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Karimi, O.

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Chapter 7

Functional polymorphisms in the blood coagulation factor *F13A1* gene and inflammatory bowel disease in the Netherlands

O. Karimi, A.A. van Bodegraven, S. Ouburg, A.S. Peña, J.B.A. Crusius

Abstract

Background: Patients with inflammatory bowel disease (IBD) have an increased risk of venous thromboembolisms and recurrent thrombotic disease compared with the general population. Plasma coagulation factor XIII activity has been reported to be decreased in IBD. Two common non-synonymous polymorphisms in the gene encoding coagulation factor XIII, A1 polypeptide *F13A1* Val34Leu and *F13A1* Pro564Leu are associated with thrombotic diseases. We investigated the role of these gene polymorphisms in the susceptibility to and severity of IBD. *Methods:* We genotyped 666 unrelated Dutch IBD patients (338 suffering from ulcerative colitis (UC) and 328 suffering from Crohn's disease (CD)) and 190 ethnically matched healthy subjects by a Polymerase Chain Reaction–Restriction Fragment Length Polymorphism method. UC was classified according to the colonic extent of inflammation. CD was classified according to the Vienna classification.

Results: The *F13A1* 34Leu allele carriage in the total IBD group showed a significant positive association in comparison to the controls (p=0.004, OR: 1.66, 95% CI: 1.18 to 2.34) and this association was maintained in both CD (p=0.011, OR: 1.63, 95% CI: 1.22 to2.38) and UC (p=0.007, OR: 1.69, 95% CI: 1.16 to2.46) when compared to controls. No significant association was found for the *F13A1* Pro564Leu polymorphism. Neither of the two polymorphisms was associated with the different clinical manifestations.

Conclusions: In the Dutch population the *F13A1* Val34Leu gene polymorphism confers an increased risk for IBD, UC and CD. A paucity of episodes of thrombotic events observed in our patients during 23 years of follow-up in UC and 19 years in CD precluded the study of the relation with thrombosis.

Introduction

Inflammatory bowel diseases (IBD) are chronic, relapsing inflammatory disorders of the gastrointestinal tract. The term encompasses ulcerative colitis (UC), Crohn's disease (CD) and a less common intermediate form. Although the precise aetiology remains to be elucidated, the current paradigm suggests a dysregulated mucosal immune response to intestinal microbes in genetically susceptible individuals.[1] Ubiquitous intestinal microbiota is an early initiating factor in the aetiopathogenesis of disease by triggering an inappropriate, overactive, and ongoing intestinal inflammation. This inflammation is continuous and confined to the colonic mucosa in UC, whereas in CD it is patchy and transmural and most commonly involves the ileum and colon but can affect any region of the gastrointestinal tract.[2]

UC and CD are complex genetic diseases with high heritability (UC:λ_s ~7-17 and CD:λ_s ~15–35).[3] Genome-wide and follow-up replicating candidate gene case-control association studies have identified 18 susceptibility loci for UC explaining ~11% of the heritability for this disease.[4-8] In 2001 linkage analysis and positional cloning led to the identification of the first CD susceptibility gene, *NOD2*, later followed by a locus at chromosome 5q31 and less consistent replication at other loci (as reviewed by Mathew et al.[9]). A meta-analysis[10] of early genome-wide association studies (GWAS) scans (up to mid-2008)[2,11,12] implicated 32 susceptibility loci accounting for ~20% of the genetic contribution to CD risk. Apart from disease-specific susceptibility loci UC and CD share loci as well as.[4,13,14] The shared susceptibility genes *IL23R*, *NKX2-3,* and *CCNY* have common denominators of intestinal inflammation.

Clinical observations showed that IBD patients have an increased risk to develop venous thromboembolic disorders compared to patients without IBD. Several studies have confirmed these observations and showed a 2-3 fold increased risk of developing venous thromboembolism (VTE) in IBD patients compared with the general and non-hospitalized patients.[15,16] Almost 80% of UC and CD patients have active disease when thromboembolism is diagnosed, which represents a significant cause of morbidity and mortality.[16] In a recent large study in the UK an overall 8.4-fold increased risk of developing VTE during acute flares of IBD as compared to the general population was reported. When compared to controls an even higher 15.8-fold risk was found in non-hospitalized patients, and a 3.2-fold higher risk in hospitalised IBD patients.[17]

Blood coagulation factor XIII (FXIII) is a protransglutaminase. After its activation, it cross-links fibrin chains and several plasma proteins to fibrin. The most important plasma protein involved in this respect is the alpha (2) plasmin inhibitor. Furthermore, FXIII catalyzes the formation of covalent ε(γ-glutamyl)lysyl bonds between fibrin strands to generate stable

fibrin networks.[18] The formation of these networks is the ultimate step of the coagulation cascade, and favours intestinal wound repair.[19] FXIII also binds antiplasmin to fibrin networks, and thus induces increased resistance against fibrinolytic degradation.[18,20] Plasma FXIII circulates as a tetramer, consisting of two identical active A-subunits and two identical carrier B-subunits. During activation of plasma FXIII, thrombin causes the N-terminal of activation-peptide of FXIII (AP-FXIII) to cleave off. Hereby FXIII converts to FXIII A'_2B_2, and by binding of Ca^{2+} the A and B subunits dissociate in the presence of fibrin.[21]

FXIII might be involved in some clinical aspects of IBD, with regard to the erosions and ulcerations of the intestinal mucosa. Repair of mucosal architecture heralds the remittance of active disease. Several studies show decreased plasma FXIII concentrations and hyperfibrinolysis in active IBD[22-25], but not in quiescent disease.[25,26] Patients with IBD also seem to be at increased risk of recurrent VTE compared to patients without IBD.[27] Furthermore, FXIII plays an important role in several aspects of the pathophysiology of IBD, as has clearly been observed in a rat model of colitis.[20] Experimental studies in rats[28,29] and some reports in humans show therapeutic benefit from addition of intravenously administered FXIII.[30,31] However, a double blind, placebo-controlled study of intravenous application of FXIII concentrate or placebo in 28 active steroid-refractory patients with UC using cessation of visible intestinal bleeding at 14 days as primary objective showed no beneficial effect.[32]

The gene encoding coagulation factor XIII, A1 polypeptide (*F13A1*) is located on chromosome 6.p24-25, includes 15 exons and 14 introns and spans over 200 kb. Many polymorphisms have been described (33). FXIII activity and its plasma concentration seem to be influenced by the *F13A1* Val34Leu and Pro564Leu single nucleotide polymorphisms (SNPs) usually indicated by the amino acid position changes.[34-37] Although the exact biochemical consequences of the *F13A1* Val34Leu and Pro564Leu SNPs are not well understood, the 34Leu allele is associated with an increased FXIIIA activation rate.[38,39] Individuals with this allele seem to have a more rapid activation and depletion of the plasma FXIIIA subunits[35] leading to decreased FXIIIA availability and thereby less stable clots with protection against thrombosis as main result.
In this study, we aimed to investigate whether the *F13A1* Val34Leu and Pro564Leu SNPs, with abilities to change physiology of circulating FXIII, are associated with IBD or with distinct clinical features of IBD.

Materials and methods

Patients and controls

Participants were recruited from the Outpatient Clinic of Gastroenterology of the VU University Medical Center, a tertiary referral centre for patients with IBD. The patient group comprised of 666 patients with IBD: 338 patients with UC and 328 patients with CD. Few patients had early onset IBD. The control group consisted of 190 healthy subjects, who have been recruited from staff and students of the same Center. All subjects were unrelated Dutch Caucasians.

IBD was classified according to disease course. Diagnosis and assessment of maximal extent of IBD were based on endoscopic, pathohistological, and radiological findings. UC is restricted to the colon, usually starting in the rectum and may spread into upper segments of the colon. The disease is classified as proctitis, when inflammation is limited to the rectum; as left sided colitis, when the extent is below the left colonic flexure; and as pancolitis, when disease reaches beyond the splenic flexure of the colon into the caecum. In some cases, UC starts as pancolitis and may regress. Additionally, there was a subgroup of UC patients who had (procto) colectomy during the course of disease, indicative of severe or intractable disease.

Classification of CD was based on three categories consisting according to the Vienna-classification.[40] This classification subdivides patients with CD according to age at diagnosis (below 40 years or from 40 years on), localization of disease (terminal ileum, colon, ileocolonic, or upper gastrointestinal tract) and disease behaviour (chronic inflammatory, stenosing, fistulising) in the disease period from diagnosis until the first surgical procedure. The demographic and clinical characteristics of patients with UC and CD are described in Table 1.

Table 1: Demographic and clinical characteristics of UC and CD patient groups and controls

	Ulcerative Colitis (UC)	Crohn's Disease (CD)	Controls
Number	338	328	190
Age at diagnosis in years	39.7	33.7	
Male/Female	148/190	99/229	86/104
Localization	Proctitis 98 (29%) Left-sided: 107 (33%) Extended colitis / pancolitis 131 (40%)		
Vienna classification		A1 (age of diagnosis < 40 years): 270 (82%) A2 (age of diagnosis ≥ 40 years): 58 (18%) B1 (chronic inflammation): 154 (47%) B2 (stenotising): 112 (34%) B3 (fistulizing): 55 (17%) L1 (terminal ileum): 78 (24%) L2 (colon): 101 (31%) L3 (colon and terminal ileum): 134 (41%) L4 (proximal gastrointestinal tract): 9 (3%)	
Years of follow-up, median (range)	23 (0-86)	19 (0-77)	
Colectomy	54 (16%)		
Intestinal surgery **	49 (14%)	127 (39%)	
Thromboembolism in history		5 (2%)	

** years of follow-up were calculated from onset of disease till last visit at our hospital; the several clinical subgroups did not differ significantly (Kruskall-Wallis). ** appendectomy excluded*
Clinical type of Crohn's disease according to the Vienna classification: A1 = diagnosis before 40 years of age, A2 = diagnosis from 40 years of age on; B1 = disease behaviour characterized by chronic inflammation, B2 = characterized by stenotising processes, and B3 = fistulizing type of disease; L1 = disease localization confined to the terminal ileum, L2 = confined to the colon, L3 = disease in colon and terminal ileum, and L4 = disease primarily in the proximal gastrointestinal tract. Clinical type ulcerative colitis according to localization: Proctitis = limited to the rectum; left sided colitis = extent is below the left colonic flexure; pancolitis = disease reaches beyond the splenic flexure of the colon into the caecum

Determination of F13A1 gene polymorphisms

Genomic DNA was extracted from peripheral blood leukocytes according to a conventional proteinase K digestion and phenol/chloroform procedure.
The *F13A1* Val34Leu polymorphism (dbSNP ID: rs5985) was analyzed as described by Balogh *et al.*[38] with forward primer 5′ ACT TCC AGG ACC GCC TTT GGA GGC 3′ and

reverse primer 5´ GTT GAC GCC CCG GGG CAC CG 3´. The underlined G in the reverse primer creates a *Cfo*I restriction site. The reaction mixture of 25μl consisted of 0.1 μg genomic DNA in 50 mM KCl, 19 mM Trizma (pH 8.5), 1.6mM $MgCl_2$, 0.5% Nonidet P-40, and 0.5% Tween 20, 200 μM each of dNTPs, 0.2 μM of each primer and 0.2U Tsp XI DNA polymerase. Optimal PCR conditions were initial denaturation at 94°C for 60 s, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 74°C for 90 s, with a final incubation at 72°C for 5 min in a GeneAmp PCR System 9700 thermal cycler. The length of the amplified PCR fragment was 114 base pairs (bp). Restriction endonuclease digestion with *Cfo*I was performed according to the manufacturer's instructions. PCR products were incubated at 37°C for two hours, followed by gel electrophoresis in 4.5% Response Research agarose and ethidium bromide staining. In 34Leu/34Leu (genotype TT) homozygotes the 114bp product remained undigested and wildtype 34Val/34Val genotypes (GG) did show a 94bp fragment after *Cfo*I digestion. Consequently, heterozygotes Val34/Leu34 (GT) revealed both the 114bp and 94bp fragments.

Analysis of the *F13A1* Pro564Leu polymorphism (rs5982) has been performed as described by Kohler *et al.*.[41] In short, the composition of the PCR mixture was as described above with a forward primer 5´ ACC TTC TAC ACC GTG GTC C 3´ allowing the detection of variants at the *Nla*IV site at position 564 and reverse primer 5´ CAG CGA GTC TCA CAA AGA ACC 3´. The program of 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 90 s, and a final incubation at 72°C for five min generated 112bp fragments. *Nla*IV digestion did result in wildtype 564Pro/564Pro (CC) genotypes characterized by three fragments of 50bp, 45bp, and 17bp. Homozygous 564Leu/564Leu (TT) subjects were distinguished by 62bp and 50bp fragments and heterozygous 564Pro/564Leu (CT) subjects by both 62bp, 50 bp, 45 bp and 17bp fragments.

Statistics

Statistical analyses were performed using Graphpad Instat statistical software (GraphPad Software, Inc. La Jolla, USA).

Comparisons of carriage of the minor alleles of the SNPs between patients and controls were performed by application of χ^2 test or Fisher's exact test, where appropriate. Genotype frequencies were compared within the CD subgroups defined by the Vienna classification. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated. A two-sided p value <0.05 was considered statistically significant.

Results

Genotyping was successful for 335 out of 338 (99%) for the Val34Leu SNP and for 328 out of 338 (97%) for the Pro564Leu SNP in patients with UC. For CD 320 out of 328 (97%) were successfully genotyped for the Val34Leu SNP and 309 out of 328 (94%) for the Pro564Leu SNP. For both SNPs success rate was 181 out of 190 (95%) in controls. The findings of genotyping the study group for the *F13A1* Val34Leu and Pro564Leu SNPs are summarized in Table 2. The genotype frequencies of both SNPs were in Hardy-Weinberg equilibrium in patients and controls (data not shown, $p>0.05$). Carriage of the minor allele 34Leu of the *F13A1* Val34Leu polymorphism shows a significant positive association with IBD when compared with controls ($p=0.004$, OR: 1.66, 95% CI: 1.18 to 2.34). This polymorphism was associated both with UC ($p=0.007$, OR: 1.69, 95% CI: 1.16 to 2.46) and with CD ($p=0.011$, OR: 1.63, 95% CI: 1.22 to 2.38).

The same analysis was performed for the *F13A1* Pro564Leu polymorphism to study its occurrence in patients with IBD, CD and UC. However, no significant differences were found when compared to controls.

In UC, when analyzing the subclassification according to maximum extent of disease we found no differences in the carriage of the minor allele nor genotype distribution of both SNPs. Also for severity of disease in UC patients, assessed by the presence of colectomy, no statistical significance differences were observed for either polymorphism.

Table 2. *F13A1* genotype distribution in ulcerative colitis and Crohn's disease patient groups subdivided according to clinical course, in all IBD patients and controls

	Val34Leu			***Pro564Leu***		
	Leu/Leu (TT)	Val/Leu (GT)	Val/Val (GG)	Leu/Leu (TT)	Pro/Leu (CT)	Pro/Pro (CC)
UC (n= 338)	31 (9.3)	130 (38.8)	174 (51.9)	16 (4.9)	101 (30.8)	211 (64.3)
Proctitis	10 (10.4)	42 (43.3)	45 (46.4)	4 (4.1)	35 (35.7)	59 (60.2)
Left-sided	8 (7.5)	41 (38.3)	58 (54.2)	5 (4.9)	28 (27.5)	69 (67.6)
Pancolitis	13 (9.9)	47 (35.9)	71 (54.2)	7 (5.5)	38 (29.7)	83 (64.8)
No colectomy	28 (9.8)	112 (39.2)	146 (51.0)	13 (4.7)	86 (30.9)	179 (64.4)
Colectomy	3 (6.1)	18 (36.7)	28 (57.1)	3 (6.0)	15 (30.0)	32 (64.0)
CD (n=328)	25 (7.8)	126 (39.4)	169 (52.8)	15 (4.9)	108 (35.0)	186 (60.1)
A1	21 (7.9)	105 (39.6)	139 (52.5)	12 (4.7)	96 (37.2)	150 (58.1)
A2	4 (7.3)	21 (38.2)	30 (54.5)	3 (5.9)	12 (23.5)	36 (70.6)
B1	11 (7.1)	61 (39.6)	82 (53.2)	5 (3.3)	53 (35.3)	92 (61.3)
B2	10 (9.0)	40 (36.0)	61 (55.0)	5 (4.6)	38 (34.9)	66 (60.6)
B3	4 (7.3)	25 (45.5)	26 (47.2)	5 (10.0)	17 (34.0)	28 (56.0)
L1	4 (5.1)	34 (43.6)	40 (51.3)	5 (6.8)	23 (31.1)	46 (62.2)
L2	7 (7.0)	38 (38.0)	55 (55.0)	5 (5.2)	29 (30.2)	62 (64.6)
L3	13 (9.8)	49 (36.8)	71 (53.4)	5 (3.8)	52 (40)	73 (56.2)
L4	1 (11.1)	5 (55.6)	3 (33.3)	0 (0.0)	4 (44.4)	5 (55.6)
IBD (n=666)	56 (8.5)	256 (39.1)	343 (52.4)	31 (4.7)	209 (32.8)	397 (62.5)
Controls (n=190)	9 (5.0)	55 (30.4)	117 (64.6)	10 (5.0)	51 (28.2)	120 (66.3)

See legend to Table 1.

Subdividing the CD patient group, based on presurgical clinical characteristics according to the Vienna classification and performing a similar analysis by analysing each of the items against the combined items for behaviour and localization (Categories B and L) did not reveal any statistical significant difference for each of the studied polymorphisms. A trend was observed for a decreased frequency of the *F13A1* Pro564Leu homozygous mutant in CD patients with fistulising type disease (B3) compared to stenotizing type of disease B2 and patients with chronic inflammation (B1) (p=0,08, OR: 2.8, 95%CI: 0.9 to 8.5). Linkage disequilibrium between the polymorphisms was low in UC, CD, IBD, and controls (r^2<0.07).

Discussion

In this study, we found a significant association between UC, CD and IBD and carriage of allele *F13A1* 34Leu but no association with the *F13A1* Pro564Leu polymorphism.

Previous studies have found genetic variants affecting the risk of thrombosis, such as factor V Leiden, factor II (prothrombin) and one of the gene polymorphisms studied by us, the *F13A1* Val34Leu. Meta-analyses of >3,000 VTE cases and almost 5,000 controls showed significant protective effects for *F13A1* Val34Leu heterozygotes and carriers, with the strongest effect for homozygotes of the *F13A1* Val34Leu allele (pooled OR=0.65) in Caucasians.[42,43] Gain-of-function mutations have been observed in procoagulant factors.[44]

The *F13A1* Val34Leu polymorphism within AP-FXIII is located critically nearby the thrombin activation site and seems to protect moderately against myocardial infarction, deep venous thrombosis and ischemic stroke as reviewed recently by Muszbek *et al*..[45] This protective effect has been attributed to structural characteristics of the bound AP-FXIII, influencing the affinity of thrombin to the bound AP-FXIII. A meta-analyses of GWAS datasets comprising more than 26,000 UC patients and controls[46] and more than 21,000 CD patients and controls[47], all of European descent, including even larger replication cohorts detected significant associations of IBD with almost 100 susceptibility genes/loci each conferring modestly increased risk of disease. According to this metaanalysis, the number of UC-associated loci is 47 explaining an estimated 16% of UC heritability whereas the number of confirmed CD–associated loci amounts to 71 explaining an estimated 23.2% of CD heritability. Twenty-eight loci represent general IBD risk loci. Major themes these genes are involved encompass IL23/Th17 signalling, autophagy and innate immunity with defective processing of intracellular bacteria in CD and barrier function in UC development. Furthermore, overlap with other gastrointestinal and with non-gastrointestinal immune-mediated diseases is apparent.[48]

Since the *F13A1* Val34Leu (rs5985) and Pro564Leu (rs5982) are present on Illumina platforms and no fair proxies are present on Affymetrix genotyping platforms (r^2=0.58 and r^2=0.28 in HapMap3 CEU reference samples, respectively) in the GWAS studies used in the meta-analyses the actual numbers were 3,291 UC patients and 12,607 controls[46] and 4,586 CD

patients and 12,119 controls.[47] These meta-analyses found no evidence for the involvement of common *F13A1* SNPs in UC and CD susceptibility including F13A1 Val34Leu ($P_{combined}$=0.20 and 0.13, respectively) or Pro564Leu ($P_{combined}$=0.57 and 0.62, respectively) (from data of the international inflammatory bowel disease consortium (IIBDGC) at www.ibdgenetics.org).

A slightly greater prevalence of *F13A1* Leu34 homozygozity in CD vs. controls has been reported in a more recent population based study.[49] However, in other small studies no differences in genotype distributions were apparent[50,51], and a somewhat larger study failed to reveal differences in allele frequencies.[52] In another small study, Koutroubakis et al. compared the prevalence of *F13A1* Val34Leu in IBD patients with vascular complications and non-IBD thrombotic patients, and found no significant differences.[53] In our patient population we have observed only 5 patients with thromboembolism during 23 years of follow-up in UC and 19 years in CD.

At present therefore, the evidence for involvement of both *F13A1* SNPs in IBD susceptibility is weak at most. Consequently, it cannot be excluded that the findings of a positive association of *F13A1* 34Leu carriers with IBD in our study results from a type II error.

Although considerable efforts in DNA sequencing in patients with atherothrombotic diseases has been performed it cannot be excluded that other *F13A1* genetic variation (rare variants with high penetrance, in/dels, inversion, translocations or copy number polymorphisms) will be discovered in future next-generation deep sequencing studies these are unlikely to notably contribute to IBD susceptibility since out of the common functional *F13A1* SNPs the Val34Leu (and the haplotype tagged by allele *F13A1* 34Leu) is the main functional polymorphism influencing FXIII activation.[54]

We did not observe statistically significant differences between the extent of UC and the studied polymorphisms, nor between the subdivisions of the Vienna classification in CD patients and the studied polymorphisms. A slight trend was observed for a decreasing carriage of the *F13A1* 564Leu/Leu genotype in penetrating CD. The absence of associations of the polymorphisms with the disease subphenotypes of UC based on maximum disease extent and need for colectomy for medically refractory disease and the absence of associations of the polymorphisms with the subtypes of the Vienna classification in CD, fit in with prior reports showing no successful replications of genotype – phenotype associations with the exception of the association between *NOD2* genotypes and structuring/penetrating, ileal CD, and the association between extensive and severe UC and the HLA-DRB1*0103 haplotype.[48] Indeed, it has been suggested that despite the potential promise of genetic markers in prediction of disease course, they may never fully predict evolution of disease, because of incomplete penetrance, their modest to low frequency and the role of other environmental factors. The place of genetic markers in prediction of disease outcome is dependent on their crosstalk with other molecular markers, clinical data and environmental factors.[55]

Genetic studies of IBD patients with an increased risk to develop venous thromboembolic disorders might follow the recommendations recently made by Muszbek et al. for the study of *F13A1* genetic variation in ischemic stroke: well-designed prospective studies with interaction of the polymorphisms with possible modifying factors, most importantly with fibrinogen level, should be investigated.[45] These studies, genome-wide or restricted to loci contributing to the risk of thrombosis, could be the focus of future studies in patients with UC and CD taking into account ethnic differences since for example the *F13A1* Val34Leu SNP is extremely rare in Asians whilst IBD seems to increase in these countries.

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