be determined in the coming years which would constitute a significant advantage for patients.

7. References

Annese V, Lombardi G, Perri F, D'Incà R, Ardizzone S, Riegler G, Giaccari S, Vecchi M, Castiglione F, Gionchetti P, Cocchiara E, Vigneri S, Latiano A, Palmieri O, Andriulli A. Variants of CARD15 are associated with an aggressive clinical course of Crohn's disease--an IG-IBD study. Am J Gastroenterol. 2005 Jan;100(1):84-92

Barrett JC, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD, Brant SR, Silverberg MS, Taylor KD, Barmada MM, Bitton A, Dassopoulos T, Datta LW, Green T, Griffiths AM, Kistner EO, Murtha MT, Regueiro MD, Rotter JI, Schumm LP, Steinhart AH, Targan SR, Xavier RJ; NIDDK IBD Genetics Consortium, Libioulle C, Sandor C, Lathrop M, Belaiche J, Dewit O, Gut I, Heath S, Laukens D, Mni M, Rutgeerts P, Van Gossum A, Zelenika D, Franchimont D, Hugot JP, de Vos M, Vermeire S, Louis E; Belgian-French IBD Consortium; Wellcome Trust Case Control Consortium, Cardon LR, Anderson CA, Drummond H, Nimmo E, Ahmad T, Prescott NJ, Onnie CM, Fisher SA, Marchini J, Ghori J, Bumpstead S, Gwilliam R, Tremelling M, Deloukas P, Mansfield J, Jewell D, Satsangi J, Mathew CG, Parkes M, Georges M, &

Daly MJ. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nat Genet. 2008 Aug;40(8):955-62

Cukovic-Cavka S, Vermeire S, Hrstic I, Claessens G, Kolacek S, Jakic- Razumovic J, Krznaric Z, Grubelic K, Radic D, Misak Z, Jadresin O, Rutgeerts P, & Vucelic B. NOD2/CARD15 mutations in Croatian patients with Crohn's disease: prevalence and genotype-phenotype relationship. Eur J Gastroenterol Hepatol 2006 Aug;18(8):895-9.

Cho J. Inflammatory bowel disease: genetic and epidemiologic considerations. World J Gastroenterol 2008 January 21; 14(3):338-347

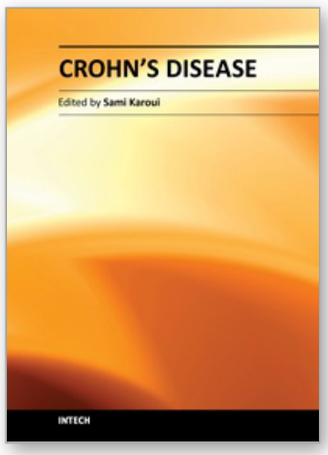
Jürgens M, Brand S, Laubender RP, Seiderer J, Glas J, Wetzke M, Wagner J, Pfennig S, Tillack C, Beigel F, Weidinger M, Schnitzler F, Kreis ME, Göke B, Lohse P, Herrmann K, Ochsenkühn T. The presence of fistulas and NOD2 homozygosity strongly predict intestinal stenosis in Crohn's disease independent of the IL23R genotype. J Gastroenterol. 2010 Jul;45(7):721-31 Epub 2010 Apr 29

Lacher M, Helmbrecht J, Schroepf S, Koletzko S, Ballauff A, Classen M, Uhlig H, Hubertus J, Hartl D, Lohse P, Schweinitz D, Kappler R. NOD2 mutations predict the risk for surgery in pediatric-onset Crohn's disease Journal of Pediatric Surgery (2010) 45, 1591–1597

Mendoza JL, Murillo LS, Fernández L, Peña AS, Lana R, Urcelay E, Cruz-Santamaría DM, de la Concha EG, Díaz-Rubio M, García-Paredes J. Prevalence of mutations of the NOD2/CARD15 gene and relation to phenotype in Spanish patients with Crohn disease. Scand J Gastroenterol. 2003 Dec;38(12):1235-40

Slomski R. 2008, Wydawnictwo Uniwersytetu Przyrodniczego w Poznaniu 2008, ISBN 978-83-7160-496-6, Poznan

Lesage S, Zouali H, Cézard JP, Colombel JF, Belaiche J, Almer S, Tysk C, O'Morain C, Gassull M, Binder V, Finkel Y, Modigliani R, Gower-Rousseau C, Macry J, Merlin F, Chamaillard M, Jannot AS, Thomas G, Hugot JP; EPWGIBD Group; EPIMAD Group; GETAID Group. CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease. Am J Hum Genet. 2002 Apr;70(4):845-57. Epub 2002 Mar 1



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In this book, several important points regarding Crohn's disease are discussed. In the first section, we focus on etiopathogeny of Crohn's disease and the recent advances in our overall understanding of the disease - specifically, the role of the gut epithelium, alterations of the epithelial crypts, and the roles of the different cytokines in the pathophysiology of Crohn's disease. In the second section, a diagnosis of Crohn's disease is discussed. Another particular area of focus is in the diagnosis of intestinal tuberculosis, and the role of mycobacterium avium in Crohn's disease. In the third and final section, the management of Crohn's disease is discussed, with a focus on recent evidence-based medicine recommendations.

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