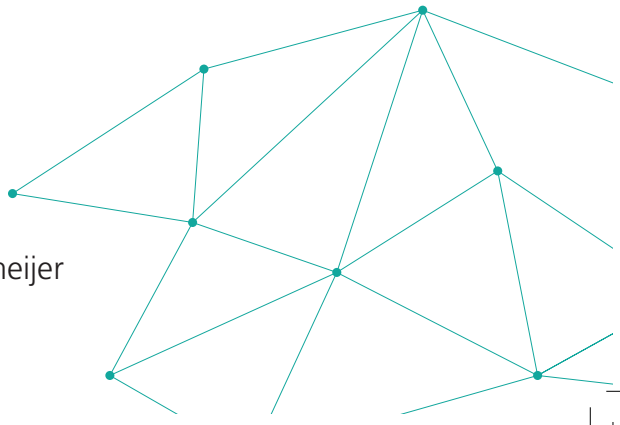
GENETIC AND IMMUNOLOGIC ASPECTS OF ACUTE PANCREATITIS

— An odyssey —



Rian M. Nijmeijer

Genetic and immunologic aspects of acute pancreatitis
An odyssey
Rian M. Nijmeijer
Thesis, Utrecht University, the Netherlands

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Genetic and Immunologic Aspects of Acute Pancreatitis

An odyssey

Genetische en Immunologische Aspecten van Acute Pancreatitis

Een odyssee

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 10 april 2014 des middags te 12.45 uur

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Copromotoren:	Dr. K.J. van Erpecum Dr. F.G. Schaap

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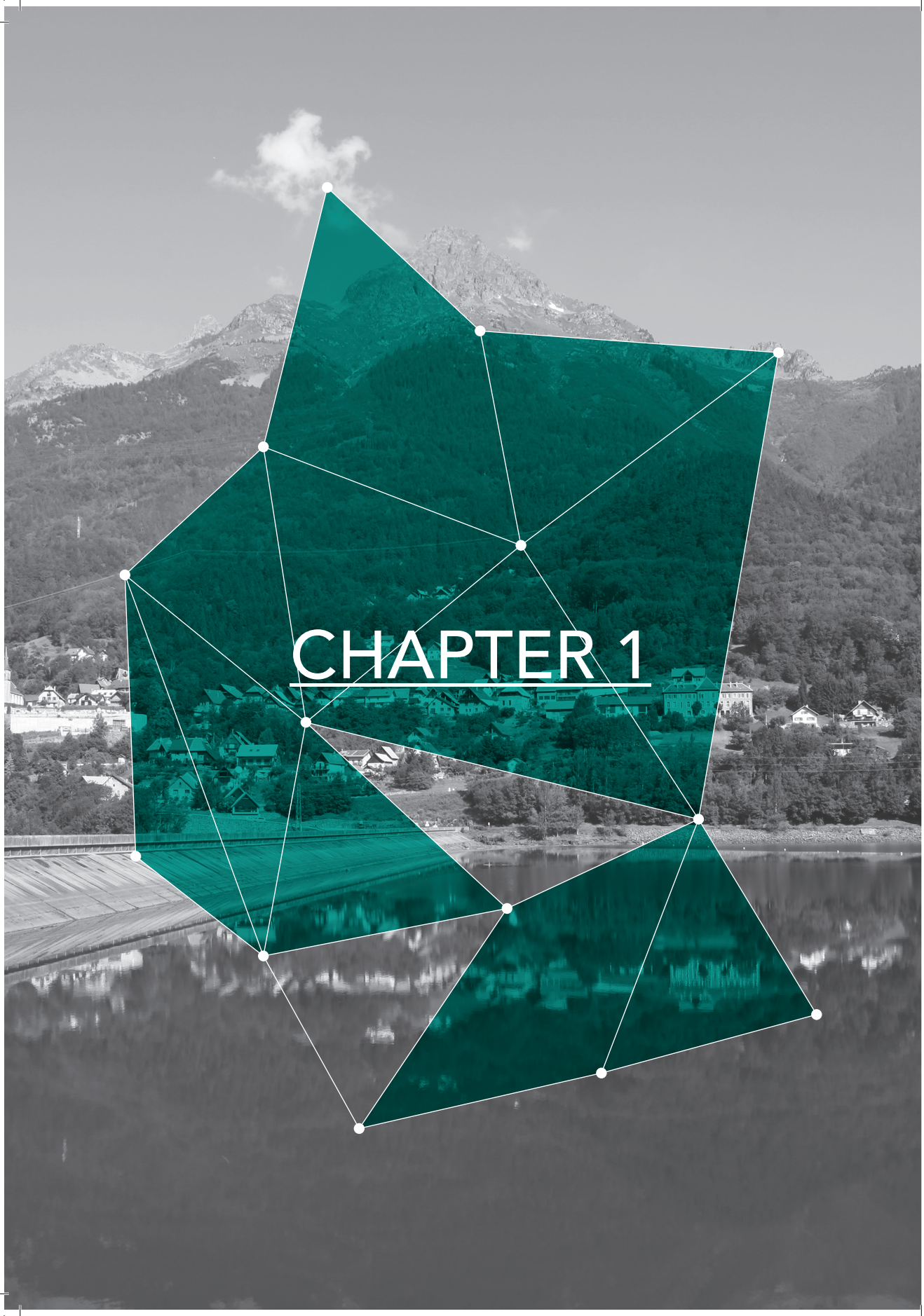

CHAPTER 1

INTRODUCTION

Acute pancreatitis is a common disease of the digestive tract with an incidence of over 280,000 hospitalizations in the United States (US) each year.[1] The median length of stay in the hospital is 4 days and the median costs are over 6,000 dollars, with an estimated aggregate cost of acute pancreatitis in the US of 2.6 billion dollars per year in inpatient costs.[1]

Acute pancreatitis is the acute inflammation of the pancreas, which is mostly caused by gallstones or alcohol abuse.[2] The incidence of the disease has increased over the years, mainly because of a rise in the incidence of gallstone disease.[3, 4] In the majority of patients, the disease is mild and self-limiting, but in a significant proportion of patients (15-25%), the disease runs a severe course.[5] Morbidity and mortality are, in the majority of cases, caused by infectious complications, of which the bacterial infection of pancreatic necrosis increases mortality the most.[6-8]

As early as 1896, the pathologist Chiari published that pancreatitis was the result of autodigestion of the pancreas[9], but despite much research work, the exact pathophysiology of the disease remains unclear. A critical event is indeed the premature activation of digestive enzymes within the pancreatic acinar cells, resulting in autodigestion of the pancreas. Whatever the initiating event, disease progression is roughly the same in every patient, starting with local inflammation of the pancreas, which initiates a generalized inflammatory response, and in some patients this leads to a final stage of (multi)organ failure and ultimately death.[10]

Acute pancreatitis often shows a biphasic course. In the first part of the disease, patients develop systemic inflammatory response syndrome (SIRS) in response to the localized inflammatory insult, which can, in severe cases, lead to development of multiple organ dysfunction and death. In the second phase, a compensatory anti-inflammatory response syndrome (CARS) develops. This can result in infectious complications including bacterial infection of pancreatic necrosis and may result in sepsis with multiple organ failure and death.[10] The severity of the disease is hard to

predict at the moment a patient is hospitalized and appears to depend upon the balance between pro-inflammatory (SIRS) and anti-inflammatory (CARS) responses in the patient.[5]

Infectious complications, including bacteraemia, pneumonia and bacterial infection of (peri)pancreatic necrosis are thought to result from bacterial translocation, where bacteria that normally reside in the gut migrate across the mucosal barrier to infect organs elsewhere. Bacterial translocation is likely facilitated both by CARS suppressing the immune system and a breech of the intestinal barrier.[11-14]

Although many causes for acute pancreatitis are known, the disease is a very complex disorder.[15] It is still unclear why some individuals with biliary disease or who consume large amounts of alcohol develop acute pancreatitis, while others do not. Furthermore, the reason why some patients develop severe acute pancreatitis with complications and have to be admitted to an intensive care unit for support, while apparently similar patients have self-limiting disease, has not yet been clarified. Genetic factors are thought to contribute to disease susceptibility and may also influence the course of the disease. Acute pancreatitis may be the result when a pathological pathway is triggered in patients who have multiple risk factors for susceptibility and disease severity.[16]

The studies that we performed for this thesis were aimed at finding genes or pathways involved in the development of acute pancreatitis and the development of complications during the disease.

The first part of this thesis focuses on the genetic variants that could potentially be involved in the susceptibility or course of acute pancreatitis. Genetic association studies performed by others focused mainly on genes linked to trypsin activation, such as the cationic trypsin gene PRSS117 and the serum protease inhibitor Kazal type 1 gene (SPINK1)[18-22], and on genes involved in innate immunity, such as TNFα and other cytokines[23-29], and genes of the Toll-like receptor pathway.[30-33] First, we investigated genes involved in mucosal barrier function (Chapter 2). Our group had already shown that in acute pancreatitis, there is a relationship between intestinal barrier dysfunction and infectious complications.[13] *MYO9B* and two tight junction adaptor genes, *PARD3* and *MAGI2*, are genes involved in the mucosal barrier function and these had been shown to be associated with celiac disease and inflammatory bowel disease, both inflammatory conditions in which intestinal permeability plays a pathophysiological role.[34-36] We therefore hypothesized that these genes might also play a role in the mucosal barrier dysfunction in acute pancreatitis.

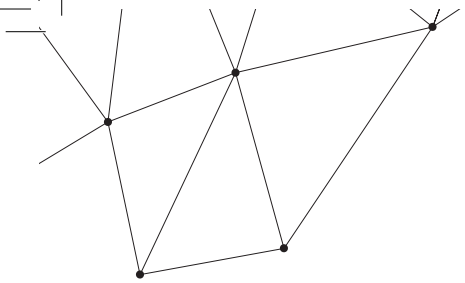
Differences in susceptibility to acute pancreatitis and the severity of the disease could potentially be explained by a difference in inflammatory response to a triggering event. The body's first line of defense is formed by the innate immune system. This system is able to sense bacteria and to kill them, but can also sense late apoptotic and necrotic cells, in this way also playing a considerable role in mucosal barrier integrity. The innate immune system is thought to be of great importance during

acute pancreatitis. Therefore, several genes involved in the innate immune system, including the Toll-like receptor pathway (Chapter 3), the mannose-binding lectin gene (*MBL2*, Chapter 4) and the nucleotide-binding oligomerization domain protein 2 (*NOD2/CARD15*, Chapter 5) were studied. Chapter 6 reports a study on the renin-angiotensin system (RAS), which is known to act as a key regulator of intravascular homeostasis, controlling extracellular fluid volume and blood pressure.[37] In several tissues including the pancreas, there are local renin-angiotensin systems. Pancreatic RAS has been implicated in the initiation and propagation of acute pancreatitis. In experimental models of acute pancreatitis increased expression of RAS components induced expression of pro-inflammatory cytokines.[38-40]

The second part of the PhD research consists of two studies that focused on two proteins we had hypothesized were involved in the pathogenesis of acute pancreatitis, FXR and FGF21. The farnesoid X receptor (FXR), a member of the nuclear receptor family, is considered the key regulator of bile acid homeostasis, but is also involved in several other processes, including fat and glucose metabolism, the maintenance of intestinal barrier integrity and prevention of bacterial translocation.[41, 42] Recently it was also shown that FXR exhibits anti-inflammatory effects by inhibiting NFκB.[43, 44] Chapter 7 reports complementary animal and human studies to study the potential role of FXR in acute pancreatitis. In a first experiment, we studied the expression of *Fxr* and Fxr-target genes in mice with experimental acute pancreatitis. A second study involved comparing the severity of acute pancreatitis in wild-type mice and mice lacking *Fxr*. Additionally, in patients with acute pancreatitis, a genetic association study was performed and the level of FGF19, one of the FXR target genes, was determined and compared to controls.

Chapter 8 looks at FGF21, a stress-induced hepatokine that affects glucose and lipid homeostasis. FGF21 was also shown to reduce severity of experimental acute pancreatitis in mice.[45] The aim of this study was an initial step towards addressing the role of FGF21 in human and mouse acute pancreatitis.

The third part of this thesis reports a study on the effect of probiotic pretreatment on mucosal barrier function in rats with experimental acute pancreatitis (Chapter 9) and one on the expression of FXR and the FXR target gene *SHP*, on the one hand, and a genetic association study of genetic variants in *FXR*, on the other, in patients with inflammatory bowel disease (Chapter 10).



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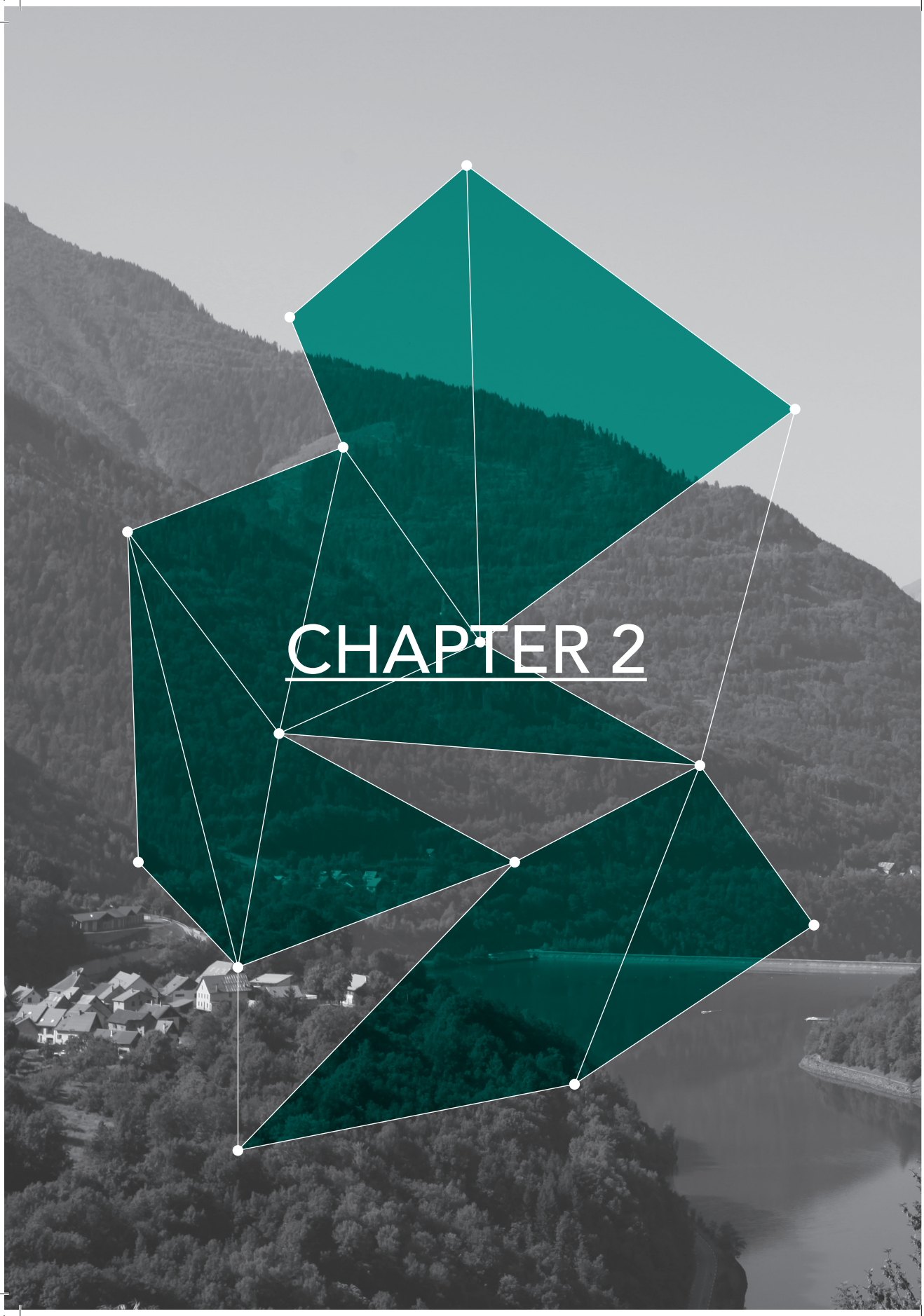
CHAPTER 2

Association analysis of genetic variants in the Myosin IXB gene in acute pancreatitis

Rian M. Nijmeijer[1], Hjalmar C. van Santvoort[1], Alexandra Zhernakova[2,3], Steffen Teller[4], Jonas A. Scheiber[4], Carolien G. de Kovel[2], Marc G. H. Besselink[1,5], Jeroen T. J. Visser[6], Femke Lutgendorff[1], Thomas L. Bollen[7], Marja A. Boermeester[5], Ger T. Rijkers[1,8,9], Frank U. Weiss[4], Julia Mayerle[4], Markus M. Lerch[4], Hein G. Gooszen[1,8], Louis M. A. Akkermans[1], Cisca Wijmenga[2,3], on behalf of the Dutch Pancreatitis Study Group

[1]Department of Surgery, University Medical Center Utrecht, Utrecht, the Netherlands; [2]Complex Genetics Group, Department of Medical Genetics, University Medical Center Utrecht, Utrecht, the Netherlands; [3]Department of Genetics, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands; [4]Department of Internal Medicine A, Ernst-Moritz-Arndt University, Greifswald, Germany; [5]Department of Surgery, Academic Medical Center, Amsterdam, the Netherlands; [6]Department of Cell Biology, Immunology Section, University of Groningen, University Medical Center Groningen, the Netherlands; [7]Department of Radiology, St Antonius Hospital, Nieuwegein, the Netherlands; [8]Department of Operating Room/Evidence Based Surgery, Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands; [9]Department of Medical Microbiology and Immunology, St Antonius Hospital, Nieuwegein, the Netherlands.
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Abstract

Introduction: Impairment of the mucosal barrier plays an important role in the pathophysiology of acute pancreatitis. The myosin IXB (*MYO9B*) gene and the two tight-junction adaptor genes, *PARD3* and *MAGI2*, have been linked to gastrointestinal permeability. Common variants of these genes are associated with celiac disease and inflammatory bowel disease, two other conditions in which intestinal permeability plays a role. We investigated genetic variation in *MYO9B*, *PARD3* and *MAGI2* for association with acute pancreatitis.

Methods: Five single nucleotide polymorphisms (SNPs) in *MYO9B*, two SNPs in *PARD3*, and three SNPs in *MAGI2* were studied in a Dutch cohort of 387 patients with acute pancreatitis and over 800 controls, and in a German cohort of 235 patients and 250 controls.

Results: Association to *MYO9B* and *PARD3* was observed in the Dutch cohort, but only one SNP in *MYO9B* and one in *MAGI2* showed association in the German cohort ($p < 0.05$). Joint analysis of the combined cohorts showed that, after correcting for multiple testing, only two SNPs in *MYO9B* remained associated (rs7259292, $p = 0.0031$, odds ratio (OR) 1.94, 95% confidence interval (95% CI) 1.35-2.78; rs1545620, $p = 0.0006$, OR 1.33, 95% CI 1.16-1.53). SNP rs1545620 is a non-synonymous SNP previously suspected to impact on ulcerative colitis. None of the SNPs showed association to disease severity or etiology.

Conclusion: Variants in *MYO9B* may be involved in acute pancreatitis, but we found no evidence for involvement of *PARD3* or *MAGI2*.

Introduction

Acute pancreatitis is an acute inflammatory condition of the pancreas, resulting in over 200,000 hospital admissions in the United States each year.[1] In most patients, it is caused by gallstone disease or alcohol abuse[2], while genetic factors are thought to contribute to disease susceptibility and may influence the clinical course of the disease[3,4]. In 20% of patients, acute pancreatitis runs a severe clinical course associated with high morbidity rates and mortality of up to 30%.[5] Nearly all the deaths are associated with infectious complications, such as bacteremia and infection of pancreatic necrosis.[6,7] To date, few studies have revealed any significant association between genetic factors and acute pancreatitis, but these studies involved relatively small cohorts. They investigated over 30 candidate genes, of which only one (SPINK1) showed consistent association with acute and recurrent acute pancreatitis.[8-12]

Failure of the gastrointestinal mucosal barrier plays an essential role in the course of acute pancreatitis, as it allows for bacterial translocation, which in turn may lead to infectious complications.[13-16] Although little is known about the exact pathophysiology of mucosal barrier failure in acute pancreatitis, it may also contribute to the development of the initial disease. Tight junction failure within the pancreas has been shown to be an extremely early event in the development of experimental acute pancreatitis in mice[17] and rats.[18,19] In a caerulein model of acute pancreatitis in rats, disruption of the actin cytoskeleton and tight junctions resulted in increased paracellular permeability.[18,20]

Genetic associations have recently been reported for two other inflammatory conditions in which intestinal permeability plays a pathophysiological role; these are celiac disease (CD) and inflammatory bowel disease (IBD). CD and its complications have been associated to both myosin IXB (*MYO9B*) and to two tight-junction adaptor genes *PARD3* and *MAGI2* [21-24], whereas IBD has repeatedly been associated to *MYO9B*[25-29] and once to *MAGI2*.[23] All three proteins are hypothesized to play a role in tight junction assembly and positioning of the tight junctions in the membrane regions of the cell, and could thus possibly play a role in intestinal barrier function.[25,30-33] In addition to CD and IBD, *MYO9B* has also been associated with susceptibility to type 1 diabetes mellitus in a Spanish cohort.[34] We know the intestinal barrier is impaired in type 1 diabetes.[35-37] Moreover, the BioBreeding diabetes prone (BBDP) rat model of diabetes, in which spontaneous development of autoimmune type 1 diabetes occurs and which is used to study the mechanisms of diabetes pathogenesis, showed an increase in intestinal permeability, even before the onset of clinical diabetes.[38,39]

Based on these genetic association studies in diseases with a compromised intestinal barrier, we hypothesized that polymorphisms in these three genes involved in mucosal barrier function might also be associated with acute pancreatitis. We therefore adopted a candidate gene approach to test genetic variants in *MYO9B*,

PAR3D and MAG12 for their potential association with acute pancreatitis in two independent cohorts: a Dutch cohort of 387 patients and more than 800 controls, and a German cohort of 235 patients and 250 controls.

Methods

Cohorts

The Dutch cohort consisted of 387 patients with acute pancreatitis and over 800 random blood bank controls. This genetic association study was part of a multicenter, randomized controlled trial (trial registry number ISRCTN38327949)[40], during which patients with a first episode of acute pancreatitis were included in a prospective database. The cohort comprised 188 randomized patients and 199 patients with acute pancreatitis who had been screened for eligibility for the PROPATRIA trial, but who were not randomized.[40] Acute pancreatitis was defined as abdominal pain in combination with a greater than three-fold elevation of serum amylase or lipase concentrations.[40] Severe acute pancreatitis was defined as acute pancreatitis with organ failure and/or local complications.[40] Infectious complications were defined as infected pancreatic necrosis, bacteremia, pneumonia, urosepsis, or infected ascites.[40] All patients or their legal representatives gave written informed consent and the ethics review boards of all 15 participating hospitals approved the protocol for this part of the study. Clinical data on the severity of disease and outcome for all patients were available from the PROPATRIA database (Table 1).[40]

Genotype data from two control cohorts were used.[21,23,25] For the single nucleotide polymorphism (SNP) typing of *MYO9B*, the controls were random hospital controls (n = 220)[21] and Dutch blood bank donors from Utrecht, Leiden and Amsterdam (n = 1323).[21,25] For the two tight junction adaptor genes (*PARD3* and *MAGI2*), only a subset of the controls was used (n = 848).[23] Characteristics of the control groups have been described previously.[21,23,25] All control genotypes were in Hardy-Weinberg equilibrium (data not shown, $p > 0.05$).

The second cohort comprised 235 German patients with acute pancreatitis and 250 German controls. The patients were prospectively enrolled in the ProZyt study.[41,42] The definitions used for acute pancreatitis and for severe acute pancreatitis were the same as for the Dutch cohort. Clinical data on the severity of disease and outcome for all patients were available from the ProZyt Study database.[41,42] All patients gave their written informed consent and the ethics review board of Greifswald University, Greifswald, Germany, approved the protocol for the study. The German controls were healthy blood bank donors (n = 250). All control genotypes were in Hardy-Weinberg equilibrium (data not shown, $p > 0.05$).

For the current genetic association study, we took peripheral blood samples from each patient. These were centrifuged at 3,000 rpm for 10 minutes and the plasma

and cell pellets were separated and stored at -80ºC. Genomic DNA was extracted from the cell pellets using DNA isolation kit I from the Magna Pure LC (initial cohort, Roche Diagnostics, Indianapolis, USA) or the Quick-gDNA MiniPrep Kit (follow-up study, Zymo Research, Irvine, California, USA).

SNP selection and genotyping

We selected five tag SNPs in *MYO9B* that had shown association with CD or IBD[21,25] (rs2305767, rs1457092, rs2305764, rs7259292 and rs1545620; Applied Biosystems, Foster City, California, USA). We also selected five SNPs from the two tight junction adaptor genes (three in *MAGI2* and two in *PARD3*) that were associated with CD and ulcerative colitis (rs10763976, rs4379776, rs6962966, rs9640699, and rs1496770).[23]

Genotyping of the two cohorts was performed independently. The Dutch cohort was genotyped in the Complex Genetics Group Laboratory, University Medical Center Utrecht, the Netherlands. The German cohort was genotyped in the Laboratory for Molecular Gastroenterology, Department of Medicine A, Greifswald Hospital, Germany. Genotyping was done using TaqMan assays (Applied Biosystems) and the genotypes were analyzed using a TaqMan 7900 HT (Applied Biosystems). Haplotypes were constructed using Haploview v4.2.[43]

Statistical analysis

For continuous values of patient characteristics (Table 1), normally distributed data were presented as mean and standard deviations (SD); all non-normally distributed data were presented as medians with an interquartile range (IQR). The association study (Table 2) was analyzed using the two-tailed chi squared test for independence of case vs. control alleles in PLINK v1.07 (http://pngu.mgh.harvard.edu/purcell/plink/).[44] For the joint analysis, allele counts for the Dutch and German cohorts were combined and a Cochran-Mantel-Haenszel analysis was done in PLINK.[44] To correct for multiple testing, 50,000 random permutations were done within each cohort, generating two empirical P-values. The first P-value was an estimate of an individual SNP's significance, the second P-value corrected for multiple testing while preserving the correlational structure between SNPs.[44] To test for heterogeneity between the Dutch and German cohorts, a Breslow-Day test was performed in PLINK.[44] Haplotype analysis was performed in Haploview v4.2.[43] Uncorrected P-values, odds ratios (OR) and 95% confidence intervals (95% CI) are shown in Table 3.

Table 1. Clinical characteristics of the two cohorts of patients with acute pancreatitis

Characteristic	Dutch acute pancreatitis patients (n = 387)	German acute pancreatitis patients (n = 235)
Male	207 (53.3%)	122 (51.9%)
Age (years, mean ± 1 SD)	56.7 (± 17.6)	52.5 (± 19.5)
Etiology of pancreatitis		
Biliary	209 (54%)	94 (40%)
Alcohol	72 (19%)	65 (28%)
Medication	14 (4%)	3 (1%)
Hypertriglyceridemia	3 (1%)	1 (0.5%)
Other	17 (4%)	52 (22%)
Unknown	72 (18%)	20 (8.5%)
Severity of pancreatitis (median, IQR)		
APACHE-II score*	7.0 (4.0-10.0)	5.0 (2.0-7.0)
Imrie score	2.0 (1.0-4.0)	1.0 (0-1.0)
CRP, highest value in first 48 hrs (mg/L)	192 (81-295)	88 (24-164)
Severe acute pancreatitis‡	104 (27%)	15 (6%)
Necrotizing pancreatitis#	84 (22%)	9 (4%)
Complications		
Infections	93 (24%)	13 (6%)
Positive blood culture	56 (15%)	10 (4%)
Organ failure during admission	58 (15%)	8 (3%)
Multi-organ failure during admission	30 (8%)	0
Mortality	20 (5%)	0

*Highest score on day of admission

‡Organ failure and/or necrosis

#Defined as: pancreatic parenchymal necrosis demonstrated on contrast-enhanced computed tomography scan

CRP, C-reactive protein; IQR, interquartile range; SD, standard deviation

Results

Polymorphisms in MYO9B may increase susceptibility to acute pancreatitis

Table 2 summarizes the results for all ten SNPs across the three genes tested. A significant association was observed for the five tagging SNPs in *MYO9B* and the two variants in *PARD3* in the Dutch cohort (most significant SNP in *MYO9B*: rs1545620, p = 2.3x10-5; most significant SNP in *PARD3*: rs4379776, p = 0.0046; Table 2). There were nine patients with CD, IBD or type 1 diabetes mellitus in this cohort. To exclude any effect from these co-morbidities, we removed these patients from the analysis.

Association analysis showed that the associations with *MYO9B* and *PARD3* remained significant in the Dutch cohort (data not shown). None of the genetic variants of *MAGI2* were associated with acute pancreatitis (Table 2). In the German cohort, an association was found for one variant in *MYO9B* and one variant in *MAGI2* (Table 2, rs7259292 and rs6962966, respectively).

We performed a joint analysis combining the Dutch and German cohorts using the Cochran-Mantel-Haenszel method with 50,000 random permutations within both cohorts. In this analysis, four of the *MYO9B* SNPs were found to be associated with acute pancreatitis. Two of these were still associated with the disease after correcting for multiple testing (rs1545620, p = 0.0006, OR 1.33, 95%CI 1.16-1.53; rs7259292, p = 0.0031, OR 1.94, 95%CI 1.35-2.78) and one showed borderline significance (rs1457092, p = 0.0557, OR 1.22, 95%CI 1.06-1.40). Both *PARD3* SNPs were associated with acute pancreatitis, but these associations did not withstand correction for multiple testing. A Breslow-Day test showed modest evidence for heterogeneity between the two cohorts for SNPs rs2305767 in *MYO9B* and rs6962966 in *MAGI2*, but these SNPs were not significant in the final analysis (Table 2).

All SNPs in *MYO9B* were located in one haploblock and were in strong linkage disequilibrium. We therefore constructed 5-SNP haplotypes using the combined genotypes of the initial and follow-up studies (Table 3). Three of the haplotypes occurred with a frequency of more than 5% in acute pancreatitis patients or controls. The haplotype CACAA occurred more often in patients than controls (37% vs. 33%, p = 0.0099, OR 1.24, 95%CI 1.05-1.46). Of the rare haplotypes with a frequency below 5%, the haplotype TACCG occurred more often in patients than controls (4.3% vs. 2.3%, p = 0.0005, OR 2.03, 95%CI 1.36-3.04). Both of these haplotypes carry the rs1545620*C allele, which is the allele stemming from the most strongly associated SNP.

To investigate an association between the three genes tested and the course of acute pancreatitis, we did a post-hoc analysis of the prevalence of all the genetic variants in four groups of patients of the combined Dutch and German cohorts. These were patients who developed severe acute pancreatitis (n = 119), infectious complications (n = 106), infected pancreatic necrosis (n = 58), or who died (n = 20). After correcting for the number of phenotypes and SNPs, the results appeared not to be significant. We also compared patients with acute biliary pancreatitis (n = 307) to patients with acute pancreatitis with non-biliary etiology, but we found no association.

Table 2. Analysis of *MYO9B*, *PARD3* and *MAGI2* SNPs in the Dutch and German cohorts and joint analysis

			Initial study			Follow-up study			Joint analysis			
			RAF patients (n = 387)	RAF controls (n > 800)	P-initial*	RAF patients (n = 235)	RAF controls (n = 250)	P follow-up*	P-joint	OR	95% CI	**P-adjusted**
rs7259292	*MYO9B*	T/C#	0.046	0.026	0.0053	0.047	0.020	0.0200	0.0003	1.94	1.35-2.78	**0.0031**
rs2305767	*MYO9B*	A/G	0.620	0.557	0.0021	0.590	0.607	0.5880	0.0211	0.85	0.74-0.98	0.1709$
rs1545620	*MYO9B*	C/A	0.448	0.364	$2.3x10^{-5}$	0.385	0.359	0.4083	$5.9x10^{5}$	1.33	1.16-1.53	**0.0006**
rs1457092	*MYO9B*	A/C	0.401	0.337	0.0011	0.344	0.345	0.9807	0.0062	1.22	1.06-1.40	0.0557
rs2305764	*MYO9B*	A/G	0.433	0.381	0.0093	0.383	0.401	0.5516	0.0614	1.14	0.99-1.31	0.4193
rs10763976	*PARD3*	A/G	0.483	0.431	0.0195	0.564	0.536	0.3857	0.0157	1.19	1.03-1.38	0.1320
rs4379776	*PARD3*	A/G	0.371	0.312	0.0046	0.353	0.341	0.6995	0.0109	1.22	1.05-1.41	0.0929
rs6962966	*MAGI2*	G/A	0.493	0.458	0.1114	0.481	0.567	0.0077	0.8450	0.99	0.85-1.14	1.0$
rs9640699	*MAGI2*	A/C	0.390	0.384	0.7708	0.391	0.413	0.4956	0.8844	0.99	0.85-1.15	1.0
rs1496770	*MAGI2*	A/G	0.407	0.400	0.7317	0.391	0.409	0.5538	0.9575	1.00	0.86-1.15	1.0

OR, odds ratio; 95% CI, 95% confidence interval; RAF, risk allele frequency.

Risk variant/second allele

* Two-tailed P-values were calculated by chi-squared test for independence of allele counts

$Heterogeneity between the cohorts (Breslow-Day test).

The risk variant was the associated allele in the Dutch cohort; the same variant frequencies were reported for the German cohort. A combined analysis of the Dutch and German results was performed using Cochran-Mantel-Haenszel analysis with 50,000 random permutations. This generated two P-values (P-joint and P-adjusted), an OR and 95% CI. P-joint shows an individual SNP's significance in the combined cohort. P-adjusted was obtained after correcting for multiple testing. SNPs rs2305767 and rs6962966 showed modest evidence for heterogeneity between the Dutch and German cohort when a Breslow-Day test was performed on this data.

Table 3. The prevalence of *MYO9B* haplotypes in the combined Dutch and German cohorts of patients with acute pancreatitis and controls reconstructed from genotyped SNPs and their association with acute pancreatitis

MYO9B haplotypes									
rs7259292	rs2305767	rs1545620	rs1457092	rs2305764	Cases (%)	Controls (%)	OR	95% CI	P-value*
C	G	A	C	G	460 (38)	900 (42)	1.00‡	–	ref
C	**A**	**C**	**A**	**A**	443 (37)	700 (33)	1.24	1.05-1.46	0.0099
C	**A**	A	C	G	190 (16)	365 (17)	1.02	0.83-1.25	0.8860
C	**A**	A	C	**A**	40 (3.3)	98 (4.6)	0.81	0.55-1.19	0.2512
T	**A**	**C**	C	G	52 (4.3)	50 (2.3)	2.03	1.36-3.04	0.0005

OR, odds ratio; 95% CI, 95% confidence interval.

* Two-tailed P-values were calculated by chi-squared test for independence of haplotype counts.

‡ This haplotype was taken as the reference.

Risk alleles are in bold and underlined.

Discussion

We performed a candidate gene study for *MYO9B*, *PARD3* and *MAGI2* looking for susceptibility to acute pancreatitis. All three genes are thought to influence intestinal permeability.[21,23,25] By analyzing a combined cohort of Dutch and German patients with acute pancreatitis, we found an association of two genetic variants in *MYO9B* for susceptibility to this disease. The SNP with the strongest association was rs1545620 (p = 0.0006, OR 1.33, 95%CI 1.16-1.53), which is a non-synonymous variant leading to an amino acid change.[25] This SNP was very strongly associated (p = 2.3x10-5, Table 2) in the Dutch cohort, but not in the German cohort. The differential association could not be attributed to heterogeneity between the cohorts.

Our analyses in two separate cohorts resulted in different findings. In the Dutch cohort, all five variants in *MYO9B* were associated with acute pancreatitis, but we were surprised to see that only one of these SNPs showed association in the German cohort. The *MAGI2* SNP rs6962966 did show heterogeneity between cohorts and did show a different pattern of association between the Dutch and German cohort, with the latter providing modest evidence for association (uncorrected p = 0.0077). While statistical power is one explanation for these differences, our findings highlight the need to replicate such results before accepting them.

Intestinal permeability is a critical factor for the course of acute pancreatitis, since a breakdown of the barrier function enables bacterial translocation, which may subsequently cause infectious complications.[13-16] We therefore explored whether the genetic variants had any relationship with the severity of disease (severe vs. mild acute pancreatitis), mortality, or the occurrence of infectious complications. These analyses revealed no associations.

One of the strengths of our study is the size of the combined cohort: 622 patients for whom clinical data were available. Most previous genetic association studies in acute pancreatitis consisted of quite small patient populations (n = 35-470). Yet, despite our relatively large cohort, our subgroup analyses did not reveal any convincing results. Future studies will need to investigate the genotypes in subgroups of patients, e.g. in those with severe acute pancreatitis. The clinical classification, however, of patients with severe acute pancreatitis into subgroups is subjective and heterogeneous, which could also account for the lack of association between genetic variants and clinical course. Finally, there could be other genetic or environmental factors that determine the course of acute pancreatitis.

The *MYO9B* gene has consistently been found to be associated with IBD in cohorts from different countries.[21,23,25-28] The rs1545620 SNP with the highest OR is a non-synonymous SNP inducing an amino acid change (Ala1011Ser) in the neck region of the *MYO9B* protein; it is necessary for the motor activity of *MYO9B* on actin filaments.[30,31] A conformational change of the protein could therefore result in lower *MYO9B* activity. This could lead to a diminished capacity for maintaining tight junction

and cytoskeleton structure.

The association of variants of *MYO9B* with acute pancreatitis points to a possible shared genetic mechanism that impairs mucosal barrier function not only in acute pancreatitis, but also in CD, IBD and type 1 diabetes mellitus. We found polymorphisms of a gene likely to be involved in maintaining tight junction function (and potentially gastrointestinal permeability) to be associated with susceptibility to acute pancreatitis rather than to the clinical course of the disease. This runs contrary to current knowledge on the pathophysiology of acute pancreatitis and we have no biological explanation for our observation. Unfortunately, there are no functional data on the role of gastrointestinal permeability and the development of acute pancreatitis. Our findings should therefore lead to experimental studies to elucidate this new, potentially important, pathophysiological concept in acute pancreatitis.

We have shown that *MYO9B* may be involved in acute pancreatitis, possibly due to its potential role in regulating the intestinal barrier function. Our results open the way to thinking about shared mechanisms leading to mucosal barrier impairment. The presence of genetic variants of *MYO9B* in an individual may be the first step that can lead to different diseases, depending on subsequent events. Whether these different outcomes are influenced by environmental factors (such as in acute pancreatitis) or by other sets of modifier genes (such as in celiac disease and inflammatory bowel disease) still needs to be determined.

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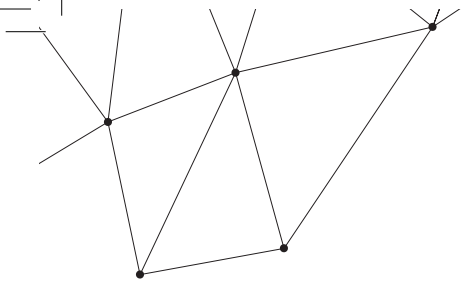

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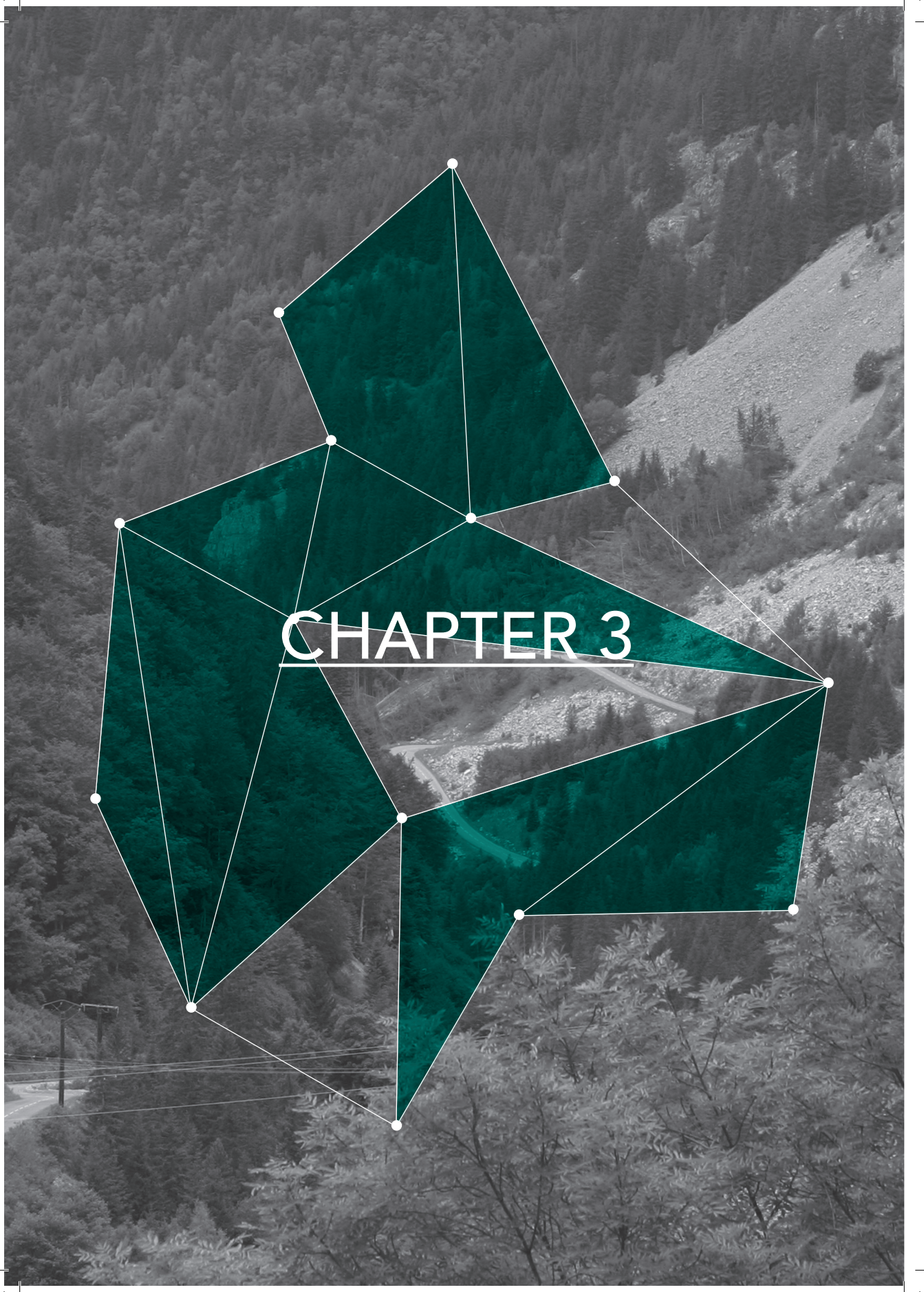

CHAPTER 3

Genetic variants in the Toll-like Receptor pathway in acute pancreatitis

Rian M. Nijmeijer[1], Hjalmar C. van Santvoort[1], Alexandra Zhernakova[2], Marc G. H. Besselink[3], Ger T. Rijkers[4,5], Hein G. Gooszen[4], Louis M. A. Akkermans[1], Cisca Wijmenga[2], Marja A. Boermeester[3], on behalf of the Dutch Pancreatitis Study Group

[1]Department of Surgery, University Medical Center Utrecht, Utrecht, the Netherlands; [2]Department of Genetics, University Medical Center Groningen and University of Groningen, Groningen, the Netherlands; [3]Department of Surgery, Academic Medical Center, Amsterdam, the Netherlands; [4]Department of Operating Rooms, University Medical Center St. Radboud, Nijmegen, the Netherlands; [5]Department of Medical Microbiology and Immunology, St. Antonius Hospital, Nieuwegein, the Netherlands.

Manuscript submitted.

Abstract

Introduction: During acute pancreatitis, failure of the gut barrier can lead to bacterial translocation with subsequent complications due to superimposed bacterial infection. Toll-like receptors (TLRs) are essential in the innate immune system's response to invading pathogenic bacteria. There are conflicting results on the role of the TLR pathway in acute pancreatitis. We aimed to determine the role of the TLR pathway by performing a candidate-pathway genetic analysis for the development, course and clinical outcomes in a large cohort of acute pancreatitis patients.

Materials & Methods: A genetic association study was performed in which 20 single nucleotide polymorphisms (SNPs) in the genes of pattern recognition receptors TLR2 and TLR4 and their associated and downstream components CD14, MyD88, IRAK3 and IRAK4 were included and studied in 387 Dutch patients with acute pancreatitis and 753 controls.

Results: One of the SNPs in *IRAK3* seemed associated with the development of acute pancreatitis (rs1152888, OR 1.47, 95% CI 1.04-2.07), but this association lost significance after correction for multiple testing. All tested TLR pathway genetic variants were not significantly different distributed between patients with severe acute pancreatitis and patients with mild acute pancreatitis, patients with (bacterial) infected pancreatic necrosis and patients without infected pancreatic necrosis, or between patients who died and patients who survived.

Conclusion: Genetic variants of key parts of the TLR pathway are unlikely to be involved in development, course and outcome of acute pancreatitis.

Introduction

Acute pancreatitis is mostly caused by gallstones and alcohol abuse.[1] Both gallstones and alcohol abuse are relatively common, whereas the incidence of acute pancreatitis is much rarer (16:100,000), suggesting that either additional environmental or modifier-genes may be involved in susceptibility to the disease. In most patients, acute pancreatitis runs a relatively mild clinical course, whereas 20% of all patients will develop severe complications such as organ failure and / or local complications.[2] At least half of deaths from acute pancreatitis are caused by infectious complications, such as secondary bacterial infection of pancreatic or peripancreatic necrosis.[3,4] Apart from having an impact on the incidence of acute pancreatitis, genetic variants may also be involved in the severity of the disease.

Failure of the gut barrier allows for bacterial translocation, which may lead to superimposed bacterial infection of pancreatic necrosis and other infectious complications.[5-7] Bacteria residing in the lumen of the gut are constantly sensed by dendritic cells of the mucosal immune system.[8] Dendritic cells express so-called pattern recognition receptors of which Toll-like receptors (TLRs) are the most important ones.[8] TLRs have many functions which collectively are essential for the maintenance of an intact gut mucosal barrier. On the other hand, TLRs can also trigger pro-inflammatory responses by underlying lamina propria immune cells.[8,9] The intestinal epithelium has to discriminate between the resident luminal bacteria to which it should not direct an acute inflammatory immune response and pathogenic bacteria that should be disposed of.[9] Inappropriate activation of the TLR signalling pathway, leading to activation of NFkB results in deleterious inflammation and tissue injury. Regulatory mechanisms, including TLR signalling, minimize the risk of such inappropriate activation in order to maintain mucosal homeostasis.[10]

Several studies have investigated the role of TLR signalling in experimental mouse models of acute pancreatitis. The results show that TLR4 plays an important role in experimental acute pancreatitis, either by regulating the severity of the disease or the infectious complications.[11-15] Recently it has been shown that genes and gene products of the TLR pathway are upregulated during acute pancreatitis, further demonstrating that several components of the TLR pathway play a key role in the innate immune response in acute pancreatitis.[16] Little is known about the TLR pathway and its genetic variants in human acute pancreatitis. We therefore performed a candidate-pathway genetic analysis for susceptibility to acute pancreatitis, the course of acute pancreatitis and the outcome of the disease in a large cohort of acute pancreatitis patients. We aimed to investigate key parts of the TLR pathway involved in inflammatory immune responses. This was done by testing for genetic association to SNPs in the pattern recognition receptor genes *TLR2* and *TLR4*, and in important associated and downstream components of the pathway, such as *CD14* for recognition of lipopolysaccharides, the gene for the adaptor molecule *MyD88*, and

the most important activator and inhibitor of this part of the TLR pathway (*IRAK4* and *IRAK3/M*).

Materials & Methods

Acute pancreatitis patients and controls

This study was part of a randomized controlled trial on the role of probiotics in lowering the infection rate in acute pancreatitis (trial registry ISRCTN38327949).[17] During this trial, patients with a first episode of acute pancreatitis were included in a prospective database. This cohort of patients has been described previously.[4] Acute pancreatitis was defined as abdominal pain in combination with a greater than three-fold elevation of serum amylase or lipase concentrations.[17] The cohort for this present study consisted of 188 randomized patients and 199 patients with acute pancreatitis that had been screened for eligibility for this trial, but who were not randomized. These patients were not randomized either because they had predicted mild acute pancreatitis or because they were reported too late (clinical symptoms of pancreatitis for more than 72 hours at time of diagnosis of predicted severe acute pancreatitis) to be included early in the disease.[17] Severe acute pancreatitis was defined as acute pancreatitis with organ failure and/or local complications.[17]
Genomic DNA of the 387 patients with acute pancreatitis was isolated using DNA isolation kit I from the Magna Pure LC (Roche Diagnostics, Indianapolis, USA). Clinical data on the course and outcome of the disease for all patients were available from the prospectively collected trial database.[17] All patients or their legal representatives gave written informed consent and the ethics review boards of the 15 participating hospitals approved the protocol for this study.

The control cohort (n = 753) consisted of Dutch blood bank donors and was a subset of the control cohorts that were previously described.[18,19] Control data for *TLR4*, *MyD88*, *CD14*, *IRAK3* and *IRAK4* were in large part already available from a previous study.[20] All *TLR2* SNPs and the rs11536898, rs5030717, rs1168771 and rs1461567 SNPs were genotyped additionally in the same set of controls for the current study. All control genotypes were in Hardy-Weinberg equilibrium (data not shown, $p > 0.05$).

SNP selection and genotyping

To study the potential involvement of the TLR pathway in acute pancreatitis, we studied an extensive part of the TLR pathway (Figure 1). Therefore, we selected tag SNPs in *TLR4* (rs1554973, rs11536889, rs12377632, rs11536898, rs4986790, rs5030717), *TLR2* (rs7656411, rs1898830, rs1816702, rs5743708), *CD14* (rs2569190), *MyD88* (rs172111, rs7744), *IRAK3* (rs1152888, rs1152912, rs1168771, rs1732886), and *IRAK4* (rs1141168, rs1461567, rs4251520) (Table 1). Call rates for

genotyping were over 95% of cases and controls, except for rs7656411 (94.3%), rs116871 and rs5743708 (both 92.7%).
Genotyping was performed in the Complex Genetics Group Laboratory, University Medical Center Utrecht. Genotyping was done using TaqMan assays (Applied Biosystems, Foster City, California, USA) and the genotypes were analyzed using a TaqMan 7900 HT (Applied Biosystems). Haplotypes were constructed using Haploview.[21]

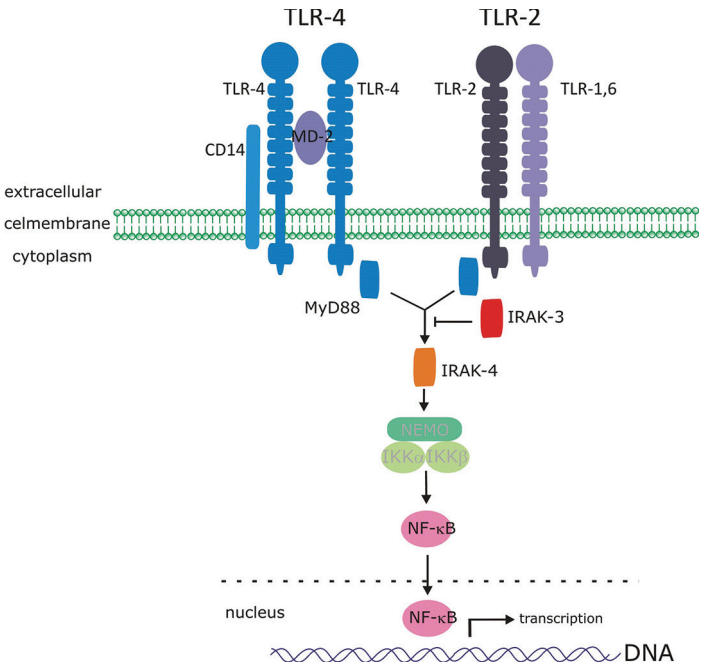


Figure 1: Part of the TLR signalling pathway.

TLR2 and TLR4 share a paWthway resulting in NFκB activation. The major components of the current association study, TLR2, TLR4, CD14, MyD88, IRAK3, and IRAK4 are all part of the innate immune system resulting in activation of NFκB.

Table 1: SNP information.

SNP number	Gene	Chromosomal location (dbSNP build 132)
rs12377632	TLR4	chr9: 120472730
rs5030717	TLR4	chr9: 120473834
rs4986790*	TLR4	chr9: 120475302
rs11536889	TLR4	chr9: 120478131
rs11536898	TLR4	chr9: 120480210
rs1554973	TLR4	chr9: 120480812
rs2569190	CD14	chr5: 140012916
rs7744	MyD88	chr3: 38184021
rs172111	MyD88	chr3: 38187994
rs1732886	IRAK3	chr12: 66583762
rs1168771	IRAK3	chr12: 66588836
rs1152888	IRAK3	chr12: 66605228
rs1152912	IRAK3	chr12: 66633908
rs1461567	IRAK4	chr12: 44164689
rs4251520	IRAK4	chr12: 44175338
rs1141168	IRAK4	chr12: 44182706
rs1898830	TLR2	chr4: 154608453
rs1816702	TLR2	chr4: 154609523
rs5743708#	TLR2	chr4: 154626317
rs7656411	TLR2	chr4: 154627655

*rs4986790: Asp299Gly; #rs5743708: Arg753Gln, R753Q

Statistical analysis

Statistical analysis was performed using 2-tailed χ^2 tests of case vs. control allele and haplotype counts in Haploview v3.2.[21] P-values, odds ratios (OR) and 95% confidence intervals (95% CI) are shown. The Bonferroni method was used to correct for multiple testing. All tables show the uncorrected P-values.

Results

A total of 387 acute pancreatitis patients and 753 healthy controls were genotyped with 20 SNPs in six genes that are key parts of the TLR pathway. Polymorphisms of TLR pathway genes were not associated with the development of acute pancreatitis. One of the SNPs in *IRAK3* displayed significant association with acute pancreatitis (Table 2; rs1152888, OR 1.47, 95% CI 1.04-2.07), but this association was not significant after

Bonferroni correction for multiple testing.

We subsequently analyzed whether polymorphisms of the genes of the TLR pathway were associated with the course of acute pancreatitis. Of the 387 acute pancreatitis patients, 104 patients had severe acute pancreatitis (27%), 56 patients had infected pancreatic necrosis (15%), and 20 patients died (5%). We compared patients with severe acute pancreatitis with patients with mild acute pancreatitis, patients with infected pancreatic necrosis with patients without infected pancreatic necrosis, and patients who died with patients who survived. We did not identify any SNPs that were associated with either severity of acute pancreatitis or with bacterial infection of pancreatic necrosis (Table 3). One of the SNPs in the *TLR4* gene, notably the Asp299Gly polymorphism, showed nominally significant association with mortality of acute pancreatitis (Table 3; rs498790, OR 2.67, 95% CI 1.03-6.92), but this association was not significant after correcting for multiple testing.

Table 2: Association of genetic variants in genes of the TLR pathway with acute pancreatitis.

			AP patients (n = 387)		Controls (n = 753)			P value*	OR	95% CI
Gene			Allele counts			Allele counts				
			Minor	Major	MAF	Minor	Major	MAF		
rs12377632	TLR4	G/A#	262	452	0.367	575	931	0.382	0.4998	0.94	0.78-1.13
rs5030717	TLR4	G/A	76	652	0.104	168	1262	0.117	0.3641	0.88	0.66-1.17
rs4986790	TLR4	G/A	48	698	0.064	114	1392	0.076	0.3264	0.85	0.60-1.20
rs11536889	TLR4	G/C	113	629	0.152	214	1292	0.142	0.5192	1.09	0.85-1.39
rs11536898	TLR4	A/C	87	635	0.120	195	1311	0.129	0.5506	0.93	0.71-1.21
rs1554973	TLR4	G/A	191	551	0.257	389	1117	0.258	0.9639	1.00	0.82-1.22
rs2569190	CD14	A/G	350	374	0.483	673	835	0.446	0.0992	1.16	0.97-1.39
rs7744	MyD88	G/A	101	633	0.138	215	1291	0.143	0.7419	0.96	0.75-1.24
rs172111	MyD88	A/G	52	694	0.070	98	1408	0.065	0.6782	1.09	0.77-1.54
rs1732886	IRAK3	G/A	201	529	0.275	450	1054	0.299	0.2444	0.89	0.73-1.08
rs1168771	IRAK3	C/T	285	437	0.395	530	846	0.385	0.6694	1.04	0.87-1.25
rs1152888	IRAK3	A/G	58	690	0.078	82	1424	0.054	0.0325	1.47	1.04-2.07
rs1152912	IRAK3	A/G	321	413	0.437	627	879	0.416	0.3452	1.09	0.91-1.30
rs1461567	IRAK4	A/G	198	524	0.274	400	1106	0.266	0.6669	1.05	0.86-1.28
rs4251520	IRAK4	G/A	92	610	0.131	175	1331	0.116	0.3189	1.15	0.88-1.51
rs1141168	IRAK4	G/A	358	386	0.481	706	800	0.469	0.5797	1.05	0.88-1.25
rs1898830	TLR2	G/A	273	453	0.376	520	972	0.349	0.2047	1.13	0.94-1.35
rs1816702	TLR2	T/C	97	635	0.133	192	1300	0.129	0.8009	1.04	0.80-1.35
rs5743708	TLR2	C/A	31	687	0.043	51	1327	0.037	0.4896	1.19	0.76-1.87
rs7656411	TLR2	G/T	167	531	0.239	334	1102	0.233	0.7333	1.04	0.84-1.29

AP = acute pancreatitis; OR = odds ratio; 95% CI = 95% confidence interval

Minor allele / major allele.

* Two-tailed P values were calculated by $\chi 2$ analysis of allele counts.

Significant P value is denoted in bold.

Table 3: Association analysis of genes of the TLR pathway with clinical subgroups of patients with acute pancreatitis.

		Patients with severe vs. patients with mild AP (n=104 and n=283, respectively)	**Patients with vs. patients without infected pancreatic necrosis** (n=56 and n=331, respectively)	**Patients who died vs. patients who did not** (n=20 and n=367, respectively)
	Gene	OR (95% CI)	OR (95% CI)	OR (95% CI)
rs12377632	TLR4	1.01 (0.72-1.41)	1.22 (0.80-1.85)	1.27 (0.65-2.48)
rs5030717	TLR4	1.31 (0.79-2.18)	1.58 (0.87-2.87)	1.08 (0.35-3.32)
rs4986790	TLR4	1.04 (0.53-2.02)	1.59 (0.59-4.28)	**2.67 (1.03-6.92)**
rs11536889	TLR4	1.13 (0.71-1.79)	1.06 (0.60-1.86)	1.40 (0.61-3.19)
rs11536898	TLR4	1.00 (0.61-1.66)	1.19 (0.65-2.17)	0.98 (0.36-2.68)
rs1554973	TLR4	1.33 (0.91-1.96)	1.30 (0.79-2.12)	1.09 (0.53-2.26)
rs2569190	CD14	1.06 (0.77-1.48)	1.05 (0.70-1.57)	1.14 (0.61-2.15)
rs7744	MyD88	1.03 (0.64-1.66)	1.35 (0.78-2.35)	0.91 (0.36-2.31)
rs172111	MyD88	1.16 (0.60-2.23)	0.99 (0.44-2.20)	1.82 (0.65-5.05)
rs1732886	IRAK3	1.45 (0.98-2.14)	1.20 (0.77-1.88)	1.43 (0.66-3.09)
rs1168771	IRAK3	1.01 (0.72-1.42)	1.08 (0.71-1.65)	1.77 (0.88-3.56)
rs1152888	IRAK3	1.45 (0.74-2.82)	1.59 (0.65-3.92)	1.13 (0.31-4.20)
rs1152912	IRAK3	1.18 (0.85-1.65)	1.24 (0.82-1.88)	1.07 (0.57-2.01)
rs1461567	IRAK4	1.09 (0.75-1.58)	0.99 (0.62-1.56)	1.15 (0.54-2.43)
rs4251520	IRAK4	1.20 (0.72-2.00)	1.25 (0.69-2.27)	1.41 (0.59-3.38)
rs1141168	IRAK4	1.01 (0.73-1.39)	1.14 (0.76-1.70)	1.72 (0.89-3.32)
rs1898830	TLR2	1.19 (0.85-1.68)	1.01 (0.66-1.55)	1.52 (0.78-2.96)
rs1816702	TLR2	1.09 (0.67-1.77)	1.40 (0.71-2.76)	1.86 (0.51-6.84)
rs5743708	TLR2	1.14 (0.49-2.62)	1.93 (0.83-4.49)	1.87 (0.49-7.11)
rs7656411	TLR2	1.05 (0.71-1.55)	1.06 (0.65-1.72)	1.54 (0.73-3.28)

AP = acute pancreatitis; OR = odds ratio; 95% CI = 95% confidence interval

Significant association is denoted in bold.

Discussion

The potential contribution of the TLR pathway in acute pancreatitis was investigated by a candidate-pathway genetic analysis. Polymorphisms of the pattern recognition receptors TLR2 and TLR4 were included, as were SNPs of the downstream or associated components CD14, MyD88, IRAK3 and IRAK4. Genetic variants were studied in a large cohort of acute pancreatitis patients and compared to a large cohort of healthy controls. We found no evidence for association between the 20 evaluated SNPs of key parts of the TLR pathway and susceptibility to develop acute pancreatitis, and the clinical course or outcome of the disease. These results suggest that these genes are not causally related to acute pancreatitis or its outcome.

Using mouse experimental models of acute pancreatitis it has been shown that TLR4 and other components of the TLR pathway play a role in the innate immune response in acute pancreatitis.[11-16] In human acute pancreatitis, early in the course of disease the expression of TLR4 on peripheral blood mononuclear cells is upregulated and TNFα concentrations in plasma are increased.[22]

The role of TLR4 has been more extensively studied in bacterial infections, sepsis, and atherosclerosis and related diseases, and variable results have been obtained.[23,24] Several groups have shown that individuals carrying the *TLR4* Asp299Gly polymorphism are at greater risk for Gram-negative bacterial infections.[25,26] However, no association was found between the Asp299Gly polymorphism and an increased incidence of systemic inflammatory response syndrome[27] or with the development or outcome of sepsis after surgery.[28]

The results of this study are in line with earlier studies investigating *TLR4* polymorphisms in Caucasian patients with acute pancreatitis, where no association with the Asp299Gly polymorphism could be detected.[29,30] Several Asian studies on *TLR4* polymorphisms in acute pancreatitis patients have been performed, but the frequency of the mutant allele of the Asp299Gly polymorphism is very low in both Chinese and Japanese populations.[31,32] In a study on 238 Chinese Han acute pancreatitis patients and 121 healthy controls the Asp299Gly polymorphism in the *TLR4* gene was not detected at all, as was the case for a Japanese cohort of 202 patients.[32,33] Another Chinese study did show association of the Asp299Gly polymorphism with infection of pancreatic necrosis, when patients were compared to healthy controls, but no correction for multiple testing was applied.[34] We could not replicate this finding. In our study, this particular SNP (rs4986790) appeared to be associated with mortality caused by acute pancreatitis, but this association was lost after correction for multiple testing.

In a Japanese study, short guanine-thymine (GT) repeats microsatellite polymorphisms in intron 2 of the *TLR2* gene were associated with both susceptibility to and severity of acute pancreatitis.[32] In the current study we did not find an association between SNPs in *TLR2* and susceptibility to or complications and the

outcome of acute pancreatitis in our Dutch cohort of acute pancreatitis patients. We, however, used an intron 2 SNP (rs1816702) rather than microsatellite markers, which complicates the comparison.

Results of studies in Caucasian and Asian people are inconsistent. Obviously, allele frequencies can vary between different ethnic populations which could explain the conflicting results.[35] Our study has some weaknesses that may have influenced the results. First of all, the tagging SNPs that we used to genotype the genes were not able to tag the complete common variation within these genes. The tagging SNPs we have used for *TLR4* could capture only 38% of common genetic variation of SNPs with a frequency over 10% with r^2 of 0.8. For *IRAK3* this was 70%, for *TLR2* and *MyD88* this was 50%, and for *IRAK4* 67%. The lack of association therefore could have been caused by lack of coverage by the tagging SNPs used here. Another reason for lack of association could also be found in a lack of power of this study. The number of acute pancreatitis patients in this study, and in most genetic association studies in acute pancreatitis, is relatively low (n = 387). This study only had 68% power to find a real difference for the *IRAK3* variant that seemed to be associated with acute pancreatitis. For all other genetic variants tested here the power was even lower. Therefore, lack of power in combination with coverage of the genes of less than 60% makes it possible that there are still variants of these genes that are associated with acute pancreatitis. However, to be clinically important the polymorphism must be relatively frequent and with a clear penetration with respect to the disease or outcome of disease. Thus, a higher study power may detect smaller differences, but with a concomitant smaller clinical significance. Possibly other polymorphisms within the innate immune system or otherwise have an overriding effect. Alternatively, etiological and / or environmental factors with an impact on acute pancreatitis may differ between the various groups.

Although we were not able to show in the current study that genes of the TLR pathway are associated with acute pancreatitis, it is possible that these SNPs do alter the penetrance of other important gene polymorphisms, modify disease manifestations and affect the severity of the disease. We have only studied genetic polymorphisms in the selected genes, but we have not investigated expression levels of the proteins of these genes on the relevant cells (dendritic cells) in the gut or on the pancreas. Additionally, it is possible that these genes harbour rare variants that do have large effect sizes. These variants would not be well tagged with the common SNPs that were selected for this study. This could be studied by next generation sequencing. It is important to keep in mind though that much larger sample sizes would be needed to ensure sufficient power to detect association with rare variants, with limited overall clinical relevance.

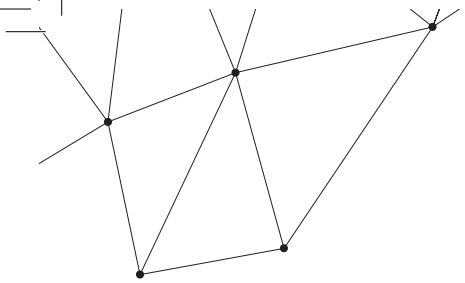
Another way to study a potential role for genes of the TLR pathway in acute pancreatitis would be an analysis of the major TLR2 and TLR4 ligands and their potential role in the disease, in particular infection of pancreas necrosis. *TLR4* and *TLR2* are both expressed on the plasma membrane of a variety of cells. TLR4

recognizes LPS on Gram-negative bacteria and TLR2 recognizes ligands of Gram-positive, mycobacteria and fungi. Next to these major ligands, there are also endogenous ligands for TLRs that are generated during inflammation or tissue injury with necrosis and that may contribute to prolonged and exaggerated inflammation, as is the case during sepsis. One can hypothesize that this could be the case in acute pancreatitis. We have not studied the ligands for the TLRs and it is still possible that the TLR pathway is functionally important in acute pancreatitis.

Genetic variants of key parts of the TLR pathway are unlikely to be involved in development, course and outcome of acute pancreatitis. We could not detect an association between development, course and outcome of acute pancreatitis and the genetic variants in *TLR2*, *TLR4*, *CD14*, *MyD88*, *IRAK-3* and *IRAK-4* that were tested in a candidate-pathway genetic analysis of the TLR pathway. This, however, does not rule out the possibility that expression and function of these genes is associated with acute pancreatitis. Since there is redundancy within the TLR pathway, it may well be that individuals harbouring combinations of genetic variants are more susceptible to complications.

Collaborators of the Dutch Pancreatitis Study Group

In addition to the authors (RMN, HCvS, MGHB, GTR, HGG, LMA, MAB), the following clinicians, members of the Dutch Pancreatitis Study Group, participated in this study. St. Antonius Hospital, Nieuwegein: B. van Ramshorst, B. L. Weusten, R. Timmer; University Medical Centre Utrecht: K. J. van Erpecum, G. A. Cirkel,V. Zeguers, A. Roeterdink, H.G. Rijnhart, M. P. Schwartz, M. S. van Leeuwen, B. U. Ridwan; Gelderse Vallei Hospital, Ede: B. J. Witteman, P. M. Kruyt; St Elisabeth Hospital, Tilburg: C. J. van Laarhoven, T. A. Drixler; University Medical Centre Groningen: V.B. Nieuwenhuijs, R. J. Ploeg, H. S. Hofker, M. R. Kruijt Spanjer, H. T. Buitenhuis, S. U. van Vliet, S. Ramcharan; Radboud University Nijmegen Medical Centre, Nijmegen: C. J. van Laarhoven, A. Nooteboom, H. van Goor, J. B. Jansen, G. T. Bongaerts, H. C. Buscher; Meander Medical Centre, Amerfoort: M. A. Brink, M. Mundt, R. Frankhuisen, E. C. Consten; Academic Medical Centre, Amsterdam: O. van Ruler, D. J. Gouma, M. J. Bruno; Maastricht University Medical Centre: C. H. Dejong, J. P. Rutten; Canisius Wilhelmina Hospital, Nijmegen: A. C. Tan, C. Rosman, L. Ootes, B. Houben; Leiden University Medical Centre, Leiden: A. F. Schaapherder, A. Haasnoot; Erasmus Medical Centre, Rotterdam: C.H. van Eijck, J. B. C. van der Wal, G. van 't Hof, E. J. Kuipers; Rijnstate Hospital, Arnhem: P. Wahab, E. J. Spillenaar Bilgen, P. van Embden; Maasstad Hospital, Rotterdam: F. J. Kubben, E. van der Harst, J. F. Lange, N. A. Wijffels, L. A. van Walraven.



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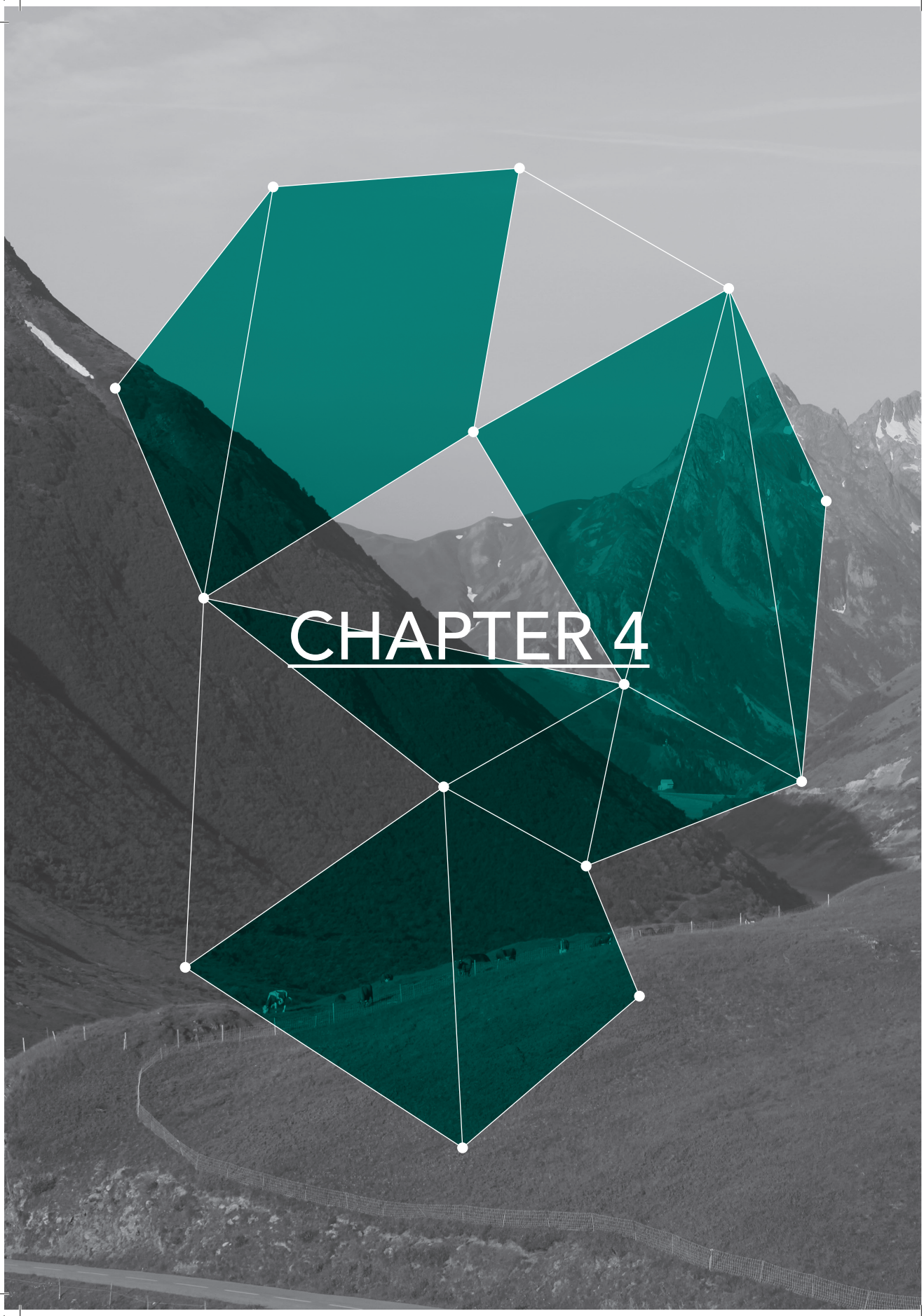
CHAPTER 4

The LYPA mannose binding lectin haplotype predisposes to acute pancreatitis

Rian M. Nijmeijer[1], Marc G.H. Besselink[2], Hjalmar C. van Santvoort[1], Ger T. Rijkers[3,4], Hein G. Gooszen[3], Cisca Wijmenga[5], Marja A. Boermeester[2], on behalf of the Dutch Pancreatitis Study Group

[1]Department of Surgery, University Medical Center Utrecht, Utrecht, the Netherlands; [2]Department of Surgery, Academic Medical Center, Amsterdam, the Netherlands; [3]Department of Operating Rooms, University Medical Center St. Radboud, Nijmegen, the Netherlands; [4]Department of Medical Microbiology and Immunology, St. Antonius Hospital, Nieuwegein, the Netherlands; [5]Department of Genetics, University Medical Center Groningen and University of Groningen, Groningen, the Netherlands.

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Abstract

Introduction: Mannose binding lectin (MBL) is a key component of innate immunity being a recognition molecule of the lectin pathway of complement. It is known to play a major role in first-line host defense during acute phase inflammatory responses. Only limited data are available on the role of MBL2 in acute pancreatitis. We aimed to study genetic variation in the MBL2 gene for association with the development, course, and outcome of acute pancreatitis.

Methods: Six single nucleotide polymorphisms in the promoter region and exon 1 of the MBL2 gene were studied in a cohort of 346 Dutch patients with acute pancreatitis and 360 controls. SNP genotyping was performed by Sanger sequencing using 3 primer sets.

Results: There was no association on single SNP level of genetic variants of exon1, the promoter region and the 5'-untranslated region with development of acute pancreatitis. The tested MBL2 genetic variants were also not associated with course or outcome of acute pancreatitis. One of the six haplotypes, the LYPA haplotype, was associated with susceptibility to acute pancreatitis (P-value 0.0003, odds ratio 2.26, 95% confidence interval 1.44-3.56).

Conclusion: One of the MBL2 haplotypes is associated with an increased susceptibility to the development but not the course or outcome of acute pancreatitis.

Introduction

Mannose-binding lectin (MBL) is a liver-derived serum protein that is a key player of the innate immune system. MBL acts as a soluble pattern recognition molecule that can recognize microorganisms, such as bacteria, yeasts, parasites and viruses and initiate the lectin pathway of the complement system and its subsequent inflammatory mechanisms. This results in an MBL-bound microorganism being taken up via complement receptors.[1] Alternatively, MBL acts as an opsonin, where phagocytic cells bind directly to complexes of MBL to microorganisms[2], after which the whole complex is directly internalized. Furthermore, it is hypothesized that the role of MBL extends beyond infection and may also play a key regulatory role in inflammation.[2] Finally, MBL can recognize and bind late apoptotic and necrotic cells[3], resulting in complement activation and apoptotic cell clearance.[2]

There are three single nucleotide polymorphisms (SNPs), located on exon 1 of *MBL2*, that result in amino acid substitutions leading to a decrease of functional MBL levels, commonly called D, B, and C.[4] In addition to these coding SNPs, there are three polymorphisms in the promoter region that also influence the serum concentration of MBL. These are the H/L variant (position -550), the X/Y variant (position -221) that has the strongest down-regulating effect on MBL concentration, and the P/Q variant, located in the 5′ untranslated part of exon 1 (position +4).

The promoter variants are in linkage disequilibrium with the variants of exon 1 and, of the 64 hypothetical haplotypes, only seven haplotypes are regularly observed.[5,6] These are four functional haplotypes with different expression levels (i.e. low-producing LXPA haplotype, high-producing HYPA and LYQA haplotypes and intermediate-producing LYPA haplotype) and three defective haplotypes (LYPB, LYQC, and HYPD).[6] Deficiency of MBL, resulting in low serum levels, is associated with an increased risk of infections.[5,6]

Acute pancreatitis is an inflammatory disorder of the pancreas, mostly caused by gallstones or alcohol abuse.[7] Acute pancreatitis is often mild and self-limiting, but in 15-25% of patients, pancreatitis is severe with an associated mortality risk of 10-24%.[8] The majority of deaths result from infectious complications, where especially bacterial infection of pancreatic necrosis results in a significant rise of morbidity and mortality.[9-11]

Acute pancreatitis is a disease with large clinical variations, which could be the result of genetic polymorphisms in genes encoding parts of the innate immune system and inflammatory cascades. Many other candidate genetic variants have been explored previously, but without a clear association.[12-16] MBL is a likely candidate, whose role in acute pancreatitis has been poorly studied. The aim of this study was to investigate whether genetic polymorphisms in the *MBL2* gene are associated with the development, severity and outcome of acute pancreatitis. We hypothesized that MBL might be involved in development and severity of acute pancreatitis, as it is an

inflammatory disorder, but MBL might also be involved in the infectious complications during the disease, such as infected pancreatic necrosis. We therefore investigated the genetic variants in the promoter region and exon 1 of the *MBL2* gene in a cohort of 346 Dutch patients with acute pancreatitis and 360 healthy controls for association with the development, course, and outcome of acute pancreatitis.

Methods

Acute pancreatitis patients and controls

This study was part of a randomized controlled trial on the role of probiotics in lowering infection rates in acute pancreatitis (trial registry ISRCTN38327949).[17] During this trial, patients with a first episode of acute pancreatitis were included in a prospective database. This cohort of patients has been described previously.[11,18] Acute pancreatitis was defined as abdominal pain in combination with a greater than three-fold elevation of serum amylase or lipase concentrations. Severe acute pancreatitis was defined as acute pancreatitis with organ failure and/or local complications.[17]

Genomic DNA of 346 patients with acute pancreatitis was isolated using DNA isolation kit I from the Magna Pure LC (Roche Diagnostics, Indianapolis, USA). Clinical data on the course and outcome of the disease for all patients were available from the prospectively collected trial database.[17] All patients or their legal representatives gave written informed consent and the ethics review boards of the 15 participating hospitals approved the protocol for this study.

The control cohort (n = 360) consisted of Dutch blood bank donors. The controls had a median age of 53 years (range: 20-70) and 39% was female.

SNP selection and genotyping

The single nucleotide polymorphisms that are associated with decreased MBL production[5,6,19-22] are situated in exon 1 of the *MBL2* gene on codons 52, 54, and 57 (genetic variants D, Arg52Cys, rs5030737; B, Gly54Asp, rs1800450; and C, Gly57Glu, rs1800451, respectively) and in the 5' UTR and promoter region of the gene (H/L, rs11003125; Y/X rs7096206; and P/Q, rs7095891). Individuals carrying wild-type alleles are referred to as AA, whereas homozygous or compound individuals are referred to as OO.[5] Call rates for genotyping were over 95% in cases and controls, except for rs7096206 (94.9%). All control genotypes were in Hardy-Weinberg equilibrium (data not shown, $p > 0.05$), except for SNP rs7096206.

Genotyping was performed partly in the Complex Genetics Group Laboratory, University Medical Center Utrecht, and partly in the laboratory of the Department of Genetics of the University Medical Center Groningen (rs11003125 in part of all samples for validation purposes and rs7096206). Genotyping was done by Sanger sequencing using three sequencing reactions. Details on primer sequences can be

found in Table 1.

Table 1. Primer sequences.

	PCR primers	Sequencing primer
Promoter region 1 –	Forward: TTAGCACTCTGCCAGGGCCAACGT	Forward
H/L variant	Reverse: TCCCATCTTTGTATCTGGGCAGCTGA	
Promoter region 2 –	Forward: CAGGTGGCAGATGGACCCGA	Reverse
Y/X variant	Reverse: TGACCCATCCCTGGCCTCTAGC	
5' UTR and exon 1 –	Forward: TAGTCACGCAGTGTCACAAGGAATGT	Forward
P/Q, D, B, C variants	Reverse: ATCCCCAGGCAGTTTCCTCTGGAAG	

In the Utrecht laboratory, for DNA amplification 50 ng of genomic DNA was used in 20 µl PCR reactions, containing a mixture of both forward and reverse primers, and all components for PCR reaction (dNTPs, MgCl, buffer and taq polymerase). Before sequencing, the PCR products were purified using LSKM Multiscreen purification plates (Millipore). In the Groningen laboratory, before sequencing, DNA amplification was performed using 25 ng of genomic DNA in 10 µl PCR reactions, also containing mastermix (AmpliTaq® Gold, Life Technologies) and 10mM primer pair mix. PCR products were amplified and purified (ExoSAP-IT, Affymetrix, Inc.).

Direct sequencing was performed using one of the primers (Table 1) on a 3730 DNA Analyzer (Applied Biosystems). Sequences were aligned and compared to *MBL2* consensus sequence using ContigExpress® software from the Vector NTI Suite 9 package. Haplotypes were constructed using Haploview.[23]

Statistical analysis

Statistical analysis was performed using 2-tailed χ^2 tests of case vs. control allele counts in PLINK.[24] Haplotypes were constructed using Haploview v3.2.[23] P-values, odds ratios (OR) and 95% confidence intervals (95% CI) are shown. The Bonferroni method was used to correct for multiple testing. All tables show the uncorrected P-values.

Results

A total of 346 acute pancreatitis patients and 360 healthy controls were genotyped with 6 SNPs in the *MBL2* gene. On single SNP level, the minor allele of one of the SNPs in exon 1 appeared to protect against acute pancreatitis (rs1800450, P 0.02, OR 0.68, 95% CI 0.49-0.95; Table 2), but this association was lost after correction for multiple testing. None of the other genotyped polymorphisms was associated with the development of acute pancreatitis.

The frequencies of exon 1 variants, which are commonly denoted as A when all wild-type alleles are present, or O in case of presence of variant alleles, differed between cases and controls (Figure 1). There were no differences between patients with mild and severe acute pancreatitis, between patients with and without infected pancreatic necrosis or between patients that died and those who survived the disease (data not shown).

We constructed 6-SNP haplotypes (Figure 2) and sought for association with the development of acute pancreatitis (Table 3 and 4). The LYPA haplotype was more prevalent among patients with acute pancreatitis than in controls (0.107 vs. 0.046, P 0.0003, OR 2.26, 95% CI 1.44-3.56). It has to be noted, however, that there is no complete linkage disequilibrium in our study between SNPs rs1800451 and rs1800450 (D'=1.0, r^2=0.003, LOD score = 0.72, Figure 2). Using sliding window haplotype analysis, we saw that the 5-SNP haplotype, where rs1800451 was excluded, was the most significant haplotype (omnibus P $8.2x10^{-5}$, data not shown), indicating that the causal variant is located on this haplotype.

Table 2. Association analysis of MBL2 SNPs in patients with acute pancreatitis and controls.

		RAF patients (n = 346)	RAF controls (n = 360)	OR	95% CI	P-value*
rs11003125	G/C#	0.352	0.369	0.93	0.75-1.16	0.5159
rs7096206	C/G	0.249	0.240	1.05	0.82-1.35	0.6772
rs7095891	T/C	0.202	0.213	0.94	0.72-1.21	0.6094
rs5030737	T/C	0.054	0.079	0.66	0.43-1.01	0.0546
rs1800450	A/G	0.096	0.135	0.68	0.49-0.95	0.0218
rs1800451	A/G	0.020	0.022	0.91	0.44-1.88	0.8018

OR, odds ratio; 95% CI, 95% confidence interval; RAF, risk allele frequency

*Two-tailed P-values were calculated by chi-squared test for independence of allele counts

#Risk variant / second allele

We subsequently analyzed whether polymorphisms in *MBL2* or the haplotypes were associated with the course and outcome of acute pancreatitis. Of the 346 acute pancreatitis patients, 92 patients had severe acute pancreatitis (27%), 51 patients had infected pancreatic necrosis (15%), and 18 patients died (4%). We compared patients with severe acute pancreatitis with patients with mild acute pancreatitis, patients with infected pancreatic necrosis with patients without infected pancreatic necrosis, and patients who died with patients who survived. We did not identify any SNPs or haplotypes that were associated with severity of acute pancreatitis, with bacterial infection of pancreatic necrosis, or with death caused by acute pancreatitis.

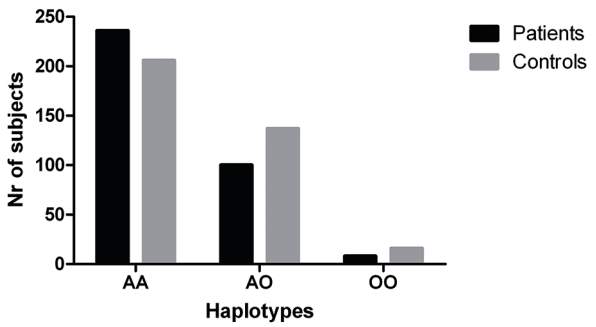

Figure 1: Prevalence of exon 1 genotype in patients and controls.
Comparison of MBL2 exon 1 genotype frequencies in patients and controls (chi squared analysis: p = 0.0062).

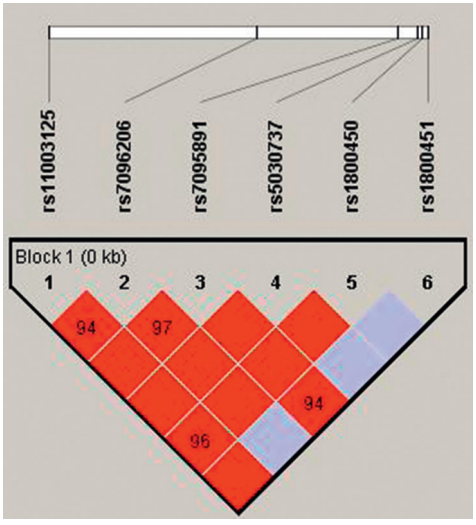

Figure 2: Haplotypes constructed from the six SNPs in the MBL2 gene. 6-SNP haplotypes were constructed using Haploview.

Table 3. Haplotypes present in the complete case-control cohort.

			Haplotype block				
H/L	**Y/X**	**P/Q**	**D**	**B**	**C**		
rs11003125	rs7096206	rs7095891	rs5030737	rs1800450	rs1800451	**Secretor haplotype**	**F**
G	G	C	C	G	G	HYPA	0.289
C	C	C	C	G	G	LXPA	0.241
C	G	T	C	G	G	LYQA	0.187
C	G	C	C	A	G	LYPB	0.114
C	G	C	C	G	G	LYPA	0.075
G	G	C	T	G	G	HYPD	0.067
C	G	T	C	G	A	LYQC	0.021

Haplotypes were constructed using Haploview. Major alleles are marked gray, minor alleles white. F, frequency in the whole cohort (cases and controls). Only haplotypes with a frequency > 0.01 are shown.

Table 4. The prevalence of *MBL2* haplotypes in patients with acute pancreatitis and controls reconstructed from genotyped SNPs and their association with acute pancreatitis

	MBL2 haplotypes										
	rs11003125	rs7096206	rs7095891	rs5030737	rs1800450	rs1800451	Cases (%)	Controls (%)	OR	95% CI	P-value*
HYPA	G	G	C	C	G	G	199 (29)	206 (29)	1.00‡	–	ref
LXPA	C	C	C	C	G	G	167 (25)	171 (24)	1.01	0.76-1.35	0.9224
LYQA	C	G	T	C	G	G	125 (18)	137 (19)	0.95	0.69-1.29	0.7212
LYPB	C	G	C	C	A	G	64 (9.4)	96 (13.3)	0.70	0.48-1.01	0.0553
LYPA	C	G	C	C	G	G	73 (10.7)	33 (4.6)	2.26	1.44-3.56	0.0003
HYPD	G	G	C	T	G	G	37 (5.4)	57 (7.9)	0.68	0.43-1.07	0.0877
LYQC	C	G	T	C	G	A	13 (1.9)	16 (2.2)	0.85	0.40-1.80	0.6548

OR, odds ratio; 95% CI, 95% confidence interval.

* Two-tailed P-values were calculated by chi-squared test for independence of haplotype counts.

‡ This haplotype was taken as the reference.

Discussion

We studied the potential contribution of the *MBL2* gene with acute pancreatitis in a cohort of 346 Dutch acute pancreatitis patients. We found no association on single SNP level with acute pancreatitis, but the LYPA haplotype was associated with an increased susceptibility to the development but not the course or outcome of acute pancreatitis. The frequency of the O haplotype, containing the genetic variants of exon 1, was increased in cases when compared to controls. When we stratified for course and outcome of acute pancreatitis, no difference was found. This indicates that the exon 1 variants by themselves do not play a major role in the pathogenesis of acute pancreatitis. None of the single SNPs, nor any of the other haplotypes was associated with course or outcome of acute pancreatitis. These results suggest that in acute pancreatitis, MBL does not alter the host's response to bacterial infection in the course of pancreatitis, but that the LYPA haplotype may have an effect on the primary insult pathway that led to acute pancreatitis.

Numerous studies have shown that genetic variants resulting in decreased promoter activity and low MBL plasma levels are associated with predisposition to infections, such as invasive pneumococcal disease[25,26], meningococcal disease[27], but also with the development of SIRS and sepsis.[25,28-30] MBL deficiency also increases susceptibility to abdominal Candida infections in patients with secondary peritonitis.[22] Receiving a liver transplant from an MBL-deficient donor leads to an increased incidence of sepsis and peritonitis in the organ recipients.[31,32] In this study we show that the LYPA haplotype confers an increased risk for acute pancreatitis, but not for the most important complication adding to mortality, namely infected pancreatic necrosis. A reason for this may be redundancy of the immune system, as was previously hypothesized in a study by Dahl *et al.* who did not find association of *MBL2* deficiency with susceptibility to infections.[33]

In one study it was suggested that patients with a non-infectious insult and high MBL producing genotype were at risk for SIRS development, requiring intensive care treatment, without having significant infectious complications.[34] It was hypothesized that high levels of functional MBL could be associated with the proinflammatory adverse effects following uncontrolled complement activation.[34] This has also been found for ischemia-reperfusion injury in patients with ischemic stroke, where patients with high MBL levels showed more local cytotoxic tissue damage than patients with functional MBL deficiency, which resulted in protection against secondary inflammatory damage of reperfused tissue and smaller infarction size.[35,36] Acute pancreatitis, although it is not a bacterial infection, has large similarities with sepsis, because in the early stage of the disease there is an enormous release of inflammatory mediators. MBL deficiency could possibly be associated with a reduced cytokine storm, leading to less severe disease. In this study, however, we could not show association between disease severity and MBL polymorphisms.

This is the first study to show a potential association of MBL with acute pancreatitis. Previously a genetic association study was carried out in a Chinese Han population, where no differences in prevalence of the different haplotypes could be shown.[16] Zhang *et al.*, however, only studied exon 1 variants and the Y/X polymorphism. Additionally, it is known that in different ethnic populations allele frequencies can differ[37], which is especially the case for MBL.[5,6] The strengths of this study are the relatively large cohort of acute pancreatitis patients in comparison to most studies on acute pancreatitis and the presence of a database containing information on the course of disease of the individual patients.[17]

This study is limited by the fact that at this point, no replication study has been performed, which would increase the strength of our findings. No plasma levels of MBL were determined, but the impact of the genetic variants on MBL plasma level is well known.[5,6,19-22] Additionally, another variant in *MBL2* has been shown to affect MBL levels.[38] This SNP (rs930507), located on exon 4 and until known only observed to occur in combination with the LXPA haplotype, was not part of this study.

The clinical impact of our finding is currently unclear. We did not find an association with the occurrence of infectious complications, such as bacterial infection of pancreatic necrosis, nor did we find an association with mortality. MBL genotyping, therefore, cannot be applied for risk stratification to identify patients that are likely to experience a severe course of disease. The lack of association may, however, be due to the smaller number of patients per group when subgroup analyses are performed. Currently, several studies into the role of the innate immune system in acute pancreatitis are being performed in various groups. Combining all results may give further insights in how the innate response system reacts to massive inflammation of the pancreas. Moreover, a genetic fingerprint of several more or less associated markers may aid to predict which patients are more vulnerable to develop a complicated course of disease.

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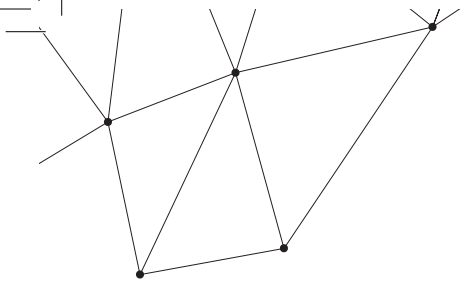

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CHAPTER 5

Nucleotide-binding oligomerization domain protein 2 (NOD2/CARD15) mutation p.R702W predisposes to a fatal outcome of severe acute pancreatitis

Annett Guenther[1], Rian M. Nijmeijer[2], Claudia Nitsche[1], Ali Aghdassi[1], Marja A. Boermeester[3], Hans-Ulrich Schulz[4], Georgios I. Papachristou[6], Julia Mayerle[1], Hjalmar C van Santvoort[2], Martin Zenker[5], Peter Simon[1], Eckhard Weber[1], David C. Whitcomb[6], Markus M. Lerch[1], Frank U. Weiss[1]

[1]Department of Medicine A, University Medicine Greifswald, Ernst-Moritz-Arndt-University Greifswald, Greifswald, Germany. [2]Department of Surgery, University Medical Center Utrecht, Utrecht, The Netherlands. [3]Department of Surgery, Academic Medical Center, Amsterdam, The Netherlands. [4]Department of Surgery and [5]Genetics, Otto-von-Guericke University Magdeburg, Magdeburg, Germany. [6]Department of Medicine, Division of Gastroenterology, Hepatology and Nutrition, University of Pittsburgh, School of Medicine, Pittsburgh, PA, USA.

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Abstract

In two cohorts comprising 941 acute pancreatitis patients (926 controls) we investigated whether mutations in nucleotide-binding oligomerization-domain-protein 2 (NOD2/CARD15) represent a risk factor for complications and mortality related to acute pancreatitis. The p.R702W mutation was found to be associated with multiple organ failure (OR: 4.25; CI 1.28-14.3; p<0.02) and mortality (OR 2.64; CI 1.35-5.05; p 0.008). Among patients who died from severe pancreatitis 40% carried the p.R702W allele with an odds ratio for death of 2.5 for heterozygous and of 9.0 for homozygous carriers. Pancreatitis patients known to carry p.R702W may require specific measures to prevent multiple organ failure.

Acute pancreatitis is the most common non-malignant disorder leading to hospital admission in the United States and remains burdened with significant mortality.[1] While most patients recover quickly from acute pancreatitis, around 20% develop complications, require intensive care treatment and have an up to 20% risk of not surviving the disease. To distinguish between patients with mild acute pancreatitis (~80%) who require little in terms of resources and those with severe disease, who need intensive care treatment, clinical classification systems and laboratory tests have been developed.[2,3,4] While these tests are useful for triaging patients who require specific therapeutic interventions, they reveal little about predisposing factors that determine disease severity or death from acute pancreatitis. The only well-established risk factor for severity to date is obesity.[5] Unlike for chronic pancreatitis[6], the identification of genetic markers for acute pancreatitis has focused on few target genes (*TLR4*, *CD14*, *TNFA*, *HSPA1B*) in relatively small cohorts and with negative or inconclusive results.[7]

In a variety of inflammatory disorders, polymorphisms in the Nucleotide-binding oligomerization domain protein 2 (NOD2/CARD15) gene have been identified as common predisposing genetic factors. NOD2/CARD15 is a member of the large family of nucleotide-binding and oligomerization domain-like receptors (NLR) which acts as intracellular sensor of pathogen-associated molecular patterns (PAMP), namely the peptidoglycan derivate muramyl-dipeptide. Its expression is found in intestinal epithelial and antigen-presenting cells where it appears to initiate or regulate innate as well as adaptive immune processes.[8]

NOD2 variants have been found associated with increasing the risk of developing Inflammatory Bowel Disease (IBD)[9], Blau-Syndrome[10] and early-onset sarcoidosis.[11] More importantly, they have been found to affect the severity of sepsis.[12] Here we investigated whether NOD2/CARD15 loss-of function mutations p.R702W , p.G908R and p.L1007fs could also affect the risk of developing acute pancreatitis or the patient's survival.

Analysis of *NOD2* mutations in European controls (1324 alleles) and patients with acute pancreatitis (1522 alleles) detected comparable allele frequencies of either the p.R702W (5.1% vs. 5.7%) mutation, the p.G908R mutation (2.2% vs. 1.1%) or the p.L1007fs mutation (2.4% vs. 2.1%) (Table 1). Conversely, in the US cohort the p.R702W mutation was found in 2.8% of controls (528 alleles) but in 8.1% of pancreatitis patients (360 alleles; OR 3.0; CI 1.52-5.96; p <0.001). The frequencies for p.G908R (1.9% vs. 0.7%, p 0.19) and L1007fs (1.7% vs. 2.5%, p 0.4) were not different between patients and controls. When the data from the US and Europe were combined for meta-analysis, carrying the p.R702W allele remained associated with a moderately increased risk for acute pancreatitis (6.1% vs. 4.5%; OR 1.39; CI 1.03-1.87; p<0.05).

Meta-analysis for association of the p.R702W allele with mild pancreatitis versus

severe pancreatitis (defined as having organ failure for longer than 48 hours) detected no significant difference (6.3% vs. 5.7%, p>0.05). When we studied patients who did not survive acute pancreatitis, 10.4% of European non-survivors (OR 2.14; CI 1.09-4.18; p<0.05) and 16.7% of US non-survivors (OR 6.84; CI 0.95-38.1; p 0.007) carried the p.R702W allele. When cohorts were combined for meta-analysis the association between death from acute pancreatitis and the p.R702W allele remained significant (11% vs. 4.5%; OR 2.64; CI 1.35-5.05; p 0.008). The meta-analysis for the NOD2 mutations p.G908R and p.L1007fs found, again, no association with non-survival in severe acute pancreatitis.

Table 1. Frequencies of the NOD2 p.R702W allele in European and US patients with acute pancreatitis

	European cohort				**US cohort**				**Meta-Analysis**				
	n	C (%)	T (%)	p^a	n	C (%)	T (%)	p^b	n	C (%)	T (%)	p^a	OR (95% CI)
Controls	1324	1256 (94.9)	68 (5.1)		528	513 (97.2)	15 (2.8)		1852	1769 (95.5)	83 (4.5)		
Acute pancreatitis	1522	1436 (94.3)	86 (5.7)	.61	360	331 (91.9)	29 (8.1)	<.001	1882	1767 (93.9)	115 (6.1)	.04	1.39 (1.03-1.87)
Non-survivors	106	95 (89.6)	11 (10.4)	.04	12	10 (83.3)	2 (16.7)	.007	118	105 (89.0)	13 (11.0)	.008	2.64 (1.35-5.05)

p^a: correction of multiple cohorts with Cochran-Mantel-Haenszel test; p^b: chi-square Pearson's test

We then stratified 167 patients with severe acute pancreatitis for which detailed complication records were available (Supplementary Table 1) and compared the incidence of specific complications between p.R702W and wild type carriers. While we could not demonstrate an association with infected necrosis, sepsis or single organ failure, the frequency of multiple organ failure in carriers of the p.R702W allele was significantly increased (OR: 4.25; CI 1.28-14.30; p 0.006) and mortality in that group was also higher (OR: 3.39; CI 0.96-11.68; p 0.03). Of note, 40% of p.R702W allele carriers died from acute pancreatitis compared with only 16.4% of patients with NOD2 wild type alleles. Finally, we applied the Armitage's trend test (Table 2) to analyze whether the mortality risk increases with the number of affected alleles. As expected the number of homozygous p.R702W carriers was small, but the risk increase was significant in European as well as US patients (p 0.002). On meta-analysis, the odds ratio to die from pancreatitis was 2.5-fold for heterozygous (CI 1.25-5.02) and 9-fold for homozygous p.R702W carriers (CI 0.8-100.93).

Table 2. Armitage's trend test for R702W alleles in Europe, US and in a meta-analysis.

	European cohort				US cohort				Meta-Analysis					
	C/C	C/T	T/T	*p*	C/C	C/T	T/T	*p*	C/C	C/T	T/T	*p*	OR C/T (95% CI)	OR T/T (95% CI)
Controls	596	64	2		249	15	0		845	79	2			
Acute pancreatitis	680	76	5	.55	152	27	1	<.001	832	103	6	.03	1.32 (0.97-1.80)	3.05 (0.61-15.14)
Non-survivors	43	9	1	.02	4	2	0	.006	47	11	1	.002	2.50 (1.25-5.02)	8.99 (0.80-100.9)

C/C, C/T, T/T: number of wildtype, heterozygous and homozygous genotypes ; *p* : Armitage's trend test ; OR: odd's ratio

Comparing 941 patients with 926 controls we found the p.R702W allele to almost triple the risk of not surviving an attack of acute pancreatitis. The risk differed slightly between European and US patients but remained significant in both individual cohorts and in the meta-analysis. Loss-of function mutations, p.G908R and p.L1007fs, that are associated with Crohn's disease and have a prevalence of >1% in the general population, conferred no such risk. The question of whether p.R702W carriers have an increased risk also of developing acute pancreatitis is not obvious. The carrier frequency among European acute pancreatitis patients (5.7%) was not significantly different from German (5.2%), Dutch (5.1%) or published Western European control cohorts.[14] The carrier frequency for p.R702W among US controls (2.8%), on the other hand, resembled that of either the Scandinavian population (1.8-2.5%), or that of cohorts with a sizable proportion of Asian or African subjects – and carrier status in the US was associated with a threefold risk of developing acute pancreatitis (8.1%). Whether or not p.R702W affects the risk of developing acute pancreatitis therefore needs to be addressed by further studies in other cohorts and/or with larger sample sizes. Our results would be compatible with a concept that disease susceptibility related to a certain genetic risk factor is depending on ethnic background or geographical factors, as it has been shown in many other complex diseases.

In Crohn's disease a gene dosage model for *NOD2* mutations was proposed because the 2 to 4-fold increased susceptibility for heterozygous mutation carriers increased another tenfold for subjects with two mutant alleles.[15] Comparably the risk of not surviving acute pancreatitis was further increased from 2.5 to 9 for homozygous carriers of the p.R702W mutation.

The question remains how the p.R702W NOD2 variant affects mortality rather than the induction of acute pancreatitis. NLR proteins have evolved to control host-pathogen interactions and their activation triggers antibacterial inflammatory responses mediated by NFkB and inflammatory caspases. Mutations in several members of NLR proteins have been linked to inflammatory diseases and sequence variant p.R702W near the LRR domain was reported to confer a loss-of-function for the NOD2 protein by impairing the production of intestinal interleukin-10.13 A

compromised immunological defense system may therefore cause the higher rates of multiple organ failure (four-fold) and mortality among p.R702W carriers. This, in turn, suggests that acute pancreatitis patients are not necessarily at an increased risk for developing infectious complications but more likely to have a compromised immune response to such infections.

Our study involves by far the largest cohort of patients with acute pancreatitis ever investigated for the presence of genetic disease susceptibility factors. It identifies NOD2 variants as being involved in disease severity rather than it's initiation, thereby affecting the survival rate of patients with acute pancreatitis. Patients known to carry a p.R702W NOD2 allele, even more those with two alleles, may require specific measures to prevent multiple organ failure early in their disease process.

Material and Methods

Classification of acute pancreatitis

Acute pancreatitis was defined as hospital admission for acute abdominal pain in combination with imaging evidence of acute pancreatitis and elevated serum amylase or lipase activities (three times upper normal limit). Severe acute pancreatitis was defined as persistent organ failure (circulatory, pulmonary, or renal) for longer than 48 hours. Diagnosis of pancreatic necrosis required imaging evidence of areas of non-enhancement on contrast-enhanced CT and infected necrosis evidence of bacterial growth in biopsy material. Sepsis was defined according to international standards16.

Patients and Control Cohorts

A total of 941 patients with acute pancreatitis and 926 healthy blood donor controls were prospectively enrolled in the trial. All patients or their legal representatives gave their written informed consent and the local ethics review boards approved the protocol. European patients were recruited from Germany (415 pancreatitis patients, 306 controls) and the Netherlands (346 pancreatitis patients, previously characterized17 and 356 controls). A replication cohort of 180 US-patients and 264 controls was recruited at the University of Pittsburgh. A total of 59 patients did not survive acute pancreatitis (overall mortality 6.3%, 22% mortality in severe acute pancreatitis).

Genotyping of genomic DNA

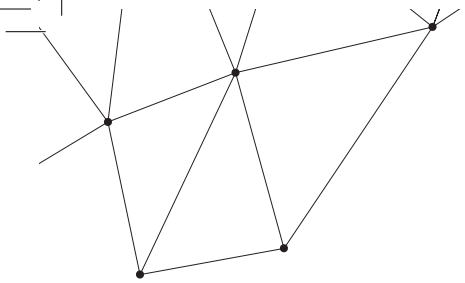
Allelic discrimination of NOD2 polymorphisms p.R702W (rs2066844), p.G908R (rs2066845) and p.L1007fs (rs5743293) was done by specific Taqman-Assays (Life-Technologies, Foster City, CA) and genomic sequencing. Statistical analysis of a genetic association was performed using the two-tailed chi-square-test for independence of

case vs. control alleles by Pearson; correction of multiple cohorts was calculated by applying the Cochran-Mantel-Haenszel test18 and considered significant when p was <0.05. OR indicates odds ratios and CI refers to 95% confidence intervals.

Supplementary Table. Frequency of complications in European patients with severe acute pancreatitis

Complication	Wild-type	R702W carrier	*pb*
Pancreatic necrosis	81.5% (123/151)	66.7% (10/15)	0.18
Infected pancreatic necrosis	51.1% (71/139)	42.9% (6/14)	0.59
Sepsis[a]	54.2% (39/72)	40.0% (2/5)	0.54
Single organ failure	69.5% (105/151)	80.0% (12/15)	0.56
Multiple organ failure	21.2% (32/151)	53.3% (8/15)	0.006
Mortality	16.4% (25/152)	40.0% (6/15)	0.03

a: no sepsis data were available for the Dutch cohort; *p*[b]: chi-square Pearson's test



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CHAPTER 6

The effect of renin angiotensin system genetic variants in acute pancreatitis

Skipworth James RA[1,2,3], Nijmeijer Rian M[4], van Santvoort Hjalmar C[10], Besselink Marc GH[5], Schulz Hans-Ulrich[6], Kivimaki Mika[7], Kumari Meena[7], Cooper Jackie A[8], Acharya Jay[8], Shankar Arjun[2], Malago' Massimo[1,2], Humphries Steve E[8], Olde Damink Steven WM[1,2,9], Montgomery Hugh M[1,3]

[1]Department of Surgery and Interventional Science, University College London, London, UK; [2]Department of Hepatopancreaticobiliary Surgery, Royal Free Hospital NHS Trust, London, UK; [3]UCL Institute of Human Health & Performance, University College London, London, UK; [4]Department of Internal Medicine, Rijnstate Hospital, Arnhem, Netherlands; [5]Department of Surgery, Academic Medical Center, Amsterdam, Netherlands; [6]General Surgery Department, Medical Faculty of Otto-von-Guericke University, Magdeburg, Germany; [7]Department of Epidemiology and Public Health, University College London, 1-19 Torrington Place, London, UK; [8]Centre for Cardiovascular Genetics, University College London, London; [9]Department of Surgery, Maastricht University Medical Centre, Maastricht, Netherlands; [10]Department of Surgery, University Medical Center Utrecht, Utrecht, Netherlands

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Abstract

Objectives: We sought association of genetic variants in the renin angiotensin system (RAS) and Vitamin D system with acute pancreatitis (AP) development and severity. Summary Background Data: The endocrine RAS is involved in circulatory homeostasis through the pressor action of angiotensin II (ang II) at its AT1 receptor (AT1R). However, local RAS regulate growth and inflammation in diverse cells and tissues, and their activity may be suppressed by Vitamin D. Intra-pancreatic ang II generation has been implicated in the development of AP.

Methods: Five hundred and forty-four Caucasian AP patients from three countries (UK 22; Germany 136; Netherlands 386) and 8487 control subjects (UK 7833, Netherlands 717) were genotyped for eight polymorphisms of the RAS/vitamin D systems, chosen based on likely functionality.

Results: The ACE I (rather than D) allele was significantly associated with alcohol-related AP when all cohorts were combined (p=0.03). The Renin rs5707 G (rather than A) allele was associated with AP (p=0.002), infected necrosis (p=0.025) and mortality (p=0.046).

Conclusions: The association of two RAS polymorphisms with AP suggests the need for further detailed analysis of the role of RAS/Vitamin D in the genesis or severity of AP, particularly given the ready potential for pharmacological manipulation of this system using existing marketed agents. However, further replication studies will be required before any such association is considered robust, particularly given the significant heterogeneity of AP causation and clinical course.

Introduction

Acute pancreatitis (AP) is a common inflammatory disorder that is mild and self-limiting in 80% of cases but which, when severe, may necessitate intensive care admission and lead to organ failure and death. The majority of cases are secondary to either gallstones or alcohol, although a multitude of causes exist. It is a common disease (current UK incidence: 150-420 cases per million per annum) and increasingly prevalent.[1] However, despite recent improvements in intensive care unit management and techniques of organ support, the severity and mortality associated with AP has not decreased since the 1970s[1]: up to 25% of patients will be diagnosed with severe disease[2-5] and approximately 4% of all patients will die.[6,7] This can be partly explained by the fact that no specific prophylactic or therapeutic agent is available, and current management is largely supportive. Better understanding of the molecular drivers of AP is essential for the identification of new therapeutic strategies.

Single-nucleotide polymorphisms (SNPs) are single base-pair variants in the DNA sequence which occur with a population frequency of >1%. Associated biological impacts may result- for instance, if the SNP alters an amino acid in the protein transcribed (thus affecting protein structure and function), or if the SNP lies in a region which affects gene transcription or mRNA stability. Associating such functional variants with specific disease phenotypes is thus one means by which to infer a causal role for the gene product in disease pathogenesis. Gene-association studies, performed to assess the molecular drivers of pancreatitis (Table 1), have generally focussed on the activation of pancreatic enzymes and pro-enzymes (one of the key steps in the initiation and propagation of pancreatic inflammation) or the process of systemic inflammation secondary to acute pancreatitis. However, small cohorts have weakened the ability to detect associations in mixed patient groups.

The renin-angiotensin system (RAS), originally described as a key regulator of intravascular homeostasis, controlling extracellular fluid volume and blood pressure[18], represents a potential target of such gene-association studies. In response to decreased afferent arteriolar pressure, decreased filtered sodium load or sympathetic nervous stimulation, the renal juxtaglomerular apparatus releases renin[19], which cleaves hepatically-derived angiotensinogen[20] to yield angiotensin I (ang I). Angiotensin-converting-enzyme (ACE) subsequently hydrolyses ang I to yield the effector peptide angiotensin II (ang II)[21], whose effects are mediated through 2 specific human receptors: the angiotensin II type 1 and 2 receptor (AT1R and AT2R).[22] However, local renin-angiotensin systems are now known to exist in diverse cells and tissues, including the pancreas[23,24], where they have paracrine and autocrine roles in the regulation of metabolism, blood flow, inflammation and healing.[25-27]

Within the pancreas, local generation of ang II influences exocrine and endocrine function through activation of the AT1R, stimulating increases in pancreatic enzyme secretion[28,29], while reducing islet blood flow and delaying insulin release.[30-32] It is

also implicated in the initiation and propagation of AP. Increased expression of RAS components is identified in experimental models of AP[33,34], where they drive activation of monocytes and macrophages[35,36], and expression of pro-inflammatory molecules such as interleukin-6 (IL-6), nuclear factor-κB (NF- κB) and monocyte chemoattractant protein-1 (MCP-1).[37] Such proinflammatory effects may occur through a number of possible AT1R-mediated mechanisms including generation of reactive oxygen species, matrix metallopeptidase-9 (MMP-9), nicotinamide adenine dinucleotide phosphate (NADPH), NF- κB tumour growth factors (TGFs) and Smad, as well as activation of human pancreatic stellate cells.[37-42] RAS inhibition has also been shown to attenuate the expression of pro-inflammatory molecules and mitigate pancreatic cellular injury.[43,44] Further, vitamin D may influence RAS activity by suppressing renin synthesis at the transcriptional level, thus acting as a negative regulator of the RAS.[45-49]

The association of AP (and its severity) with specific variants in key RAS/Vitamin D pathway genes would infer a causal role for such systems in AP pathogenesis. Given the postulated role for RAS/Vit D in AP pathogenesis, we hypothesized that such gene associations would be identified. We thus sought association of common functional

Table 1. Selected recent genetic associations with various clinical forms of pancreatitis

Gene	Protein	No. Pts	Association	Authors
PRSS1	Cationic Trypsinogen	108 HCP; 415 ICP; 82 Controls	CP	Teich *et al*, 2002[8]
SPINK1	Pancreatic Secretory Trypsin Inhibitor	1. 32 CP; 117 Controls 2. 198 Mixed CP; 290 Controls	1. CP 2. Hereditary CP	Kaneko *et al*, 2001[9] Chandak *et al*, 2004[10]
CFTR	Cystic Fibrosis Transmembrane Regulator	1. 67 ICP; 60 Controls 2. 25 CP; 236 Controls	1. Idiopathic CP 2. CP	Weiss *et al*, 2005[11] Tzetis *et al*, 2007[12]
CTLA-4	Cytotoxic T Lymphocyte-Associated Antigen 4	46 AIP; 78 CCP; 200 Controls	Autoimmune Pancreatitis	Chang *et al*, 2007[13]
IL-8	Interleukin-8	92 AP; 200 Controls	Severity of AP	Hofner *et al*, 2006[14]
TGF-β1	Transforming Growth Factor β1	28 CP; 94 Controls	Fibrosis in CP	Bendicho *et al*, 2005[15]
TLR-4	Toll-Like Receptor-4	521 AP; 120 Controls	No association with AP	Guenther *et al*, 2010[16]
TNF-α	Tumour Necrosis Factor α	77AP; 71 Controls	AP Severity	Balog *et al*, 2005[18]

AP- Acute Pancreatitis; CP- Chronic Pancreatitis; A IP- Autoimmune Pancreatitis; H CP- Hereditary Chronic Pancreatitis; ICP- Idiopathic Chronic Pancreatitis; C CP- Chronic Calcific Pancreatitis

variants in the RAS and Vitamin D systems with AP, and its severity, in order to clarify the possible role for RAS in AP pathogenesis; and in particular to elucidate whether genotypes associated with higher RAS activity were associated with the development or severity of acute pancreatitis.

Methods

Subjects

Blood samples were taken from AP patients from 3 Northern European countries, following local ethical approval (The Joint UCL/UCLH Committees on the Ethics of Human Research (Committee A); Reference No. 08/H0714/90) and written informed consent: (i) University College London Hospitals (UCLH, London, UK), between 2006 and 2009; (ii) Magdeburg University Hospital (Magdeburg, Germany) between 1996 and 2003; (iii) Eight university medical centres and seven major teaching hospitals in the Netherlands between 2004 and 2007, with blood stored at University Medical Centre Utrecht (Utrecht, Netherlands), as part of the PROPATRIA trial, a multicenter, randomized controlled trial (trial registry number ISRCTN38327949).[50]

AP was defined as upper abdominal pain in combination with serum amylase or lipase concentrations raised to at least three times the upper limit of normal. Prospective data, including demographics, predicted severity score (e.g. acute physiology and chronic health evaluation II (APACHE II)), necrosis and in-hospital mortality, were collected. Patients were classified as having actual (rather than predicted) severe AP in the presence of organ failure of over 48 hours duration and/or local pancreatic or peri-pancreatic complications such as necrosis, fluid collections and pseudocysts (as defined by the revised Atlanta classification[51]).

Controls were 2766 (2711 following quality control) healthy UK Caucasian males who had participated in the second Northwick Park Heart Study (NPHSII) (aged 51 to 60 years, recruited from 9 general medical practices within the UK) for assessment of ACE (rs4646996) and CYP2R1 (rs10741657) genotypes, and 5059 (1334 women and 3725 men) UK civil servants from the Whitehall II study (WHII; aged 35-55 years and working in the London offices of 20 Whitehall departments)[52] for analysis of other genetic variants. Full details of the genotyping and quality control have been published previously.[53] In summary, DNA from WHII was extracted from 6156 individuals from whole blood samples using magnetic bead technology (Medical Solutions, Nottingham, UK) and normalised to a concentration of 50ng/μl. Custom SNP arrays were designed by the Institute of Translational Medicine and Therapeutics, the Broad Institute and the National Heart Lung and Blood Institute supported Candidate-gene Association Resource Consortium (HumanCVD BeadChip)[54] on 5592 of these samples. After restriction to White/European groups and quality control, 5059 samples were utilised for analysis.

Seven hundred and seventeen samples were also obtained from blood-bank donors within the Netherlands and utilised as controls for all genotypes (kindly provided by Prof. C. Wijmenga, Department of Genetics, University Medical Center Groningen, Groningen, the Netherlands).

Genetic Analysis

DNA was extracted from UK subjects utilising the 'salting-out' technique[55]; from German subjects using Qiagen® DNA extraction kits (Qiagen, Hilden, Germany); and using the DNA isolation kit I from the Magna Pure LC (Roche Diagnostics, Indianapolis, USA) for the Dutch cohort. Common functional polymorphisms in genes of the human RAS and Vitamin D systems, or those previously associated with pancreatic disease, were selected by review of the published literature (Table 2). Subjects were genotyped for all pre-selected variants, with genetic analysis performed at University College London (London, UK).

Table 2. A table demonstrating further information on the genetic variants investigated in this study. All variants listed here have previously been associated directly with RAS, indirectly with RAS (vitamin D-metabolising system), or with forms of pancreatic disease. Allele frequencies are based on Caucasian data from HapMap and ABI AoD.

Gene	Gene Symbol	Chrom Location	SNP Name	ID Number	DNA Change	Allele Frequencies	Known Effect
Angiotensin Converting Enzyme 1	*ACE*	17q23.3	I/D	rs4646994	----------	0.54D:0.46I	D Allele: Higher Levels of Tissue & Circulating ACE[56]
Angiotensin II Receptor Type 1	*ATR1*	3q24	A1166C	rs5186	A>C	0.75A/0.25C	C Allele: Increased Receptor Activity[57,58]
Angiotensin II Receptor Type 2	*ATR2*	Xq23	1675	rs1403543	G>A	0.61A:0.39G	G Allele: Increased Receptor Expression[57]
Angiotensinogen	*AGT*	1q42.2	M235T	rs699	C>T	0.62T:0.38C	T Allele: Higher Levels of AGT, Low Prorenin, Susceptibility to DM[59]
Renin	*REN*	1q32	----------	rs5707	G>T	T0.87:G0.13	G Allele: Increased Levels of Renin[60]
Vitamin D Receptor	*VDR*	12q13.11	FokI	rs2228570	C>T	0.56C:0.44T	T Allele: Increased Suceptibility to DM[61]
Vitamin D Hydoroxylase	*CYP2R1*	11p15.2	CYP2R1	rs10741657	A>G	0.63G/0.37A	G Allele: Increased Susceptibility to DM[62]
Vitamin D Binding Protein	*GC*	4q12	----------	rs7041	G>T	0.58G:0.43T	1S & 1S-2: Increased Levels of Circulating Vitamin D Levels[63,64]

SNP- Single Nucleotide Polymorphism; **Chrom**- Chromosome; **DM**- Diabetes Mellitus

DNA was measured and standardised using a Nanodrop® 8000 (Thermo Scientific; Waltham, Ma, USA) spectrophotometer and Beckman Coulter Biomek® 2000 (Biodirect; Taunton, Ma, USA) respectively, to a concentration of 15ng/µl stock, prior to further dilution to 5ng/µl working stocks. The ACE insertion/deletion polymorphism was assessed via initial PCR amplification of DNA and subsequent identification of differences in DNA size utilising 7.5% microplate array diagonal gel electrophoresis (MADGE). The remaining genotypes were determined by polymerase chain reaction amplification (PCR), utilising custom-prepared TaqMan® SNP genotyping assay kits (Applied Biosystems; Carlsbad, Ca, USA).

Statistical Analysis

Patient data were anonymised. Allele frequencies were tested for deviation from Hardy-Weinberg equilibrium using a χ^2 goodness-of-fit test and all allele frequencies were in keeping with existing published population data. Odds ratios and p values were calculated for an additive genetic effect using logistic regression models with adjustment for age, sex and region. For ACE and rs5707 genotypes we also tested the recessive model. Analysis was performed using Stata Version 11 (StataCorp, Texas, USA) and a p value of < 0.05 was considered statistically significant. No adjustment was made for multiple comparisons. Power calculations were performed using Quanto (http://hydra.usc.edu/gxe/). Detectable odds ratios were calculated for the sample size of 544 cases assuming an additive effect. In addition we calculated the number of AP cases that would be required to detect an effect of the size observed in this study. The ratio of controls to cases was 5.4 for (rs4646996) and CYP2R1 (rs10741657), 1.4 for AT^2R (rs1403543) and AGT (rs699) and 10.6 for the remaining SNPs.

Results

Combined study groups (Table 3)

Five hundred and forty-four Caucasian patients (304 [55.9%] male) with acute pancreatitis (UK n=22, Germany n=136, Netherlands n=386), and 8487 control subjects (UK NPHS-II n=2711, UK WHII n=5059, Netherlands n=717) were genotyped. Median patient age was 56 years (17-91years). Two hundred and sixty nine (49.5%) cases were secondary to biliary disease, 118 (21.7%) to alcohol and 157 (28.9%) to other causes. One hundred and seventy three (31.8%) patients overall had severe acute pancreatitis (based upon revised Atlanta criteria[51]; UK n=4, Germany n=65, Netherlands n=104) and 38 (7.0%) died (Germany n=18 and Netherlands n=20). Infected necrosis occurred in 58 (14.2%) patients in UK and Dutch cohorts (UK n=2 and Netherlands n=56). Data on infected necrosis were not available in the German cohort.

Table 3. Baseline characteristics of the 3 study cohorts under investigation.

Study Cohort	Study Group (n=)	Controls (n=)	Aetiology (n=)	Severe AP (n=)	Infected Necrosis (n=)	Mortality (n=)
UK	22	5067 (WHII) 2766 (NPHSII)	9 (40.9%) ETOH 8 (36.4%) Biliary 5 (22.7%) Other	4 (18.2%)	2 (9.1%)	0 (0%)
Netherlands	386	717	72 (18.7%) ETOH 209 (54.1%) Biliary 105 (27.2%) Other	104 (26.9%)	56 (14.5%)	20 (5.2%)
Germany	136	N/A	37 (27.2%) ETOH 53 (39.0%) Biliary 46 (33.8%) Other	65 (47.8%)	N/A	18 (13.2%)

Genotype distributions for cases and controls are shown in Table 4 for the overall cohort. The G allele of the renin rs5707 SNP was associated with AP (p=0.003), infected necrosis (p=0.02) and mortality (p=0.003) in the Dutch cohort, where 60% of deaths occurred secondary to infected necrosis (Table 5). These findings were replicated when the cohorts were combined (AP (OR (95% CI) = 2.19 (1.34-3.60) p=0.002), infected necrosis (2.75 (1.13-6.68) p=0.025) and mortality (2.66 (1.02-6.95) p=0.046) for the recessive model). There were no other significant genotype associations with the development of AP, severe AP or mortality from AP.

Table 4. Comparison of genotype frequency between combined AP cohorts and controls.

Gene				P value*	No. AP patients required for statistical power
ACE (rs4646994)	**II**	**ID**	**DD**		
Controls[1,3]	839 (24.5)	1656 (48.4)	928 (27.1)	0.63	11167
AP	132 (25.1)	273 (52.0)	120 (22.9)		
OR (95%	1.00	1.22 (0.92-1.60)	0.92 (0.67-1.26)		
AT1R (rs5186)	**AA**	**AC**	**CC**		
Controls[2,3]	2778 (48.4)	2433 (42.4)	530 (9.2)	0.08	803
AP	266 (52.8)	187 (37.1)	51 (10.1)		
OR (95% CI)	1.00	0.74 (0.58-0.94)	0.87 (0.58-1.30)		
AT2R (rs1403543)	**AA**	**AG**	**GG**		
Controls[3]	280 (39.8)	146 (20.8)	277 (39.4)	0.31	5114
AP	199 (40.5)	119 (24.2)	174 (35.4)		
OR (95% CI)	1.00	0.88 (0.60-1.29)	0.87 (0.67-1.14)		
CYP2R1 (rs10741657)	**GG**	**GA**	**AA**		
Controls[1,3]	1235 (37.1)	1576 (47.4)	517 (15.5)	0.35	3264
AP	181 (37.7)	227 (47.3)	72 (15.0)		
OR (95% CI)	1.00	1.10 (0.85-1.41)	1.17 (0.82-1.67)		
AGT (rs699)	**AA**	**AG**	**GG**		
Controls[2,3]	2104 (36.6)	2701 (47.0)	945 (16.4)	0.15	1182
AP	190 (37.6)	238 (47.1)	77 (15.3)		
OR (95% CI)	1.00	1.15 (0.90-1.47)	1.27 (0.89-1.81)		
Renin (rs5707)	**AA**	**AC**	**CC**		
Controls[2,3]	3476 (60.6)	2004 (35.0)	253 (4.4)	0.06	647
AP	314 (61.2)	159 (31.0)	40 (7.8)		
OR (95% CI)	1.00	0.99 (0.77-1.27)	2.18 (1.32-3.61)		
VDR (rs2228570)	**GG**	**GA**	**AA**		
Controls[3]	293 (41.6)	304 (43.2)	107 (15.2)	0.18	2220
AP	176 (36.2)	326 (48.6)	74 (15.2)		
OR (95% CI)	1.00	1.28 (0.99-1.66)	1.17 (0.82-1.67)		
GC (rs7041)	**CC**	**CA**	**AA**		
Controls[2,3]	1752 (30.5)	2912 (50.7)	1075 (18.7)	0.29	1975
AP	165 (33.1)	244 (48.9)	90 (18.0)		
OR (95% CI)	1.00	0.77 (0.59-1.00)	0.88 (0.63-1.25)		

*additive genetic model adjusted for age, sex, region.

Table 5a. Effect of Renin G allele upon outcome from acute pancreatitis in Dutch AP samples and controls.

	Rs5707	AA	AC	CC	P value*
Overall	Controls	443 (63.9)	224 (32.3)	26 (3.8)	0.003
	AP	214 (59.9)	115 (32.2)	28 (7.8)	
	OR (95% CI)	1.00	1.06 (0.79-1.42)	2.34 (1.30-4.23)	
Infected Necrosis	No	183 (59.6)	104 (33.9)	20 (6.5)	0.02
	Yes	31 (62.0)	11 (22.0)	8 (16.0)	
	OR (95% CI)	1.00	0.64 (0.31-1.33)	2.42 (0.97-6.02)	
Mortality	No	204	112	23	0.003
	Yes	10	3	5	
	OR (95% CI)	1.00	0.53 (0.09-2.12)	4.67 (1.14-16.97)	

*recessive model

Table 5b. Effect of Renin G allele upon outcome from acute pancreatitis in combined cohort samples and controls.

	Rs5707	AA	AC	CC	P value*
Overall	Controls	3476 (60.6)	2004 (35.0)	253 (4.4)	0.002
	AP	314 (61.2)	159 (31.0)	40 (7.8)	
	OR (95% CI)	1.00	0.99 (0.77-1.27)	2.18 (1.32-3.61)	
Infected Necrosis	No	191 (60.3)	106 (33.4)	20 (6.3)	0.025
	Yes	33 (63.5)	11 (21.2)	8 (15.4)	
	OR (95% CI)	1.00	0.61 (0.30-1.27)	2.37 (0.96-5.88)	
Mortality	No	290 (60.8)	153 (32.1)	34 (7.1)	0.046
	Yes	24 (66.7)	6 (16.7)	6 (16.7)	
	OR (95% CI)	1.00	0.47 (0.19-1.19)	2.18 (0.82-5.80)	

*recessive model

Alcohol-related acute pancreatitis

One hundred and eighteen patients (21.7% of total) had alcohol-related AP (9 UK, 37 Germany, 72 Netherlands). Median age was 47 years (22-88 years) and 98 (83.1%) were male. Of Dutch and UK patients, 11 (14.1%) had infected necrosis (10 Dutch). Fifty (42.4%) patients had actual severe pancreatitis (1 UK, 28 Germany, 21 Dutch), and five (4.2%) died (3 Germany, 2 Netherlands). The ACE I (rather than D) allele (rs4646994) was associated with alcohol-related AP when the cohorts were combined (OR (95% CI) 0.57 (0.34-0.95) p=0.03 recessive model for DD vs. I I/ID). There were no other significant associations (Table 6).

Table 6. Comparison of genotype frequency between patients with AP secondary to alcohol and combined controls.

Gene				**P value***
ACE (rs4646994)	**II**	**ID**	**DD**	
Alcohol	32 (27.6)	65 (56.0)	19 (16.4)	0.18 *(Additive model)*
Control[1,3]	839 (24.5)	1656 (48.4)	928 (27.1)	0.03 *(Recessive model)*
OR (95% CI)	1.00	1.17 (0.74-1.86)	0.63 (0.34-1.15)	
AT1R (rs5186)	**AA**	**AC**	**CC**	
Alcohol	57 (50.9)	41 (36.6)	14 (12.5)	0.85
Control[2,3]	2778 (48.4)	2433 (42.4)	530 (9.2)	
	1.00	0.75 (0.49-1.16)	1.23 (0.65-2.33)	
AT2R (rs1403543)	**AA**	**AG**	**GG**	
Alcohol	46 (14.1)	10 (6.4)	45 (14.0)	0.79
Control[3]	280 (39.8)	146 (20.8)	277 (39.4)	
	1.00	1.17 (0.44-3.08)	0.94 (0.60-1.47)	
CYP2R1 (rs10741657)	**GG**	**GA**	**AA**	
Alcohol	34 (33.3)	47 (46.1)	21 (20.6)	0.18
Control[1,3]	1235 (37.1)	1576 (47.4)	517 (15.5)	
	1.00	1.16 (0.73-1.86)	1.52 (0.84-2.76)	
AGT (rs699)	**AA**	**AG**	**GG**	
Alcohol	45 (40.2)	57 (50.9)	10 (8.9)	0.92
Control[2,3]	2104 (36.6)	2701 (47.0)	945 (16.4)	
	1.00	1.18 (0.78-1.81)	0.79 (0.38-1.64)	
Renin (rs5707)	**AA**	**AC**	**CC**	
Alcohol	72 (64.9)	34 (30.6)	5 (4.5)	0.95
Control[2,3]	3476 (60.6)	2004 (35.0)	253 (4.4)	
	1.00	0.93 (0.60-1.45)	1.15 (0.43-3.10)	
VDR (rs2228570)	**GG**	**GA**	**AA**	
Alcohol	34 (30.6)	60 (54.1)	17 (15.3)	0.17
Control[3]	293 (41.6)	304 (43.2)	107 (15.2)	
	1.00	1.76 (1.09-2.85)	1.30 (0.68-2.51)	
GC (rs7041)	**CC**	**CA**	**AA**	
Alcohol	38 (34.2)	57 (51.4)	16 (14.4)	0.30
Control[2,3]	1752 (30.5)	2912 (50.7)	1075 (18.7)	
	1.00	0.87 (0.56-1.36)	0.72 (0.39-1.35)	

[1]NPHSII; [2]WHII; [3]Netherlands Blood Bank

*additive genetic model adjusted for age, sex, region.

Biliary acute pancreatitis

Two hundred and seventy patients had biliary AP (49.4% of total: 8 UK, 53 Germany, 209 Netherlands). Median age was 62 years (18-91 years) and 115 (42.6%) patients were male. Of Dutch and UK subjects, 26 (12.1%) patients developed infected necrosis (25 Dutch, 1UK). Seventy (25.9%) had actual severe AP (2 UK, 19 Germany, 49 Netherlands) and 17 (6.3%) died (8 Germany, 9 Netherlands). No significant associations with genotype were identified (Table 7).

Discussion

To our knowledge, this is the largest study thus far investigating the association of RAS genotype with acute pancreatitis. The human ACE gene has a genetic variant in which the absence (Deletion, D allele) rather than the presence (Insertion, I allele) of a 287 base pair fragment is associated with higher circulating[65] and tissue ACE activity such as that in myocardium[66], and inflammatory cells.[67] Our analysis of 544 AP patients and 8487 controls demonstrated an association of the ACE I allele (lower ACE activity) with alcohol-related acute pancreatitis. To date, no other studies have identified an association of ACE genotype with the development or severity of acute[68], chronic[69-72], familial[70] or tropical calcific pancreatitis.[73] However, these studies were small, incorporated mixed aetiologies and disease-types (acute, chronic, familial and tropical calcific pancreatitis), and did not address other RAS variants (see Table 8). We sought to resolve these issues by using larger sample sizes; the comparison of single aetiologies; the investigation of the effects of multiple RAS genotypes; investigation of alternate systems that may affect the RAS pathway (e.g. the vitamin D pathway); and use of multiple cohorts. However, still larger sample sizes may be required to detect effect sizes of the magnitude found (see Table 4) as the sample size utilised here only had sufficient power to detect an odds ratio in the range of 1.20-1.26.

Table 7. Comparison of genotype frequency between patients with AP secondary to biliary pathology and combined controls.

Gene				P value*
ACE (rs4646994)	**II**	**ID**	**DD**	
Biliary	72 (27.6)	125 (47.9)	64 (24.5)	0.48
Control[1,3]	839 (24.5)	1656 (48.4)	928 (27.1)	
	1.00	0.95 (0.66-1.37)	0.86 (0.56-1.31)	
AT1R (rs5186)	**AA**	**AC**	**CC**	
Biliary	123 (50.4)	95 (38.9)	26 (10.7)	0.16
Control[2,3]	2778 (48.4)	2433 (42.4)	530 (9.2)	
	1.00	0.76 (0.54-1.06)	0.80 (0.47-1.36)	
AT2R (rs1403543)	**AA**	**AG**	**GG**	
Biliary	97 (39.6)	74 (30.2)	74 (30.2)	0.14
Control[3]	280 (39.8)	146 (20.8)	277 (39.4)	
	1.00	0.76 (0.47-1.23)	0.76 (0.53-1.10)	
CYP2R1 (rs10741657)	**GG**	**GA**	**AA**	
Biliary	96 (41.4)	108 (46.6)	28 (12.1)	0.62
Control[1,3]	1235 (37.1)	1576 (47.4)	517 (15.5)	
	1.00	1.01 (0.72-1.42)	0.84 (0.50-1.41)	
AGT (rs699)	**AA**	**AG**	**GG**	
Biliary	97 (39.4)	110 (44.7)	39 (15.9)	0.58
Control[2,3]	2104 (36.6)	2701 (47.0)	945 (16.4)	
	1.00	1.02 (0.73-1.43)	1.17 (0.73-1.88)	
Renin (rs5707)	**AA**	**AC**	**CC**	
Biliary	161 (63.9)	71 (28.2)	20 (7.9)	0.59
Control[2,3]	3476 (60.6)	2004 (35.0)	253 (4.4)	
	1.00	0.79 (0.56-1.12)	2.11 (1.09-4.08)	
VDR (rs2228570)	**GG**	**GA**	**AA**	
Biliary	96 (39.7)	109 (45.0)	37 (15.3)	0.60
Control[3]	293 (41.6)	304 (43.2)	107 (15.2)	
	1.00	1.02 (0.72-1.44)	1.16 (0.72-1.44)	
GC (rs7041)	**CC**	**CA**	**AA**	
Biliary	78 (32.0)	121 (49.6)	45 (18.4)	0.36
Control[2,3]	1752 (30.5)	2912 (50.7)	1075 (18.7)	
	1.00	0.73 (0.51-1.04)	0.86 (0.54-1.38)	

[1]NPHSII; [2]WHII; [3]Netherlands Blood Bank

*additive genetic model adjusted for age, sex, region.

Table 8. Previous studies investigating the effect of RAS polymorphisms on outcome from pancreatitis.

Gene; SNP	Study Cohorts	Significant Associations	Authors
ACE; I/D	79 AP / 95 Controls	None	Oruc *et al*, 2009[68]
ACE; I/D	98 AP & 789 CP / 1294 Controls	None	Hucl *et al*, 2009[69]
ACE; I/D	104 CP + 51 FP / 163 Controls	None	Oruc *et al*, 2004[70]
ACE; I/D	55 CP / 128 Controls	None	Lukic *et al*, 2011[72]
ACE; I/D	91 TCP / 99 Controls	None	Bhaskar *et al*, 2006[73]

AP- Acute Pancreatitis; **CP**- Chronic Pancreatitis; **FP**- Familial Pancreatitis; **TCP**- Tropical Calcific Pancreatitis

Alcohol has also been shown to directly activate RAS in animal models of alcoholic cardiomyopathy.[74] Despite this, our study demonstrated an association of the ACE I allele (lower ACE activity) rather than the postulated D-allele (i.e. high activity) with alcohol-related acute pancreatitis. This finding seems contrary to the hypothesis that increasing activity of the RAS may lead to increasing likelihood of developing acute pancreatitis or severe disease. However, ACE inhibitor use has been associated with pancreatitis[75], and multiple angiotensin I processing enzymes are now known to exist (e.g. chymase, chymotrypsin, tonin, aminopeptidase A, B and N, prolylendopeptidase, and neutral endopeptidase). Further, ACE2, a homologue of ACE with 42% sequence homology[76] has recently been discovered and its primary product, Ang (1-7), acts through the Mas receptor[77] to negatively regulate the RAS; thereby counter-balancing ACE action. Thus, ACE levels, as assessed by RAS genotypes, may not truly represent overall RAS or ACE activity at a local or systemic level, and further studies may require serum assays or pancreatic biopsy samples for direct assessment of tissue ACE levels.

When the cohorts were combined, the renin rs5707 G allele was associated with AP ($p=0.002$), infected necrosis ($p=0.025$) and mortality ($p=0.046$). The renin rs5707 G allele has previously been associated with hypertension and diabetes, and has been hypothesised to increase the activity of the RAS.[60] However, although this finding was also present in the Dutch cohort, we could not replicate this finding in cohorts from other countries. Nor was there biological consistency through disease association with other RAS genotypes, or those of (the putatively RAS-regulatory) Vitamin D system investigated here. Such lack of consistency and replication is likely to be secondary to heterogeneity in AP causation and allele frequencies between populations, as well as small individual cohort sample sizes, particularly when single aetiologies were examined. Similarly, the patient cohort contained a high proportion of Dutch and German individuals, whilst the control group was UK-dominated. However, all cohorts were derived from North-European, Caucasian populations with similar ancestry and any minor allelic variations are therefore unlikely to influence the significance of results. Meanwhile, the medical systems and diagnostic criteria utilised in each country were similar, although any national differences in diagnosis, management and

outcome would require analysis via long-term, prospective, national registries.

Although multiple causes exist, a common pathophysiological pathway in AP involves premature activation of various proteolytic pancreatic enzymes such as trypsin, chymotrypsin, carboxypeptidase and kallikrein. Chymotrypsin is capable of converting angiotensinogen to ang I, and trypsin to catalyse ang II to ang III and IV.[78] Therefore, pancreatic enzyme activity may be another crucial factor in the activation of RAS during AP - an effect which may swamp that of RAS genotype. Further, the activation of this common AP-initiating pathway occurs via different mechanisms in biliary- and alcohol-related pancreatitis, and the heterogeneous proportion of each in the three cohorts may further explain the inconsistency of genetic association between the cohorts from various geographical regions (UK: 40.9% ETOH, 36.4% biliary, 22.7% other; Netherlands: 18.7% ETOH, 54.1% biliary, 27.2% other; Germany: 27.2% ETOH, 39.0% biliary, 33.8% other).

However, such data must be interpreted with caution, and cannot be taken as proof of a role for RAS or Vitamin D in the genesis or severity of AP, perhaps in part due to the study limitations, including the problem of multiple comparison and a relatively small sample size, but also possibly in part due to heterogeneity of AP causation and variations in clinical course. Adjustment for multiple comparison, whilst necessary, may also make the discarding of a 'true positive' finding more likely than that of a 'false positive'. In addition, uniformity in assessment of disease severity is difficult to achieve[79], and heterogeneity, by its definition, will also weaken power in genetic studies. Further studies should concentrate on large, well-structured study cohorts with clear phenotypes and substantial numbers of individuals in any aetiological group, as well as with tight coconstraints on defined severity, in order to circumvent these issues. In addition, association of any phenotype with one allelic variant may, of course, occur by chance. In addition, the gene variant under study may (through strong linkage disequilibrium) mark activity in an adjacent gene (through which any observed associations are in fact mediated). For reasons such as this, findings of candidate gene association studies require replication if to be considered robust, with subsequent fine-mapping of the genes required.

Further studies should concentrate on large, well-structured study cohorts with clear phenotypes, to attempt to circumvent these issues. However, the association of two RAS polymorphisms ACE I and Renin rs5707 G ACE I with AP in this study does suggest that this issue warrants further detailed analysis, given the ready potential for pharmacological manipulation of this system using existing marketed agents. Such roles are also worthy of active investigation in diverse pancreatic disease states.

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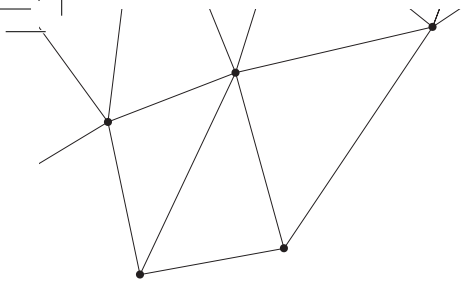

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CHAPTER 7

Impact of global Fxr deficiency on experimental acute pancreatitis and genetic variation in the FXR locus in human acute pancreatitis

Rian M. Nijmeijer[1], Frank G. Schaap[2,3], Alexander J.J. Smits[4], Andreas E. Kremer[2,5], Louis M.A. Akkermans[1], Alfons B.A. Kroese[1,6], Ger. T. Rijkers[7,8], Marguerite E.I. Schipper[4], André Verheem[1], Cisca Wijmenga[9], Hein G. Gooszen[6], Karel J. van Erpecum[10]

Departments of [1]Surgery, [4]Pathology and [10]Gastroenterology and Hepatology, University Medical Center Utrecht, Utrecht, the Netherlands; [2]Tytgat Institute for Liver and Intestinal Research, Academic Medical Center, Amsterdam, the Netherlands; [3]Department of General Surgery, Maastricht University, Maastricht, The Netherlands; [5]Department of Medicine 1, Friedrich-Alexander-University of Erlangen-Nuremberg, Erlangen, Germany; [6]Neurotoxicology Research Group, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, the Netherlands; [7]Department of Operating Rooms, University Medical Center St. Radboud, Nijmegen, the Netherlands; [8]Department of Medical Microbiology and Immunology, St. Antonius Hospital, Nieuwegein, the Netherlands; [9]Department of Genetics, University Medical Centrum Groningen, University of Groningen, Groningen, the Netherlands.

Manuscript submitted

Abstract

Background: Infectious complications often occur in acute pancreatitis, related to impaired intestinal barrier function, with prolonged disease course and even mortality as a result. The bile salt nuclear receptor farnesoid X receptor (FXR), which is expressed in the ileum, liver and other organs including the pancreas, exhibits anti-inflammatory effects by inhibiting NF-κB activation and is implicated in maintaining intestinal barrier integrity and preventing bacterial overgrowth and translocation. Here, with the aid of complementary animal and human experiments, we explore the potential role of FXR in acute pancreatitis.

Methods: Experimental acute pancreatitis was induced using the CCK-analogue cerulein in wild-type and $Fxr^{-/-}$ mice. Severity of acute pancreatitis was assessed using histology and a semi-quantitative scoring system. Ileal permeability was analyzed *in vitro* by Ussing chambers and an *in vivo* permeability assay. Gene expression of *Fxr* and *Fxr* target genes was studied by quantitative RT-PCR. Serum FGF19 levels were determined by ELISA in acute pancreatitis patients and healthy volunteers. A genetic association study in 387 acute pancreatitis patients and 853 controls was performed using 9 tagging single nucleotide polymorphisms (SNPs) covering the complete *FXR* gene and two additional functional SNPs.

Results: In wild-type mice with acute pancreatitis, ileal transepithelial resistance was reduced and ileal mRNA expression of Fxr¬ target genes *Fgf15*, *SHP*, and *IBABP* was altered. Nevertheless, $Fxr^{-/-}$ mice did not exhibit a more severe acute pancreatitis than wild-type mice. In patients with acute pancreatitis, FGF19 levels were lower than in controls. However, there were no associations of *FXR* SNPs or haplotypes with susceptibility to acute pancreatitis, or its course, outcome or etiology.

Conclusion: We found no evidence for a major role of FXR in acute human or murine pancreatitis. The observed altered Fxr activity during the course of disease may be a secondary phenomenon.

Introduction

Acute pancreatitis (AP) is the acute inflammation of the pancreas, and is mostly caused by gallstones or alcohol abuse.[1] In the majority of patients the course of the disease is mild, but in around 20% of patients, AP is severe with organ failure and/or local complications.[2] Mortality from AP is especially caused by infectious complications, such as bacterial infection of pancreatic necrosis.[3,4] Failure of the intestinal barrier function plays a critical role, as it allows for bacterial translocation, facilitating such infectious complications.[5-8]

The intracellular bile salt receptor farnesoid X receptor (FXR) is mainly expressed in ileum and liver, and to some extent in other organs, such as the pancreas[9], with little information available on its function in the latter organ. FXR is considered the master regulator of bile acid homeostasis, which regulates various genes encoding for bile acid transport proteins, including apical sodium-dependent bile acid transporter (ASBT) and ileal bile acid binding protein (IBABP).[10,11] Also, the enterokine fibroblast growth factor 15 (Fgf15, human orthologue FGF19), whose expression is controlled by FXR, exerts a negative feedback regulation of hepatic bile salt neo-synthesis and, at least in mice, induces gallbladder refilling at the end of the postprandial phase.[12]

More recently, FXR has been implicated in the regulation of fat and glucose metabolism, in the maintenance of intestinal barrier integrity and prevention of intestinal bacterial overgrowth, by affecting putative FXR-dependent genes such as *angiogenin-1*, *iNOS*, *CAR12* and *IL18*.[13] In patients with Crohn's colitis, who show impaired antibacterial defense and impaired intestinal barrier function, *FXR* expression was altered in areas of inflamed mucosa.[14] Furthermore, we recently showed in two murine models for colitis that administering the semi-synthetic FXR agonist INT747 (Obeticholic acid®) ameliorates intestinal inflammation, improving colitis symptoms, preserving intestinal barrier function, and reducing goblet cell loss.[15] The underlying mechanism for these anti-inflammatory effects is thought to be inhibition of NF-κB.[16] We also recently detected impaired mRNA expression of FXR target genes in the ileum of patients with clinically quiescent Crohn's colitis.[17] FGF19 signaling has been implicated in regulating inflammation by antagonizing NF-κB signaling in FGF19 target tissues, which may include the pancreas.[18,19]

Because of its role in intestinal barrier function, i.e. prevention of bacterial translocation and modulation of inflammation, we hypothesized that FXR might play an important role in AP. Deficiency of FXR could result in increased severity of the pancreatitis, increased bacterial translocation, and infectious complications. In this study, we therefore explored, with the aid of complementary animal and human experiments, whether FXR could affect AP.

Materials & Methods

Animals

In the first series of experiments, we used adult male wild-type C57BL/6 mice of 10-12 weeks and 20-30 grams of weight (Harlan, Horst, the Netherlands). For the second series of experiments, mice with global Fxr deficiency ($Fxr^{-/-}$) on a C57BL/6 genetic background[20] were obtained by breeding of heterozygous mice. We used male adult $Fxr^{-/-}$ and wild-type C57BL/6 littermates of 11-16 weeks and 25-35 grams of weight. All mice were kept under constant housing conditions (22°C, 60% relative humidity and a 12-hour light/dark cycle) for at least two weeks prior to the start of the experiment, and had free access to water and food (CRM (E), B.M.I. – Technilab, Someren, the Netherlands) throughout the experiment.

Animal experiments

AP was induced by ten intraperitoneal injections with an hourly interval of cerulein, a CCK analogue (Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands; 50 µg/kg in 0.9% NaCl). Controls received an equal volume of saline. In an initial experiment, pancreatic injury was assessed 24 and 72 hrs after induction of AP. For this purpose, 30 wild-type mice (Harlan) were randomly allocated to a control group (n=10, sacrificed after 72 hrs) and two experimental groups that were terminated after 24 hrs (early pancreatitis, n=10) and 72 hrs (late pancreatitis, n=10). To assess the impact of Fxr deficiency on AP, $Fxr^{+/+}$ (wild-type) and $Fxr^{-/-}$ mice received control (n=5) or cerulein (n=10 per genotype) treatment and were sacrificed after 24 hrs.

Animals were terminated by cervical dislocation or by cardiac puncture under isoflurane anesthesia. For histopathologic evaluation, parts of the ileum and pancreas were fixated in 4% formaldehyde. For RNA isolation, parts of the ileum and liver were immediately snap frozen in liquid nitrogen and stored at -80°C. Plasma samples were stored at -80°C for determination of amylase and bilirubin by standard clinical chemical assays.

The experimental design was approved by the animal experiments committee of Utrecht University, Utrecht, the Netherlands (2007.III.09.117; 2009.III.08.074).

Histopathology

After fixation in 4% formaldehyde, tissues were embedded in paraffin and cut in serial sections of 4 µm for hematoxylin and eosin (H&E) staining. Qualitative assessment of the severity of AP was performed in the initial experiment and, in the second experiment, a slightly modified semi-quantitative scoring system was used.[21,22] The following items were scored: edema (0-4 points), number of neutrophils in the edema (0-4 points), pancreatic ductal pathology (inflammatory cells; present=1; absent=0), intralobular inflammatory infiltrate (0-3 points) and peripheral necrosis of pancreatic tissue (0-4 points). The maximum composite score was 16. To assess the

ileal brush border in the second experiment, PAS-diastase staining was performed. Histopathological evaluation was performed by two experienced pathologists (AJJS, MEIS), blinded for experimental study groups.

Measurement of transepithelial electrical resistance

In the initial experiment, a 4 cm segment of the distal ileum was removed for electrical resistance measurements in Ussing chambers, as described elsewhere.[22] Briefly, flat sheets of mucosa were mounted in Ussing chambers with both sides of the epithelium in contact with Krebs-Ringer's solution, stirred and gassed with humidified carbogen at 37°C. Three ileal samples per animal were used. The transepithelial potential difference Vte (mV) was continuously monitored and transepithelial electrical resistance R ($\Omega.cm^2$) was calculated.[22] The reported values for the resistance were obtained at the end of the 20 min equilibration period. At the end of the experiment, viability of the tissue segments was confirmed based on carbachol-induced voltage increase.[22]

In vivo intestinal permeability assay

In the second experiment, intestinal permeability was assessed with fluorescein isothiocyanate (FITC)-conjugated dextran as previously described.[15] Briefly, two hours before termination, mice were gavaged with 0.6 mg/g body weight of FITC-conjugated dextran (MW 3,000-5,000 Da; Sigma-Aldrich). After termination, FITC fluorescence was measured in plasma with the aid of a fluorometer (BMG Polarstar Galaxy, MTX Lab Systems, Inc., Vienna, Virginia, USA) and compared to a calibration line of standard concentrations of FITC-conjugated dextran.

Analysis of gene expression

Total RNA was isolated from murine ileum and liver (RNeasy Midi Kit, Qiagen, Hilden, Germany). RNA integrity was tested by RNA gel electrophoresis. cDNA was synthesized from total RNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). Quantitative RT-PCR was performed using SYBR Green Supermix (BioRad) on an iCycler iQ system using diluted cDNA as template (primer sequences are provided in Supplementary Table 1). Expression levels were estimated using the comparative threshold cycle method. Cyclophilin was used as housekeeping gene, with similar expression levels in ileum and liver under all experimental conditions.

Determination of plasma FGF19 levels in patients with acute pancreatitis

FGF19 levels were determined by ELISA in plasma samples of 15 randomly selected patients with predicted severe AP.[23] Patients were participants in an earlier clinical trial (trial registry ISRCTN38327949) and were fed by continuous enteral nutrition.[24] Clinical data were available from the prospectively collected trial database.[24] As a control group, FGF19 levels were also determined in a group of 28 healthy volunteers

receiving an oral fat load.[25] In this group, fed FGF19 levels were calculated as the average of postprandrial FGF19 levels at 2, 3, 4, and 6 hrs.

Genetic association study

For the genetic association study, a previously described cohort of 387 patients with a first episode of AP was used.[26] All patients or their legal representatives gave their written informed consent, and the ethics review boards of all participating hospitals approved the study protocol. Genomic DNA was isolated from whole blood using a DNA isolation kit I (Magna Pure LC, Roche Diagnostics, Indianapolis, USA). Clinical data on the severity of disease and outcome of all patients were available from the prospectively collected trial database.[24] The controls consisted of 853 healthy, voluntary, Dutch blood donors.[27] All control genotypes were in Hardy-Weinberg equilibrium (data not shown, $p > 0.05$). Call rates for all SNPs were > 95%.

Nine tagging single nucleotide polymorphisms (SNPs) covering the complete FXR gene were selected using Haploview v4.2.[28] In addition, two functional SNPs affecting FXR expression (-1G/T, rs56163822) and FXR function (518T/C, rs61755050) were analyzed.[29] Details of the SNPs studied are given in Supplementary Table 2. Genotyping was performed using TaqMan assays on a TaqMan 7900 HT (Applied Biosystems, Foster City, California, USA). Haplotype analysis was performed in Haploview.[28]

Statistical analysis

Statistical analyses were performed using GraphPad PRISM software (Graphpad Software, La Jolla, CA, USA). Electrical resistance, histology scores, and clinical parameters were compared using one-way ANOVA with Tukey's post-hoc test or the non-parametric Kruskal-Wallis test with Dunn's post-hoc test where appropriate. Differences in gene expression levels were evaluated using the non-parametric Kruskal-Wallis test with Dunn's post-hoc test. Plasma FGF19 levels were compared between AP patients and healthy controls by ANOVA with Tukey's post-hoc test. Statistical analysis of the genetic association study was performed using two-tailed chi-squared for independence tests of case versus control allele and haplotype counts in Haploview v4.2.[28] Uncorrected P-values, odds ratios (OR) and 95% confidence intervals (95% CI) are given (Table 2 and 3). The Bonferroni method was used to correct for multiple testing. Data of continuous values are shown as mean ± standard deviation (SD). P-values below 0.05 were considered statistically significant.

Results

Acute pancreatitis results in decreased transepithelial resistance and altered expression of Fxr targets in the ileum

Pancreatic injury was initially assessed in wild-type mice sacrificed at 24 hrs (early AP) or 72 hrs (late AP) after induction of AP. Plasma amylase levels were twice as high in the early and late AP mice compared to the control group (mean ± SEM, 4521 ± 527 U/L vs. 2186 ± 109 U/L, $p < 0.001$). Histopathological examination of the pancreas revealed edema, influx of neutrophils and necrosis in all mice of the early pancreatitis group (Figure 1A). In contrast, the pancreata of all mice in the late pancreatitis group showed no signs of edema or necrosis and displayed infiltration of lymphocytes and fibroblasts rather than neutrophils. There were no histopathological abnormalities in the control group. Histopathological examination of the ileum revealed normal enterocytes without any signs of inflammatory infiltrate in all groups. Nevertheless, the transepithelial electrical resistance of the ileum was approximately half in the early AP group compared to controls and the late AP group (Figure 1B). This indicates that AP induces a transient increase in ileal permeability.

Impaired intestinal barrier function in patients with inflammatory bowel disease is accompanied by reduced ileal expression of FXR targets.[17] Ileal gene expression in mice was therefore analyzed to test the consequences of an AP-induced decline of transepithelial electrical resistance. *Fxr* mRNA expression was comparable between the three experimental groups, as was mRNA expression of the Fxr-target gene *Asbt* (Figure 1C). In contrast, mRNA expression of Fxr-target genes *Shp*, *Fgf15* and *Ibabp* was reduced in the early AP group compared to control mice (Figure 1C). In the late pancreatitis group, expression of all Fxr target genes was normalized (Figure 1C). *Fxr* and its target genes are exclusively expressed in the villous lining of differentiated enterocytes.[13] We therefore also assessed mRNA expression of *Villin*, which is expressed exclusively in these differentiated enterocytes.[17] mRNA expression of *Villin* showed no differences between the groups, including the early AP group (Figure 1C), indicating that no intestinal damage was present in this mouse model of AP. Regarding Fxr-dependent genes implicated in intestinal barrier function[13]: *Angiogenin-1* (*Ang1*) mRNA expression in the ileum was reduced in the early pancreatitis group, whereas *iNos*, *Car12* and *Il18* were similar in all groups (Table 1).

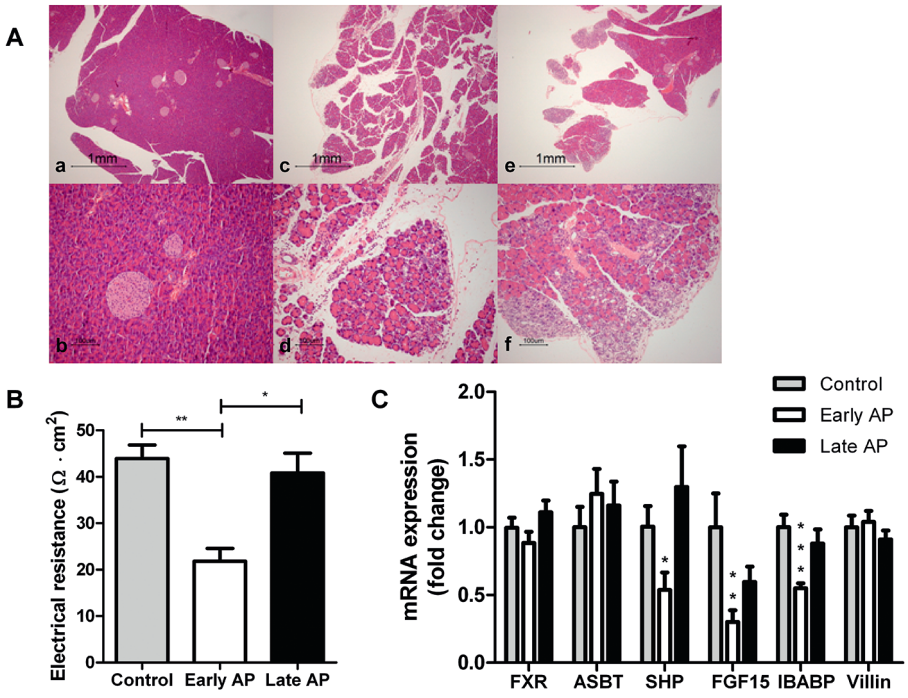

Figure 1. A – Representative pancreatic histology of wild-type mice from the control group (a, b) and mice with early (c, d) and late (e, f) acute pancreatitis (H&E staining, 20x and 100x magnifications consecutively). Control mice have normal pancreatic morphology, whereas mice of the early pancreatitis group exhibit edema, influx of neutrophils and necrosis. Mice of the late pancreatitis group have no edema or necrosis, but show influx of lymphocytes and fibroblasts. B – Transepithelial electrical resistance of the ileum measured by Ussing chamber experiments. The resistance of the ileum was lower in the early pancreatitis group in comparison to both controls and the late pancreatitis group. C – Ileal mRNA expression of *Fxr* and FXR target-genes *Asbt*, *Shp*, *Fgf15*, and *Ibabp*, and *Villin* in wild-type mice of the control group, and the early and late pancreatitis groups. Expression of *Fxr*, *Asbt* and *Villin* did not differ between experimental groups. Expression of the other Fxr target genes was lower in early acute pancreatitis, but not in late pancreatitis. Bars indicate means and SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In the liver, mRNA expression of *Fxr* and its target gene *Shp* were diminished after 24 hours, but normalized after 72 hours (Table 1). *Fgf15* could not be detected in the liver. Hepatic *iNos* expression was also lowered after 24 hours, whereas *Ang1* expression increased in the early phase and returned to baseline expression in the late phase (Table 1).

Table 1. Gene expression levels in mouse ileum and liver following induction of acute pancreatitis.

Organ	Gene	Control group	AP 24 hr	AP 72 hr
Ileum	iNos	1.00 ± 0.11	1.10 ± 0.16	1.14 ± 0.12
	Ang1	1.00 ± 0.06	0.74 ± 0.04**	0.94 ± 0.06
	Car12	1.00 ± 0.07	0.81 ± 0.04	1.16 ± 0.13
	IL18	1.00 ± 0.07	0.97 ± 0.10	1.68 ± 0.70
Liver	FXR	1.00 ± 0.04	0.75 ± 0.03***	1.06 ± 0.06
	SHP	1.00 ± 0.07	0.75 ± 0.07*	1.03 ± 0.16
	iNOS	1.00 ± 0.05	0.81 ± 0.03***	1.09 ± 0.05
	ANG1	1.00 ± 0.08	1.38 ± 0.05***	1.00 ± 0.07

Expression levels were normalized to cyclophilin expression, and values are given as means ± SEM for the experimental groups: control group (n = 10), AP 24 hr (n = 10), and AP 72 hr (n = 9). Both AP groups are compared to the control group; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Deficiency of Fxr does not lead to more severe acute pancreatitis in mice

The above findings indicate that ileal Fxr activity is disturbed in the early phase of murine AP (i.e. 24 hrs after induction). To test whether Fxr dysfunction contributes to the pathology of AP, mice deficient for Fxr were given ten hourly injections of cerulein to induce AP and sacrificed after 24 hrs. Weight loss due to pancreatitis induction did not differ between wild-type and $Fxr^{-/-}$ mice (mean ± SEM: 6.1 ± 0.37 and 5.2 ± 1.78 % of body weight, respectively, $p = 0.48$). In both wild-type and $Fxr^{-/-}$ pancreatitis groups, plasma amylase levels were significantly higher than in corresponding groups without AP (mean ± SEM; wild-type controls 2160 ± 149 U/L, wild-type AP 8013 ± 923 U/L, $p < 0.01$; $Fxr^{-/-}$ controls without AP 2285 ± 96 U/L, $Fxr^{-/-}$ AP 6801 ± 671 U/L, $p < 0.01$).

In order to identify whether cholestasis was present in these mice as a sign of post-hepatic bile duct obstruction by the inflamed pancreas, we determined plasma bilirubin levels. In wild-type and $Fxr^{-/-}$ mice, AP did not affect the plasma bilirubin levels (mean ± SEM; wild-type controls 1.8 ± 0.5 µmol/L, wild-type AP 1.5 ± 0.2 µmol/L; $Fxr^{-/-}$ without AP 8.0 ± 2.1 µmol/L, $Fxr^{-/-}$ with AP 5.8 ± 2.6 µmol/L). Fxr deficiency resulted in elevated bilirubin levels ($p < 0.05$). As a potential explanation for this phenomenon, we found elevated hepatic expression of the basolateral bilirubin glucuronide efflux pump Mrp1 in $Fxr^{-/-}$ mice (data not shown).[30]

We subsequently investigated whether Fxr deficiency affects epithelial permeability. Plasma levels of FITC-conjugated dextran were not increased by AP induction in either wild-type or $Fxr^{-/-}$ mice. Nevertheless, $Fxr^{-/-}$ mice had significantly higher plasma levels of FITC-conjugated dextran than wild-type mice (mean ± SEM: wild-type, 3.41 ± 0.61 µg/ml vs. $Fxr^{-/-}$, 7.45 ± 2.31 µg/ml, $p < 0.05$), indicating that loss of Fxr leads to increased intestinal permeability.

Upon histopathological examination, wild-type and Fxr$^{-/-}$ control mice did not exhibit edema, influx of inflammatory cells, or necrosis of the pancreas. In contrast, all mice in the pancreatitis groups showed clear signs of AP: interlobular and/or interacinar edema, influx of neutrophils, and necrosis (Figure 2A). Pancreatitis severity scores were similar in wild-type and Fxr$^{-/-}$ mice (composite pancreatitis severity score, mean ± SEM: 9.9 ± 0.8 vs. 11.3 ± 0.7, p=0.27; Figure 2B). When the individual components of the severity score (presence of edema, inflammatory infiltrate, and necrosis), were analyzed, there were also no differences found between wild-type and Fxr$^{-/-}$ mice (data not shown).

As expected, AP did not affect the expression of Fxr in the ileum of wild-type mice (results not shown). In contrast to the results depicted in Figure 1C, the effects of AP on expression of Fxr targets *Shp* and *Ibabp* in the ileum of wild-type mice did not reach significance in this experiment (results not shown). Nevertheless, a consistent decrease in expression of Fxr target *Fgf15* in the ileum was still noted following induction of AP (Figure 2C). Deficiency of Fxr resulted in reduced ileal expression of Fxr targets *Shp* and *Fgf15* in mock-treated mice, with AP having no additional suppressive effect (results not shown and Figure 2C). *Fxr* mRNA could still be detected, albeit at a lower level, in the ileum of Fxr$^{-/-}$ mice. The strategy used for disruption of the Fxr gene in these mice[20] results in a non-functional transcript as is evident from the near absence of ileal Ibabp expression in Fxr$^{-/-}$ mice (results not shown).

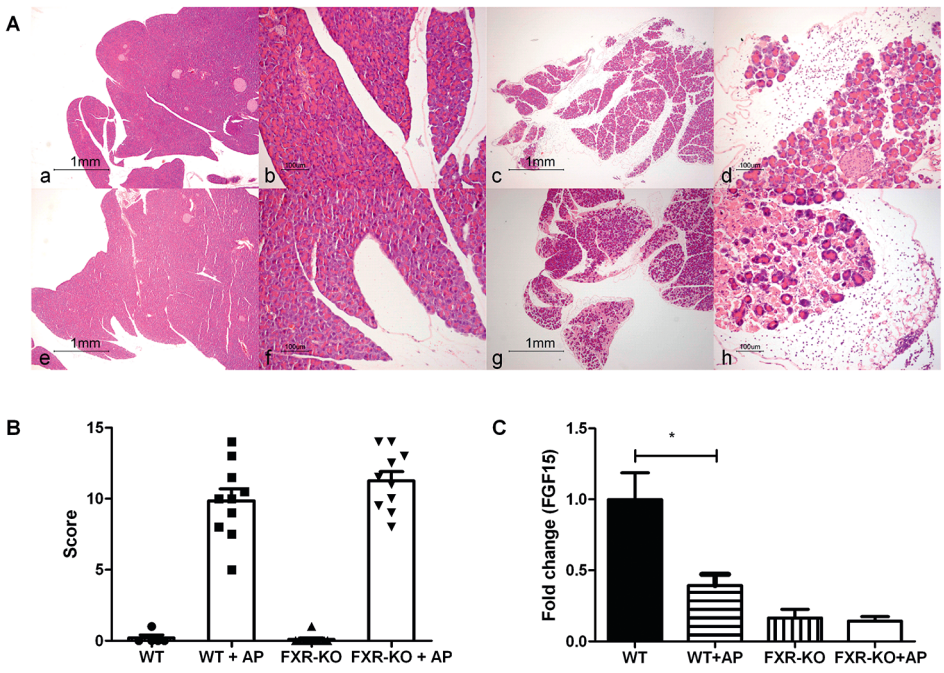

Figure 2. A - Representative pancreatic histology following induction of acute pancreatitis (H&E staining, 20x and 100x consecutive magnifications for each experimental group): wild-type control (a,b); wild-type acute pancreatitis (c,d); $Fxr^{-/-}$ control (e,f); $Fxr^{-/-}$ acute pancreatitis (g,h). B – Semi-quantitative composite pancreatitis severity score of histopatho¬logical examination of pancreas samples from wild-type and $Fxr^{-/-}$ mice with and without acute pancreatitis. Absence of *Fxr* does not result in more severe acute pancreatitis. C – Ileal mRNA expression of *Fgf15* in wild-type and $Fxr^{-/-}$ mice with and without early acute pancreatitis. *Fgf15* expression was decreased in wild-type mice with acute pancreatitis. Bars indicate mean and SEM.

Upon histopathological examination, there were no inflammatory infiltrates in the ileum of wild-type or $Fxr^{-/-}$ mice with and without AP and PAS-diastase staining showed intact brush borders (results not shown). There were no differences in mRNA expression of inflammatory genes *Car12* and *iNos* in the enterocyte (results not shown). As an additional marker of pro-inflammatory response, we determined Tnf-α mRNA expression, but found no differences in expression (results not shown). These findings indicate that there were no signs or very limited signs of inflammation on the molecular level in the ileum.

Plasma FGF19 levels are lowered in patients with acute pancreatitis

To obtain an impression of FXR activation in patients with AP, we studied plasma FGF19 levels in patients with predicted severe pancreatitis. Plasma FGF19 levels in AP patients under digestive conditions were significantly lower than in healthy volunteers after ingestion of a single bolus of fat (0.33 ± 0.19 vs. 0.62 ± 0.30 ng/mL, $p < 0.001$; Figure 3). This suggests that in patients with AP, FGF19 may be decreased in a similar way as to that observed in the AP mouse model.

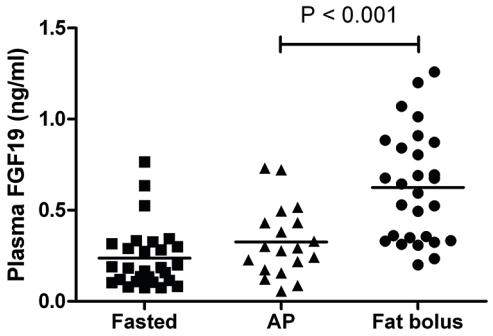

Figure 3. FGF19 plasma levels in patients with predicted severe acute pancreatitis during continuous enteral nutrition. For comparison, FGF19 plasma levels of healthy controls in the fasting state and after a single bolus of fat are also shown. The postprandial levels represent the average of plasma FGF19 levels at 2, 3, 4 and 6 hours after fat ingestion. There appears to be a blunted FGF19 release in the pancreatitis group. Bars indicate mean and SD.

Genetic polymorphisms in FXR are not associated with acute pancreatitis

To study a potential association between AP and *FXR*, 387 patients with AP and 853 controls were genotyped for 9 tagging and 2 functional SNPs in the *FXR* locus. An association with AP was seen for one of the variants (rs10860603, p = 0.0364, OR 1.30, 95% CI 1.02-1.65) (Table 2). This association did not, however, withstand correction for the number of tested SNPs (Pcorrected = 0.40). There was no association of haplotypes of *FXR* with AP (data not shown).

To investigate a potential association between *FXR* and the course and outcome of AP, we studied the prevalence of the genetic variants in patients with a severe course versus a mild course of AP, patients with infected pancreatic necrosis versus patients without it, and patients who died from the pancreatitis versus those who survived (Table 3). One of the tag SNPs seemed to be associated with a severe course of AP (rs10860603, p=0.0368, OR 1.61, 95% CI 1.00-2.60) and one with infection of pancreatic necrosis (rs11110395, p=0.0099, OR 2.55, 95% CI 1.28-5.07). Haplotypes containing the same risk allele also seemed to show association (data not shown). Another tag SNP showed a significant difference between patients who died and those who survived (rs11837065, p=0.0272, OR 2.09, 95% CI 1.07-4.06). After Bonferroni correction for multiple testing, however, there were no associations of SNPs or haplotypes in the *FXR* gene with course or outcome of AP. Finally, we compared patients with biliary AP to patients with AP of non-biliary origin. None of the SNPs was associated with a biliary cause of AP (Table 3).

Table 2. Association of genetic variants of FXR with acute pancreatitis

		Acute pancreatitis patients			**Controls**			**P value***	**OR**	**95% CI**
		Allele counts			Allele counts					
		Major	Minor	MAF	Major	Minor	MAF			
-1 G>T	C/A#	732	14	0.981	1588	36	0.978	0.5926	1.14	0.62-2.10
518 T>C	A/G	743	5	0.993	1616	6	0.996	0.3203	1.86	0.60-5.82
rs11837065	C/T	452	250	0.644	1014	592	0.631	0.5663	1.05	0.88-1.27
rs12313471	A/G	699	39	0.947	1548	76	0.953	0.5268	1.15	0.77-1.71
rs11110390	C/T	497	247	0.668	1070	544	0.663	0.8088	1.02	0.85-1.23
rs4764980	G/A	384	354	0.520	832	778	0.517	0.8728	1.01	0.85-1.21
rs11110395	G/T	692	44	0.940	1538	84	0.948	0.4272	1.18	0.81-1.71
rs17030285	C/G	610	102	0.857	1415	215	0.868	0.4599	1.11	0.86-1.42
rs11610264	T/C	536	200	0.728	1160	458	0.717	0.5703	1.06	0.87-1.28
rs10860603	G/A	616	122	0.835	1398	214	0.867	0.0364	1.30	1.02-1.65
rs35739	T/C	398	350	0.532	900	712	0.558	0.2334	1.11	0.93-1.32

OR = odds ratio; 95% CI = 95% confidence interval

Major allele/minor allele; MAF = major allele frequency

*Two-tailed p values were calculated by $\chi 2$ analysis of allele counts

Table 3. Association analysis of genetic variants in FXR with subgroups of acute pancreatitis patients

	Patients with severe vs. patients with mild AP	**Patients with infected pancreatic necrosis vs. patients without it**	**Patients who died vs. surviving patients**	**Patients with biliary vs. non-biliary acute pancreatitis**
	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
-1 G>T	1.63 (0.42-6.42)	1.18 (0.22-6.46)	2.62 (0.47-14.59)	1.47 (0.51-4.24)
518 T>C	2.28 (0.13-41.40)	1.01 (0.06-18.33)	0.33 (0.02-6.10)	1.54 (0.30-7.84)
rs11837065	1.35 (0.96-1.90)	1.18 (0.76-1.83)	2.09 (1.07-4.06)#	1.00 (0.73-1.37)
rs12313471	1.02 (0.50-2.11)	1.61 (0.53-4.92)	0.71 (0.19-2.65)	1.02 (0.54-1.94)
rs11110390	1.24 (0.88-1.73)	1.27 (0.83-1.94)	1.69 (0.88-3.24)	1.07 (0.79-1.45)
rs4764980	1.17 (0.85-1.62)	1.35 (0.89-2.04)	1.57 (0.81-3.06)	1.09 (0.82-1.46)
rs11110395	1.64 (0.87-3.07)	2.55 (1.28-5.07)*	1.20 (0.23-6.35)	1.41 (0.76-2.62)
rs17030285	1.35 (0.82-2.20)	1.01 (0.55-1.84)	1.20 (0.44-3.28)	1.17 (0.77-1.77)
rs11610264	1.03 (0.72-1.47)	1.22 (0.76-1.96)	0.99 (0.48-2.05)	1.00 (0.72-1.39)
rs10860603	1.61 (1.00-2.60)^	1.18 (0.66-2.10)	1.14 (0.45-2.88)	1.31 (0.88-1.94)
rs35739	1.16 (0.84-1.60)	1.11 (0.74-1.67)	1.20 (0.63-2.31)	1.18 (0.89-1.58)

OR = odds ratio; 95% CI = 95% confidence interval

^rs10860603, 88.1% vs. 81.7%, p = 0.0368; *rs11110395, 11.5% vs. 5.1%, p = 0.0099; #rs11837065, 52.8% vs. 34.7%, p = 0.0272.

Discussion

Because of the role of FXR in intestinal barrier function, namely prevention of bacterial translocation and modulation of inflammation, we hypothesized that FXR might play an important role in AP. Deficiency of FXR might lead to increased severity of pancreatitis, increased bacterial translocation and subsequent infectious complications. In this study, we therefore explored, with the aid of complementary animal and human experiments, whether FXR could affect AP. We observed that induction of AP by repeated administration of a supraphysiological dose of the CCK-analogue cerulein was accompanied by perturbed Fxr activity in the ileum. However, the ileal Fxr pathway appears to have no major pathogenic role in this model of AP, as indicated by similar pancreatic histopathology following induction of AP in mice with global deficiency of *Fxr* and in wild-type controls. Moreover, a case-control association study indicated that genetic variation in the *FXR* locus is not associated with the risk, etiology or outcome of AP in human subjects. The collective findings of our study indicate that FXR is not a major player in the pathogenesis of AP.

In an initial experiment in wild-type mice, we observed that expression of ileal Fxr target genes *Fgf15*, *Shp* and *Ibabp* (Figure 1C) was disturbed at 24 hrs after induction of AP, while expression levels recovered at the time point that histopathological damage of the pancreas had largely resolved (i.e. 72 hrs after AP induction). Of note, decline of Fxr activity was shown through decreased expression of Fxr target

genes, without change of *Fxr* expression. This phenomenon is in line with previous data obtained in patients with Crohn's disease, in animal colitis models, and *in vitro* and *ex vivo* models, where *FXR* expression itself was not significantly changed by pro-inflammatory cytokines. These findings indicate that the inhibition of FXR target gene expression is due to decreased FXR activity.[31] Decline in ileal FXR target gene expression is likely to be due to impaired delivery of its activating bile salt ligands. It is well known that under pro-inflammatory conditions such as AP, small intestinal motility is decreased, both under fasting and fed conditions, with decreased ileal bile salt delivery as a result.[32] Since AP did not affect bilirubin levels in our wild-type and $Fxr^{-/-}$ mice, post-hepatic obstruction by the inflamed pancreas is unlikely. The transient decline in ileal *Fgf15* expression likely accounted for de-repression of hepatic *Cyp7a1*, as higher expression of this bile salt synthetic gene was found after 24 hrs (data not shown). In our Ussing chamber experiments in wild-type mice, impaired ileal Fxr activation was accompanied by decreased transepithelial resistance at the early time point (Figure 1B), indicative for increased intestinal permeability, without ileal inflammation. In contrast, in our second series of experiments, plasma levels of FITC-conjugated dextran were not increased by AP induction in either wild-type or $Fxr^{-/-}$ mice. These findings indicate that transepithelial resistance measurements are a more sensitive marker for disturbed intestinal permeability than FITC-conjugated dextran. Nevertheless, $Fxr^{-/-}$ mice had significantly higher plasma levels of FITC-conjugated dextran than wild-type mice, which was in line with the increased intestinal permeability in $Fxr^{-/-}$ mice previously reported.[13]

Although transient Fxr dysfunction was apparent in the early phase of acute murine pancreatitis, this likely did not have a pathogenic contribution, as the severity of AP was similar in mice with genetic disruption of *Fxr* and controls (Figure 2A and B). The lack of effect upon loss-of-function may relate to decreased intestinal transport of activating bile salt ligands to Fxr in the ileum, as discussed above, and other Fxr expressing tissues of wild-type mice, and result in a phenotype resembling that of the true Fxr-deficient mouse. If this interpretation is correct, gain-of-function studies (e.g. Fxr agonism) may, in theory, be more suitable to address the role of Fxr in AP. Likewise, other models of AP that do not rely on overstimulation of the gallbladder and exocrine pancreas function could, in theory, be employed to further delineate a role of Fxr in AP. Nevertheless, our combined human and murine data strongly argue against a critical role of Fxr in human AP.

In line with our data in wild-type mice, our analysis of non-fasted serum FGF19 levels suggests that ileal FXR dysfunction could also occur in patients with AP. Being a bile salt-regulated enterokine, circulating FGF19 levels increase postprandially in healthy controls (Figure 3). FGF19 levels in enterally fed patients with AP, however, are close to values observed in fasted controls. A possible role of FXR in human AP was further addressed by studying genetic variation at the *FXR* locus in a cohort of 387 cases and 853 controls. None of the 11 SNPs tested (9 tagging and 2 functional

variants), nor the inferred haplotypes, were independently associated with AP. Likewise, none of the variants were independently associated with the risk or course of AP, nor in haplotypes. Thus, genetic variation in the *FXR* locus does not predispose to, or have a major impact on the course of AP in human subjects.

Of note, genetic variation in the *FXR* locus did not differ between subjects with a biliary (i.e. gallstone) or non-biliary (mainly alcohol) cause of AP. $Fxr^{-/-}$ mice on a lithogenic diet are highly susceptible to cholesterol gallstone formation due to altered biliary lipid composition[33], although data on the role of FXR in human cholesterol gallstone formation are extremely limited. In female, non-obese gallstone patients, decreased expression of *FXR* and its target genes *ASBT*, ileal lipid binding protein (ILBP) and OSTα-OSTβ (all involved in bile acid transport) has been described in the enterocyte.[34,35] These findings suggest an intestinal defect with decreased absorption and subsequently a diminished bile acid pool.[36] Data on *FXR* gene polymorphisms in biliary disease show conflicting results. In a Mexican population, the most commonly found *FXR* haplotype was associated with gallstone prevalence in males, whereas no association was found in German and Chilean populations.[37] Our data yielding an absence of association of *FXR* polymorphisms or haplotypes contribute to knowledge in the subgroup of patients with gallstone pancreatitis. This subgroup is noteworthy for the presence of small gallstones and biliary sludge.[38]

In conclusion, loss-of-function of Fxr did not affect the severity of pancreatitis in the relatively mild model of cerulein-induced AP (fast recovery, no infection, mild histopathological abnormalities). Moreover, our genetic study does not support a major role for variation in the FXR locus as a determinant of human AP.

Acknowledgments

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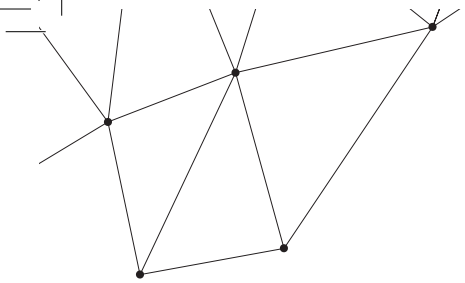
Supplementary Table 1. Primer sequences

Gene	Forward primer	Reverse primer
FXR	5'tgagaacccacagcatttcg3'	5'gcgtggtgatggttgaatgtc3'
SHP	5'cgatcctcttcaacccagatg3'	5'agggctccaagacttcacaca3'
FGF15	5'aaaacgaacgaaatttgttggaa3'	5`acgtccttgatggcaatcg3`
IBABP	5'ttgagagtgagaagaattacgatgagt3'	5'tttcaatcacgtctccctggaa3'
ASBT	5'tgactcgggaacgattgtg3'	5'ggaataacaagagcaaccagagaa3'
iNOS	5'caggaggagagagatccgattta3'	5'gcattagcatggaagcaaaga3'
ANG1	5'agcgaatggaagcccttaca3'	5'ctcatcgaagtggaccggca3'
IL18	5'ccgcctcaaaccttcca3'	5'catggcagccattgttcct3'
CAR12	5'ctcagacctgtaccctgacttca3'	5'gagcctatctcaataagaacagcaa3'
TNF-α	5'catcttctcaaaattcgagtgacaa3'	5'tgggagtagacaaggtacaaccc3'
Cyclophilin	5'ggagatggcacaggaggaa3'	5'gcccgtagtgcttcagctt3'

Supplementary Table 2. SNP information

	SNP number	Chromosomal location (12)
Tagging SNPs	rs11837065	100859983
	rs12313471	100864393
	rs11110390	100874901
	rs4764980	100885107
	rs11110395	100888664
	rs17030285	100930213
	rs11610264	100932375
	rs10860603	100943948
	rs35739	100948515
Functional SNPs	-1g>t, rs56163822	exon 3
	518t>c, rs61755050	exon 5



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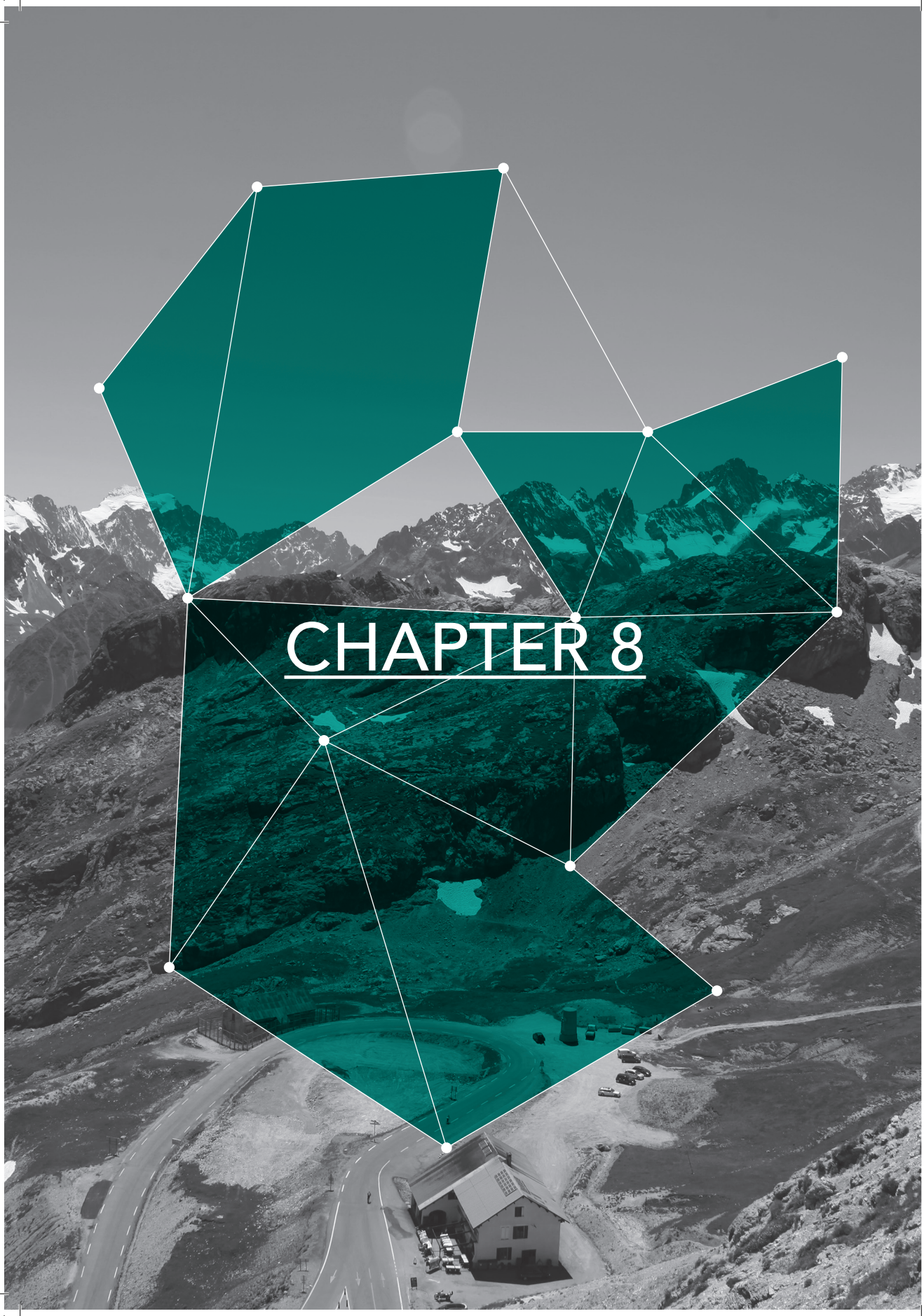
CHAPTER 8

Elevation of Fibroblast Growth Factor 21 (FGF21) in experimental and human acute pancreatitis

Rian M. Nijmeijer[1*], Andreas E. Kremer[2,3*], Stefan Holdenrieder[4], Hein G. Gooszen[5], Cisca Wijmenga[6], Peter L.M. Jansen[2,7,8], Karel J. van Erpecum[9], Frank G. Schaap[2,8]

*These authors share first authorship

Departments of [1]Surgery and [9]Gastroenterology and Hepatology, University Medical Center Utrecht, Utrecht, The Netherlands. [2]Tytgat Institute for Liver and Intestinal Research, and [8]Department of Gastroenterology and Hepatology, Academic Medical Center, Amsterdam, The Netherlands. [3]Department of Medicine 1, Friedrich-Alexander University of Erlangen-Nuremberg, Erlangen, Germany. [4]Institute of Clinical Chemistry, University Hospital Munich-Grosshadern, Munich, Germany. [5]Department of Operating Rooms, University Medical Center St. Radboud, Nijmegen, The Netherlands. [6]Department of Genetics, University Medical Centrum Groningen, University of Groningen, Groningen, The Netherlands. [8]Department of General Surgery, Maastricht University, Maastricht, The Netherlands.

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Abstract

Introduction: Fibroblast Growth Factor 21 (FGF21) is an endocrine factor that affects glucose and lipid homeostasis via effects on white adipose tissue and possibly other tissues. In mice, FGF21 reduces the severity of cerulein-induced acute pancreatitis (AP). It is unclear whether this protective action results from a direct effect on the pancreas or is related to general metabolic improvements that influence pancreatic recovery after an insult.

Aim of the Study: To gain insight into the role of FGF21 in human AP, we determined serum FGF21 levels in a total of 219 sera from AP patients at 0-9 days after onset of symptoms, and in 61 healthy controls. Additional study groups consisted of patients with elevated CRP or elevated pancreatic lipase of various etiology. In a case-control study, a total of 462 patients with AP and 1080 healthy controls were genotyped for four SNPs in the *FGF21* locus.

Results: Median FGF21 level in controls was 0.110 ng/mL (range 0.020-0.486). Median FGF21 level was significantly elevated at 3 (0.226 ng/mL, range 0.018-18.0, n=57, P<0.01) and 4 (0.290 ng/mL, range 0.020-1.28, n=37, P<0.01) days after onset of symptoms. Marked intra-individual variation in FGF21 levels was noted at most time points after onset of AP symptoms. FGF21 elevation was not specific for AP, and was also observed in patients with elevated CRP or elevated pancreatic lipase, irrespective of the underlying cause. Only one out of the four studied SNPs was detected in cases and controls. This variant (rs739320) did not show association with acute pancreatitis or severity, infectious complications and outcome of the disease.

Conclusion: Serum FGF21 levels are elevated during the course of AP, but this elevation is not specific for this disease. Further studies are required to shed light on the origin, stimuli and functionality of this elevation.

Introduction

Sudden inflammation of the pancreas (acute pancreatitis, AP) is a potentially fatal disorder most often caused by alcohol abuse or gallstones.[1,2] Pending the response to pancreatic cell injury, the course of the disease is mild (mainly inflammation of reversible nature) or severe (predominantly necrosis). Mild AP is characterized by inflammation and edema of the pancreas, and the disease is managed by conservative treatment (*e.g.* fasting and intravenous rehydration).[3] In approximately one fifth of the patients, the course of the disease is severe with organ failure and/or infectious complications requiring ICU admission and/or surgical intervention. Apart from alcohol and gallstones, obesity is recognized as an independent risk factor for the development, and predictor of the severity of AP.[1,2] Moreover, genetic susceptibility contributes to this complex inflammatory disorder.[4]

Fibroblast Growth Factor 21 (FGF21) is a member of the FGF19 subfamily of FGFs that are characterized by an endocrine mode of action.[5] Although the liver is held responsible for the bulk of FGF21 in the circulation, FGF21 is expressed in several other tissues including white and brown adipose tissue, skeletal muscle and the pancreas. FGF21 has favorable metabolic actions, and is currently undergoing clinical evaluation for improvement of glucose control and dyslipidemia in obese subjects with type 2 diabetes.[6] The direct action of FGF21 is restricted to cell types expressing the *c* isoform of FGF Receptor 1-3 (in particular FGFR1c) and the obligate signaling cofactor βKlotho.[7,8] White adipose tissue represents a key target tissue of FGF21, where it augments glucose uptake and governs lipolysis.[7,5]

Mice with transgenic overexpression of *FGF21* show reduced severity of cerulein-induced AP, a model of mild edematous AP.[9,10] Conversely, genetic deficiency of *Fgf21* resulted in a more severe disease phenotype. It is currently unresolved if the protective action of Fgf21 results from a direct effect on the pancreas, or is related to general metabolic improvements that influence pancreatic recovery after an insult. Both pancreatic acinar and β cells have been reported to respond directly to FGF21.[9,11] FGF21 signaling in acinar cells has been associated with reduced expression of proinflammatory and profibrotic genes.[9] In β cells, FGF21 treatment is related with improved β cell function[11] and enhanced engraftment of transplanted islets.[12] Apart from a direct mode of action, FGF21 may exert indirect effects on pancreatic function via its metabolic target tissues. For instance, FGF21 reduces lipolysis in white adipose tissue and accordingly reduces circulating levels of potentially lipotoxic free fatty acids that can give rise to β cell dysfunction.[13] Genetic loss of *Fgf21* gave rise to ectopic accumulation of triglycerides in the pancreas, likely through de-repression of lipolysis in adipose stores, and this may exacerbate pancreatic injury caused by cerulein administration.[9]

Although the molecular mechanisms are poorly defined, gain- and loss-of-function studies in mice clearly depict a role of FGF21 in experimental AP. The role of FGF21

in human AP has not been explored yet. As an initial step to address a possible contribution to the human pathology, we determined serum FGF21 levels during the course of AP, and studied genetic variation at the FGF21 locus in a cohort of patients with AP.

Materials and Methods

Measurement of FGF21

FGF21 levels were determined by a sandwich ELISA specific for human FGF21 as detailed elsewhere.[14] Serum FGF21 levels were determined in the following study groups. (**A**) Patients with a first episode of predicted severe acute pancreatitis (APACHE II score ≥8, n=219) from a previously published[15] and unpublished cohort. Availability of an adequate volume of serum for FGF21 analysis was the sole selection criterion. Blood samples were originally taken at admission to the hospital and this ranged from 0-9 days after onset of symptoms. (**B**) Surplus sera of 85 consecutive patients with elevated serum CRP levels (*i.e.* CRP >0.5 mg/dL) upon biochemical evaluation at the Department of Clinical Chemistry (University Hospital Munich-Grosshadern). (**C**) Surplus sera of 180 consecutive patients with elevated pancreatic lipase (i.e. >60 U/L) upon biochemical evaluation at the Department of Clinical Chemistry (University Hospital Munich-Grosshadern). Patients in groups B and C had diverse underlying disease. (**D**) A series of healthy adult volunteers of either gender (n=61).

Genetic association study

A total of 462 patients with AP and 1080 healthy controls were genotyped for four SNPs in the *FGF21* locus. Cases consisted of a previously described cohort of patients (n=346)[16] and an additional cohort of 116 patients with a first episode of AP. Clinical data on severity of disease and outcome were available from the prospectively collected trial database of the former cohort.[15] The controls consisted of healthy Dutch blood donors (39% females) with a median age of 52 (range: 19-70).[17] All subjects or their legal representatives gave their written informed consent.

Genomic DNA was isolated from whole blood using DNA isolation kit I (Magna Pure LC, Roche Diagnostics, Indianapolis, USA). A total of four FGF21 SNPs were studied including three variants (rs739320, rs36123953, rs3745708) that resulted in single nonsynonymous substitutions and were predicted to alter FGF21 function, and one promoter variant (rs2231858) that was located in close proximity (4 nt distance from the core motif) to an element involved in regulation of FGF21 by intracellular stress.[18]

Genotyping of rs36123953 and rs2231858 was performed using TaqMan assays on a TaqMan 7900 HT (Applied Biosystems, Foster City, California, USA).

Genotyping of rs739320 and rs3745708 was performed by Sanger sequencing. For this purpose, DNA amplification was performed using 25 ng of genomic DNA in 10 µl PCR reactions containing mastermix (AmpliTaq® Gold, Life Technologies) and forward (5'ACAAGTCCCCACACCGGGACC3') and reverse (5'AGCGTAGCTGGGGCTTCGGC3') primers. After purification of the amplicon (ExoSAP-IT, Affymetrix, Inc.), direct sequencing was performed using the *reverse* primer on a 3730 DNA Analyzer (Applied Biosystems). Sequences were aligned and compared to the *FGF21* consensus sequence (Genbank NM_019113) using the ContigExpress® software from the Vector NTI Suite 9 package.

Statistical analysis

Non-parametric ANOVA with Dunn's multiple comparison *post-hoc* testing was employed to evaluate differences between controls and patient groups, or between controls and patients with AP at the various time points after onset of symptoms. Statistical analysis of the genetic association study was performed using two-tailed chi squared for independence tests of case vs. control allele counts in Haploview v3.32.[19]

Results

FGF21 levels in patients with acute pancreatitis

Serum FGF21 levels were determined in healthy controls (n=61) and in patients with AP (n=219) at several days after onset of symptoms (Figure 1). Median FGF21 level in controls was 0.110 ng/mL (range 0.020-0.486). Median FGF21 level was significantly elevated at 3 (0.226 ng/mL, range 0.018-18.0, n=57, P<0.01) and 4 (0.290 ng/mL, range 0.020-1.28, n=37, P<0.01) days after onset of symptoms. Median FGF21 levels were highest at 3-4 days after onset of symptoms before gradually leveling off. Note that limited sample size precluded statistical interference at 0, 8 and 9 days after onset of symptoms. Marked elevation of serum FGF21 was noted in individual patients at most time points after onset of symptoms, with 18.0 ng/mL being the highest level observed in a patient. However, FGF21 elevation was not a general phenomenon in AP patients, with a substantial number of patients having (sub)normal levels. Patients with severe AP (30.3% of cases) had higher FGF21 levels (median 0.26 ng/mL, range 0.020-18.0, P=0.015) than patients with a mild disease course (median 0.16 ng/mL, range 0.018-9.34). Patients having inflammatory necrosis (15.6% of cases) displayed higher FGF21 levels (median 0.53 ng/mL, range 0.026-9.66; P=0.036) relative to patients without such complications (median 0.16 ng/mL, range 0.018-18.0). Patients succumbing to AP (9.0% of cases) had higher FGF21 levels (median 0.68 ng/mL, range 0.020-18.0, P=0.034) than surviving patients (median 0.17 ng/mL, range 0.018-16.0).

It cannot be ruled out that proteases released from the damaged pancreas may have resulted in (partial) degradation of FGF21 in these historical samples. However, analysis of fresh serum samples of patients suspected to have AP, revealed that sample storage for up to 2 weeks at room temperature had no effect on absolute FGF21 level (data not shown). Thus, elevated FGF21 levels are apparent during the course of AP.

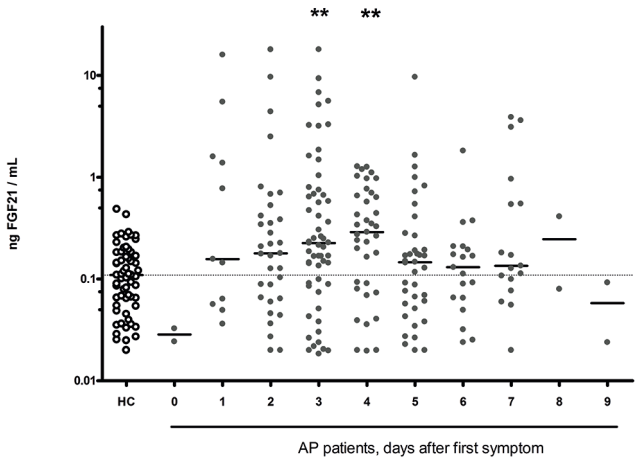

Figure 1. Serum FGF21 is elevated during the course of acute pancreatitis. FGF21 levels were determined in sera of healthy controls (HC, n=61), and in patients with acute pancreatitis (n=219) at the indicated time points after onset of symptoms. Median values are represented by solid bars (AP patients) or a dotted line (HC). Note the drastic elevation of FGF21 in some patients. **denotes statistical significance (P<0.01) between controls and AP patients at the specified time point after onset of symptoms.

FGF21 levels in subjects with elevated CRP or pancreatic lipase

To assess whether the observed elevation of FGF21 is specific for acute pancreatitis or whether it is a more general inflammation-related phenomenon, serum FGF21 levels were evaluated in a group of patients with elevated CRP (median 15.3 mg/dL, range 0.6-37.6), irrespective of the underlying cause (Figure 2). A notable elevation of serum FGF21 was also apparent in this study group (median 0.610 ng/mL, range 0.022-36.2, n=84, P<0.001), with the maximum level (36.2 ng/mL) observed in a patient with necrotising pancreatitis. Patients with notable FGF21 elevation could not be simply categorized according to diagnoses or intervention type (data not shown). Serum FGF21 showed a modest positive correlation with serum CRP levels (Spearman's Rho +0.35, P=0.001).

To further explore the specificity of elevated FGF21 in AP, serum FGF21 was determined in a group of patients with elevated pancreatic lipase (median 223 U/L, range 62-1591), irrespective of the underlying cause (Figure 2). Median CRP level in this patient group was 4.5 mg/dL (range 0.1-25.0). Also in this study group, serum FGF21 were markedly elevated (median 0.544 ng/mL, range 0.017-35.7,

P<0.001) and patients with marked FGF21 elevation did not cluster into discrete categories (data not shown). No correlation was found between serum FGF21 and pancreatic lipase. A modest association between serum FGF21 and CRP was apparent (Spearman's Rho +0.23, P=0.003).

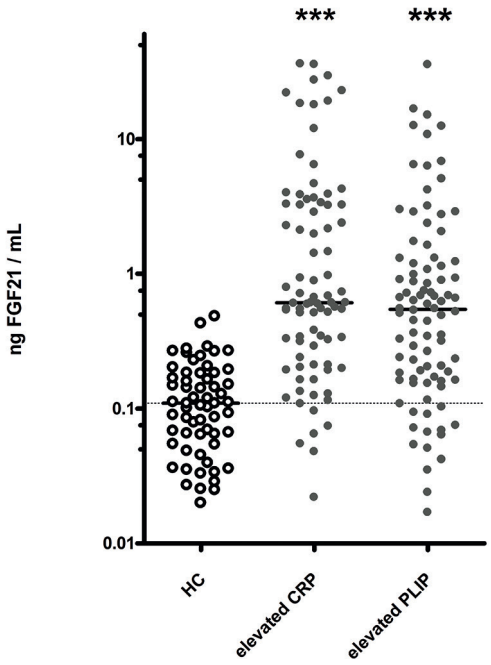

Figure 2. Increased serum FGF21 in patients with elevated CRP or Pancreatic Lipase. FGF21 levels were determined in sera of healthy controls (HC, n=61), in patients with elevated CRP (n=84), and in patients with elevated pancreatic lipase (PLIP, n=180). Median values are represented by solid bars (patients) or a dotted line (HC). ***denotes statistical significance (P<0.001) between controls and patients.

Genetic association study – selection of SNPs

Apart from genome-wide association studies, only a single dedicated study deals with the role of genetic variation in the *FGF21* locus in human pathology, specifically metabolic disease.[20] Hence, little information is available on functionality and allele frequency of the over 200 SNPs at the *FGF21* gene locus that have been deposited in public databases. For this study we selected four SNPs that were predicted to be functional. For this purpose, the SNP database[21] was searched for variants that resulted in substitutions that altered the amino acid sequence of *FGF21* (Figure 3). Two methods were employed to predict the effect of these nonsynonymous changes.[22,23] Three SNPs predicted to be functional by at least one of these methods were selected for further consideration. A fourth SNP was evaluated as it was in close proximity (separated by 4 nt from the core of the element) to a promoter element that

is essential for stress-induced FGF21 expression and may interfere with this regulatory mechanism.[18]

Variant	SNP id	PolyPhen prediction	SIFT prediction	MAF
L174P	**rs739320**	Probably damaging	Tolerated	0.29
G141S	rs41308776	Benign	Tolerated	0.01
R47Q	**rs36123953**	Possibly damaging	Affects protein function	0.0.26
A109T	rs3745712	Benign	Tolerated	Unknown
A109D	rs3745711	Benign	Tolerated	Unknown
A173P	rs885662	Benign	Tolerated	0.01
P178L	**rs3745708**	Probably damaging	Affects protein function	Unknown
Promoter	**rs2231858**	Probably involved in induction of ER stress		Unknown

Figure 3. Single nucleotide FGF21 polymorphisms included in the genetic association study. Selected SNPs are indicated in bold, and were chosen based on predicted (PolyPhen, SIFT) functionality. Pending availability at the time of SNP database access, the minor allele frequency (MAF) is shown.

Genetic variation in FGF21 in acute pancreatitis

A case-control study was conducted to evaluate the role of genetic variation at the *FGF21* locus in human AP, with a total of 462 patients with AP and 1080 healthy controls being genotyped. Only one out of the four studied SNPs was detected in cases and controls. This variant (rs739320) did not show association with acute pancreatitis (minor allele frequency 0.355 in patients, 0.371 in controls, P>0.05) or severity, infectious complications and outcome of the disease (data not shown). The control genotypes for rs739320 were in Hardy-Weinberg equilibrium (data not shown, P>0.05). The minor alleles of the other three SNPs were not detected in any of the cases or controls, and this is likely due to the low frequency of the minor alleles in the European population. For both rs36123953 and rs2231858, the minor allele frequency in Europeans is less than 0.1%.[21] There is no frequency data available for SNP rs3745708.

Discussion

Observations in animal models of experimental acute pancreatitis led us to question whether FGF21 has a pancreatoprotective role in humans as well. As a first step towards addressing this issue, we studied serum FGF21 levels and genetic variation in the *FGF21* gene in patients with AP. The main finding of this study is that serum FGF21 is elevated in AP, however, the elevation is not specific for AP and rather appears to reflect a general inflammatory process.

Serum FGF21 levels are elevated during the course of AP, specifically at days 3 and 4 after onset of symptoms (Figure 1). At these, as well as on the other studied time points, there is a huge inter-individual variation in FGF21 levels. It is currently unclear if this variation is related to the disease process. What could be the origin of elevated FGF21 in AP? In mice, *Fgf21* is highly expressed in the pancreas[8] and transiently induced in the early phase of mild edematous AP.[9] However, it has not been reported if this momentarily upregulation of pancreatic expression resulted in changes in serum Fgf21 levels, nor has it been studied whether expression of *Fgf21* in other tissues was affected by induction of AP. In a rat model of AP that consisted of bile salt infusion in the pancreatic duct followed by secretagogue treatment, we observed elevation of serum Fgf21 from 24 hrs after initiation of AP onward (Nijmeijer and Schaap, unpublished observations). Thus, FGF21 elevation is observed during the course of both human and experimental AP. Like in humans, increased circulating Fgf21 is not only observed in experimental AP but also in other conditions accompanied by inflammation.[24] Unfortunately, tissues from abovementioned rat study are no longer available to address the source(s) of elevated Fgf21 experimentally. The pancreas in man is not amenable to be biopsied in the phase of acute inflammation, animal experiments are thus required to shed light on the tissues contributing to elevated serum Fgf21 in AP. Although the pancreas may be the origin of elevated serum FGF21, preliminary immunohistochemical analysis indicates a similar pattern (ductular positivity) and intensity of staining of archival sections of inflamed and non-inflamed human pancreas (Komuta and Schaap, unpublished observations).

In addition to its origin(s), the stimuli that results in elevation of serum FGF21 in AP are unknown. It is conceivable that inflammatory stimuli are involved in this process. In mice, such triggers were shown to elevate circulating Fgf21 levels through divergent effects on Fgf21 gene expression in the liver (repression) and white adipose tissue and skeletal muscle (induction).[24] In line with an inflammatory trigger, elevation of serum FGF21 was also observed in patients with elevated CRP caused by various underlying disease, or in patients having elevated pancreatic lipase unrelated to AP (Figure 2). Moreover, serum FGF21 and CRP levels were positively correlated. Regulation of FGF21 gene expression is complex and (patho)physiological (e.g. starvation/fatty acids, cold exposure, intracellular stress) stimuli have been identified that modulate its expression.[25,26,18] It remains to be determined by which molecular mechanisms, both direct and via cross-talk with other regulatory pathways, inflammation affects serum FGF21 levels. Such insight may also shed light on the highly variable outcome of inflammation in terms of FGF21 level, which shows a high interindividual variation.

The elevation of serum Fgf21 by inflammatory stimuli protected *ob/ob* mice from sepsis-related toxicity.[24] It will be interesting to learn if elevation of FGF21 in AP has a protective effect. If this proves to be the case, genetic variation in FGF21 may influence susceptibility to, or affect the course of AP. However, our case-control genetic study does not provide support for such notion. Only one out of four minor

variants of the studied FGF21 SNPs could be detected in controls and patients AP, and this variant (rs739320) was not associated with AP or the clinical course of the disease.

In summary, our study revealed that serum FGF21 is elevated in patients with AP, but such increase is not restricted to this disease. Further studies are required to shed light on the origin, stimuli and functionality of this elevation.

Acknowledgements

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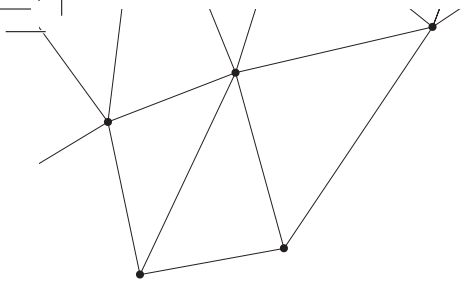

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CHAPTER 9

Probiotics prevent intestinal barrier dysfunction in acute pancreatitis in rats via induction of ileal mucosal glutathione biosynthesis

Femke Lutgendorff[1,2], Rian M. Nijmeijer[2], Per A. Sandstrom[1], Lena M. Trulsson[1], Karl-Eric Magnusson[3], Harro M. Timmerman[2], L. Paul van Minnen[2], Ger T. Rijkers[2], Hein G. Gooszen[2], Louis M. A. Akkermans[2], Johan D. Soderholm[1]

[1]Department of Clinical and Experimental Medicine, Division of Surgery, Linkoping University, Linkoping, Sweden; [2]Gastrointestinal Research Unit, Department of Surgery, University Medical Center, Utrecht, the Netherlands; [3]Department of Clinical and Experimental Medicine, Division of Medical Microbiology, Linkoping University, Linkoping, Sweden.

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Abstract

Background: During acute pancreatitis (AP), oxidative stress contributes to intestinal barrier failure. We studied actions of multispecies probiotics on barrier dysfunction and oxidative stress in experimental AP.

Methodology/Principal Findings: Fifty-three male Spraque-Dawley rats were randomly allocated into five groups: 1) controls, non-operated, 2) sham-operated, 3) AP, 4) AP and probiotics and 5) AP and placebo. AP was induced by intraductal glycodeoxycholate infusion and intravenous cerulein (6 h). Daily probiotics or placebo were administered intragastrically, starting five days prior to AP. After cerulein infusion, ileal mucosa was collected for measurements of *E. coli* K12 and ^{51}Cr-EDTA passage in Ussing chambers. Tight junction proteins were investigated by confocal immunofluorescence imaging. Ileal mucosal apoptosis, lipid peroxidation, and glutathione levels were determined and glutamate-cysteine-ligase activity and expression were quantified. AP-induced barrier dysfunction was characterized by epithelial cell apoptosis and alterations of tight junction proteins (i.e. disruption of occludin and claudin-1 and up-regulation of claudin-2) and correlated with lipid peroxidation ($r>0.8$). Probiotic pre-treatment diminished the AP-induced increase in *E. coli* passage (probiotics 57.4 ±33.5 vs. placebo 223.7 ± 93.7 a.u.; $P<0.001$), ^{51}Cr-EDTA flux (16.7±10.1 vs. 32.1±10.0 cm/s10^{-6}; $P<0.005$), apoptosis, lipid peroxidation (0.42±0.13 vs. 1.62±0.53 pmol MDA/mg protein; $P<0.001$), and prevented tight junction protein disruption. AP-induced decline in glutathione was not only prevented (14.33±1.47 vs. 8.82±1.30 nmol/mg protein, $P<0.001$), but probiotics even increased mucosal glutathione compared with sham rats (14.33±1.47 vs. 10.70±1.74 nmol/mg protein, $P<0.001$). Glutamate-cysteine-ligase activity, which is rate-limiting in glutathione biosynthesis, was enhanced in probiotic pre-treated animals (probiotics 2.88±1.21 vs. placebo 1.94±0.55 nmol/min/mg protein; $P<0.05$) coinciding with an increase in mRNA expression of glutamate-cysteine-ligase catalytic (GCLc) and modifier (GCLm) subunits.

Conclusions: Probiotic pre-treatment diminished AP-induced intestinal barrier dysfunction and prevented oxidative stress via mechanisms mainly involving mucosal glutathione biosynthesis.

Introduction

Multi-organ-failure and systemic inflammatory response syndrome (SIRS) remain major causes of mortality at intensive care units.[1] There is compelling evidence for an important role of the gut in the origin and development of critical illness.[2,3] Gut barrier dysfunction can propagate a pathophysiological state leading to increased mortality. Deitch *et al.*[4] demonstrated for example, that shock-induced intestinal hypoperfusion leads to release of reactive oxygen species (ROS) and oxidative stress resulting in barrier failure and release of pro-inflammatory mediators, enhancing a subsequent SIRS. Evidence suggests that ROS disrupt epithelial tight junctions (TJs)[5,6] leading to barrier dysfunction.[7] Furthermore, ROS cause epithelial cell apoptosis[8] contributing to mucosal barrier failure[9–11] and associated mortality[12,13] in experimental studies Moreover, clinical evidence shows that increased intestinal apoptosis is a prominent event in patients who succumb from sepsis.[14] The mucosal barrier may be further compromised by overgrowth of enteric pathogens e.g. *Escherichia coli*[15] or by other opportunistic pathogens which switch on their virulence genes upon intestinal hypoxia[16], suggesting an important role for intestinal microbiota in gut-derived sepsis.[2] Taken together in critically ill patients, SIRS may be driven by an oxidative stress-induced disruption of the equilibrium of the otherwise symbiotic three-way partnership between intestinal microbiota, epithelium, and immune system.

Conversely, a moderate increase in intracellular ROS concentrations may paradoxically afford protection against oxidative stress via upregulation of oxidative defense mechanisms. Indeed, *de novo* synthesis of the most important endogenous antioxidant, glutathione (GSH) is found to be enhanced after low dose H^2O^2[17] and is also increased by other weak oxidative agents.[18]

Severe acute pancreatitis (AP), which is characterized by intestinal barrier dysfunction and not seldom leading to SIRS, represents aclinical disease in which maintenance of this equilibrium is severely disturbed.[3] Since commensal bacteria are believed to be a crucial part of host homeostasis, recent studies have looked at effects of probiotics in recreating equilibrium.[19–21] Our group previously developed a probiotic combination designed to prevent infectious complications in critical illness based on anti-inflammatory and microbiota modulating capacities.[22] Five-day pre-treatment with these multispecies probiotics attenuated bacterial translocation and reduced the mortality in experimental AP in rats[23], but recently we also demonstrated in a double-blind clinical study that these probiotics, contrary to any expectations, doubled the mortality compared with placebo in 298 patients with predicted severe AP.[24] These results painfully showed the need to study mechanisms of action of probiotics in critical illnesses. The objective of this study was to characterize the intestinal mucosal barrier in experimental AP and to explore mechanisms by which multispecies probiotics affect barrier function under these circumstances. We found that probiotics maintained the mucosal barrier in AP by up-regulation of the rate-limiting step in

glutathione (GSH) biosynthesis.

Materials and Methods

Rats

Male specific pathogen-free Sprague-Dawley rats (B&K, Sollentuna, Sweden, 250–350 g, 50–70 days of age) were maintained under constant conditions with a 12-hour light/dark cycle and free access to water and standard rat pellets. Rats were acclimatized for one week prior to surgery and randomly allocated into five groups: 1) non-operated controls (n= 5); 2) sham-procedure (n= 12); 3) AP (n= 12); 4) AP, placebo (n= 12); 5) AP, probiotics (n= 12). The experimental design (fig 1) was in accordance with guidelines of the Linko¨ping University Animal Welfare Committee, following European legislation (2003/65/EC).

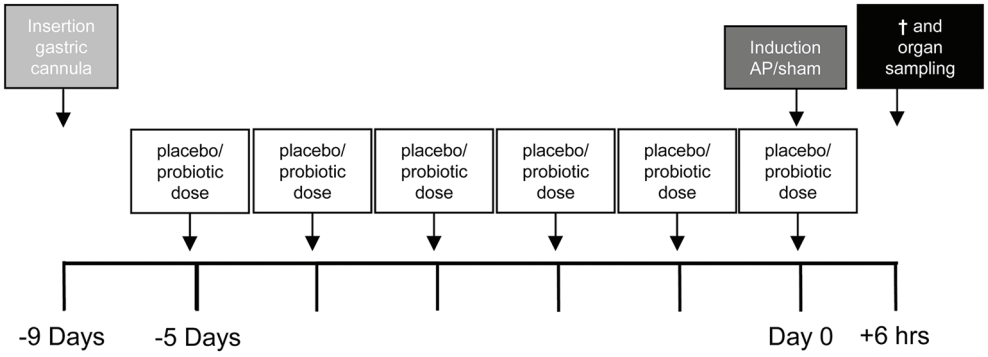


Figure 1. Experimental design. At the start of the experiment, animals were fitted with a gastric cannula, except for control animals. Probiotics and placebo were administered daily to the probiotics and placebo group, starting 5 days prior to induction of acute pancreatitis (AP). At day 0, AP or sham-procedure was performed. After the six hours of cerulein infusion, animals were anesthetized for removal of organ samples.

Probiotics

The multispecies probiotics consisted of six viable, freeze-dried strains: *Lactobacillus acidophilus* (W70), *Lactobacillus casei* (W56), *Lactobacillus salivarius* (W24), *Lactococcus lactis* (W58), *Bifidobacterium bifidum* (W23), and *Bifidobacterium lactis* (W52) (previously classified as *Bifidobacterium infantis*) (Ecologic® 641, Winclove Bio Industries, Amsterdam, the Netherlands). Placebo, that consisted of the carrier of the probiotic product, i.e. cornstarch and maltodextran, was packed in identical coded sachets to guarantee blinding. Before daily administration, probiotic or placebo formulas were reconstituted in sterile water, for 15 min at 37°C. A single probiotic dose of 1.0 ml contained $5x10^9$ colony forming units (CFU) of bacteria.

Surgical procedures

Under general anesthesia (2% isoflurane), a permanent gastric cannula was fitted in all rats, as performed previously.[23] Animals were allowed to recover for four days prior to the start of daily probiotics or placebo administrations through the cannula and then after five days of pre-treatment AP was induced as described by Schmidt *et al*.[25] Briefly, pressure controlled (35<mmHg) retrograde infusion of 0.5 ml sterilized glycodeoxycholic acid (Sigma-Aldrich, Zwijndrecht, the Netherlands) into the biliopancreatic duct was followed by intravenous cerulein (5 µg/kg/h, 1 ml/h, for 6 h, Sigma-Aldrich). During the sham procedure, cannulation of the biliopancreatic duct without infusion of glycodeoxycholic acid was followed by intravenous saline (1 ml/h, 6 h). Three animals needed to be excluded due to detachment of the gastric cannula and two due to anaesthesiological failures.

Collection of samples

Whole blood was sampled by tail vein puncture, before start of treatment and before induction of acute pancreatitis. After cerulein or saline infusion, rats were anaesthetized and 15 cm distal ileum, the pancreatic tail and whole blood were collected. Ten cm of ileum was used for Ussing chamber experiments and immediately submersed into ice-cold oxygenated Kreb's buffer (115 mM NaCl, 1.25 mM $CaCl^2$, 1.2 mM $MgCl^2$, 2mM KH^2PO^4, and 25 mM $NaHCO_3$, pH 7.35). The remainder was flushed with cold Kreb's buffer to remove adherent bacteria, stripped of the external muscle, freeze-dried and stored at -70°C until analyzed. Histological assessment verified that no bacteria remained associated with the tissue samples. Samples for histological and immunohistochemical examinations were formalin fixed, and embedded in optimum cutting temperature compound (Histolab, Vastra Frolunda, Sweden). All analyses were run in duplicates.

Ussing chamber experiments

Mucosal permeability was measured as previously described.[26] Briefly, ileum, stripped of external muscle while immersed in Kreb's buffer, was mounted into Ussing chambers (Harvard Apparatus Inc., Holliston, MA, USA[27]) where 9.6 mm^2 tissue was exposed to 3 ml (1.5 ml each half-chamber) circulating, oxygenated Kreb's solution at 37°C. The serosal buffer contained 10 mM glucose as energy source and was osmotically balanced by 10 mM mannitol in the mucosal buffer. Chambers contained agar-salt bridges to monitor potential difference across the tissue for vitality assessment. Baseline values for short circuit current (Isc), indicating net ion secretion, and conductance (passive ion flux), were recorded at equilibrium, 40 min after mounting.

Transepithelial transport of macromolecules was assessed by measuring horseradish peroxidase (HRP) (Sigma-Aldrich), as model antigen, and ^{51}Cr-EDTA (Perkin-Elmer, Boston, MA, USA) flux, as paracellular probe. HRP and ^{51}Cr-EDTA were

added to the mucosal side to a final concentration of 10^{-5} M and 34 mC^{i}/ml, respectively. Serosal samples (300 µl) were collected at 0, 30, 60, 90 and 120 min after start and were used to analyze transepithelial fluxes of ^{51}Cr-EDTA, expressed as cm/s•10^{-6}, using a gamma-counter (1282 Compugamma, LKB, Bromma, Sweden). HRP-activity was determined as previously described[26], and transepithelial HRP flux was expressed as pmol•cm^{-2}•h^{-1}. Permeability was calculated in 3 ileal samples per rat during the 30–120 min period for both markers.

To assess bacterial passage, fluorescent *E.coli* K12 (1x10^{8} CFU/ml, Molecular Probes, Leiden, the Netherlands), killed by paraformaldehyde to stop reproduction without loss of antigenicity[28], were added after equilibration, to the mucosal side. After 120 min, the entire volume of serosal compartments was analyzed at 488 nm in a fluorimeter (Cary Eclipse, Varian, Victoria, Australia). One unit corresponds to 3.0•10^{3} CFU/ml.[26]

Immunohistochemistry

Frozen ileum sections (5 µm) of 4 rats per group were incubated with 5% bovine serum albumin, washed and incubated with a primary antibody (1:50 rabbit anti-rat occludin, mouse anti-rat claudin-1 or mouse anti-rat claudin-2; Zymed Laboratories, San Francisco, CA, USA) for 1 h at room temperature. Following extensive washes, slides were incubated with Alexa Fluor®488 goat anti-mouse or anti-rabbit immunoglobulin-G (1:500 dilution, Jackson ImmunoResearch Europe Ltd, London, United Kingdom) for 1 h at room temperature.

Apoptotic cells were detected by 'in-situ cell death detection kit' (Roche Diagnostics, Bromma, Sweden). Frozen ileum sections (5 µm) were permeabilized in 0.1 mol/l sodium citrate for 2 min on ice and incubated in terminal-deoxynucleotidyl-transferasemediated-dUTP-nick-end-labeling (TUNEL) reaction mixture for 1 h at 37°C.

All sections were counterstained with 0.5 µM 4',6-diamidineo-2-phenylindole (DAPI) for 10 min, mounted in antifading Fluorescent Mounting Medium (DakoCytomation, Stockholm, Sweden) and examined using confocal imaging with a 2-photon BioRad Radiance 2000 microscope (Carl Zeiss, Jena, Germany), equipped with high numerical aperture (NA = 1.4) 60x and 100x oil immersion objectives. Each test included negative controls. Image acquisition settings were identical for each experiment. Apoptotic rate was determined by counting the number of TUNEL^{+} cells/100 epithelial cells in 4 sections from 4 rats per group.

DNA-fragmentation assay

Histone-associated DNA-fragmentation was determined in ileal homogenate corresponding to 50 µg freeze-dried mucosa as previously described[29], using Cell Death Detection ELISA ^{PLUS} (Roche Diagnostics). Results are normalized to protein content, as measured according to Bradford's method[30] and expressed as ratio to control animals.

Histological measurements of mucosal damage

Coded ileal sections were haematoxylin-eosin (H&E) stained and the degree of mucosal damage was determined in 4 tissue sections per rat, by a pathologist blinded to the experimental design. Histopathological grading, from 0 (normal mucosa) to 5 (severe mucosal damage), was performed according to criteria by Chiu *et al*.[31]

To confirm pancreatitis, histological analysis of H&E stained pancreatic sections was performed in 4 tissue sections per rat, utilizing Spormann's criteria.[32]

Lipid peroxidation

To assess oxidative damage, malondialdehyde (MDA) concentration was determined, using a lipid peroxidation assay (LPO-586; Byoxitech, OXIS International, Portland, OR, USA). Ileal mucosa was homogenized in 5 mM butylated hydroxytoluene to prevent sample oxidation. Supernatants were used to determine MDA levels according to manufacturer's instructions. Results were normalized to protein contents of the crude homogenates.

Glutathione assay

To estimate the antioxidative capacity, reduced and oxidized GSH contents were determined in ileal tissue and plasma using a commercially available assay (Glutathione Assay Kit II, Merck Chemicals, Hull, United Kingdom). To ensure absence of adherent bacteria, samples were flushed with cold Kreb's buffer, and microscopically examined. Freeze-dried ileal mucosa was homogenized in acid medium (0.2 M 2-(N-morpholino) ethanesulphonic acid, 0.05 M phosphate, and 1 mM EDTA, pH 6.0), centrifuged (10 min, 10,000x*g*) and supernatants were collected. After protein determination, supernatants corresponding with 1 mg protein, and plasma aliquots were deproteinized with 5% metaphosphoric acid (Sigma-Aldrich Chemie BV) and 4 M triethanolamine (Sigma-Aldrich Chemie BV) and plasma samples were lyophilized.

Individual bacterial strains from the used probiotics were grown in de Man-Rogosa-Sharpe (MRS) broth at 37°C for 24 h, under strict anaerobic conditions. To determine GSH release during bacterial growth, samples were taken at 0, 6, and 24 h, centrifuged (4,000x*g* for 10 min at 4°C) and supernatants were collected. For determination of bacterial GSH content, bacteria were collected after 24 h of cultivation and disrupted by sonication (Bransonic 3200, Branson Ultrasonics b.v., Soest, the Netherlands) on ice for 10 min with 3 sec cooling interval per min. Suspensions were centrifuged, yielding a cell-free extract.

Cell-free extracts, tissue supernatants and plasma samples were analyzed for total GSH according to the protocol provided by the manufacturer. To quantify oxidized GSH (GSSG), 2-vinylpyridine was added to the acidic medium to derivatize GSH. GSH levels were calculated by subtracting the amount of GSSG from the total GSH content and normalized to protein content.

Cysteine

Mucosal cysteine was determined using the spectrophotometric method developed by Gaitonde[33] and expressed as nmol/mg protein.

Glutamate-cysteine-ligase

Biosynthesis of GSH was analyzed by quantification of glutamate-cysteine-ligase (GCL, EC: 6.3.2.2) activity as previously described.[34] For determination of systemic GSH biosynthesis, erythrocytes were obtained by centrifugation of ETDA blood samples at 900xg for 3 min and after washing 3 times with 5 volumes of cold isotonic NaCl solution. Erythrocytes were lysed by the addition of 50 mmol Tris-HCl buffer (pH 7.4), containing 1 mmol EDTA, and by sonication for 2x20 seconds. The erythrocyte membranes were removed by centrifugation at 18,000x*g* for 40 min. For determination of local intestinal mucosal GCL activity, ileal tissues were homogenized in 250 mM sucrose containing 20 mM Tris, 1 mM EDTA, 20 mM boric acid, 2 mM serine, pH 7.4. GCL activity was determined as the difference between γ-glutamylcysteine (GC) synthesis in unblocked and GC synthesis in samples blocked with 200 mM 5-sulfosalicylic acid dehydrate and expressed as nmol (GC)/min/mg protein.

mRNA expression analysis

Total RNA was isolated from ileal mucosa using the RNeasy Midi Kit (Qiagen, Hilden, Germany) and spectrophotometrically quantified, showing A260/A280 ratios within normal range. Subsequently, the integrity of total RNA was checked by denaturing agarose gel electrophoresis. First strand cDNA was synthesized from total RNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA) and quantitative RT-PCR was performed using the iCycler iQ system (BioRad). RT-PCR with mRNA-specific primers for the catalytic (GCLC) and modifier (GCLM) subunits of GCL and 18S rRNA as a reference gene was performed (GCLC-forward 5'-ggcgatgttcttgaaactctg-3', GCLCreverse 5'-cagagggttgggtggttg-3'; GCLM-forward 5'-ctgactcacaatgacccaaaag-3', GCLM-reverse 5'-ttcaatgtcagggatgctttc-3'; 18S rRNA-forward 5'-aatcagttatggttcctttgtcg-3', 18S rRNA-reverse 5'-gctctagaattaccacagttatccaa-3'; Sigma-Aldrich) and mRNA levels were quantified using SYBR Green based detection.

Prior to real-time PCR analysis cDNA samples were diluted 1:25, except for 18S rRNA which was diluted 1:1000, with RNasefree water. PCR reactions were set up in a volume of 25 µl, containing 5 µl of diluted cDNA, 12.5 µl of 2xiQ SYBR Green Supermix (BioRad) and 300 nM of the forward and reverse primer each. Thermal cycling conditions were 95°C for 3 min as initial denaturation and enzyme-activating step followed by 40 cycles of 95°C for 15 s denaturation, 60°C for 30 s annealing and 72°C for 30 s extension. After amplification a melting curve analysis was performed by increasing the temperature by 0.5°C increments from 55°C to 95°C and measuring fluorescence at each temperature for a period of 10 s. All cDNA samples

were analyzed in triplicate and each run contained a relative standard curve. Purified PCR products were used to generate the relative standard curves, consisting of serial dilutions. Levels of GCLC and GCLM mRNA were quantified using the comparative threshold cycle method, normalized to18S rRNA expression and expressed as ratio to controls (a.u.).

Statistical Analysis

Normal distribution was assessed using Shapiro–Wilk's test. Parametric values are presented as mean (SD). Statistical analysis was performed by ANOVA followed by Tukey's HSD test. Nonparametric values are given as median (25–75th interquartile range). Comparisons between two groups were done by Mann-Whitney U-test and between multiple groups by Kruskal-Wallis. Spearman's rank correlation coefficients were computed for correlation analyses. Considering Bonferroni's correction, $P<0.01$ was considered significant.

Results

Acute pancreatitis induced severe ileal mucosal barrier dysfunction

Mortality due to AP did not occur. Pancreatitis was confirmed by histological scoring of pancreatic injury[32] (sham-operated 0 (0-0) *vs.* after induction of pancreatitis 3 (2–5.1); $P<0.001$).

Pancreatitis induced increased permeability to HRP and ^{51}Cr-EDTA (fig 2A, B), accompanied by increase in baseline conductance, representing paracellular ion flux (fig 2C) and elevation of Isc, indicative of ion secretion (fig 2D). Moreover, transepithelial bacterial passage increased by as much as 7-fold in animals subjected to AP (fig 2E) and tissues from rats in the pancreatitis group responded to *E. coli* K12 added to the luminal buffer with an enhanced elevation in conductance (fig 2F). These data suggest a combined perturbation of paracellular andtranscellular pathways.

To further characterize the paracellular pathway TJ protein expression was studied in tissue sections. In sham-operated animals, occludin was localized in the cytoplasm of epithelial cells and along the basolateral membrane with an enrichment of occludin at the apical surface (fig 3). In AP partial disruption of occludin was seen in crypts as well as in villi. Claudin-1 staining pattern was diffuse with predominantly intracellular localization in sham-operated animals (fig 3). AP caused decreased staining intensity and aggregation of claudin-1 within the cytosol. Claudin-1 was not detected near areas of epithelial disruption, suggesting that detachment of enterocytes was preceded by loss of claudin-1.Contrary to occludin and claudin-1, the pore-forming TJ protein, claudin-2[7], was only scarcely detectable in crypts of shamoperated animals, whereas rats from the AP group showed intense staining of claudin-2 both in crypt

and surface epithelium (fig 3).

It is reported that intestinal epithelial apoptosis contributes to mucosal barrier dysfunction.[9–11] Confocal microscopy with immunofluorescent TUNEL staining (fig 4A) revealed AP-induced epithelial cell apoptosis compared to sham animals (25.8 (24.4–26.3) *vs.* 2.60 (2.47–2.73) TUNEL$^+$ cells/100 epithelial cells; P<0.001). Mucosal DNA-fragmentation corroborated these findings (fig 4B). Moreover, DNA-fragmentation

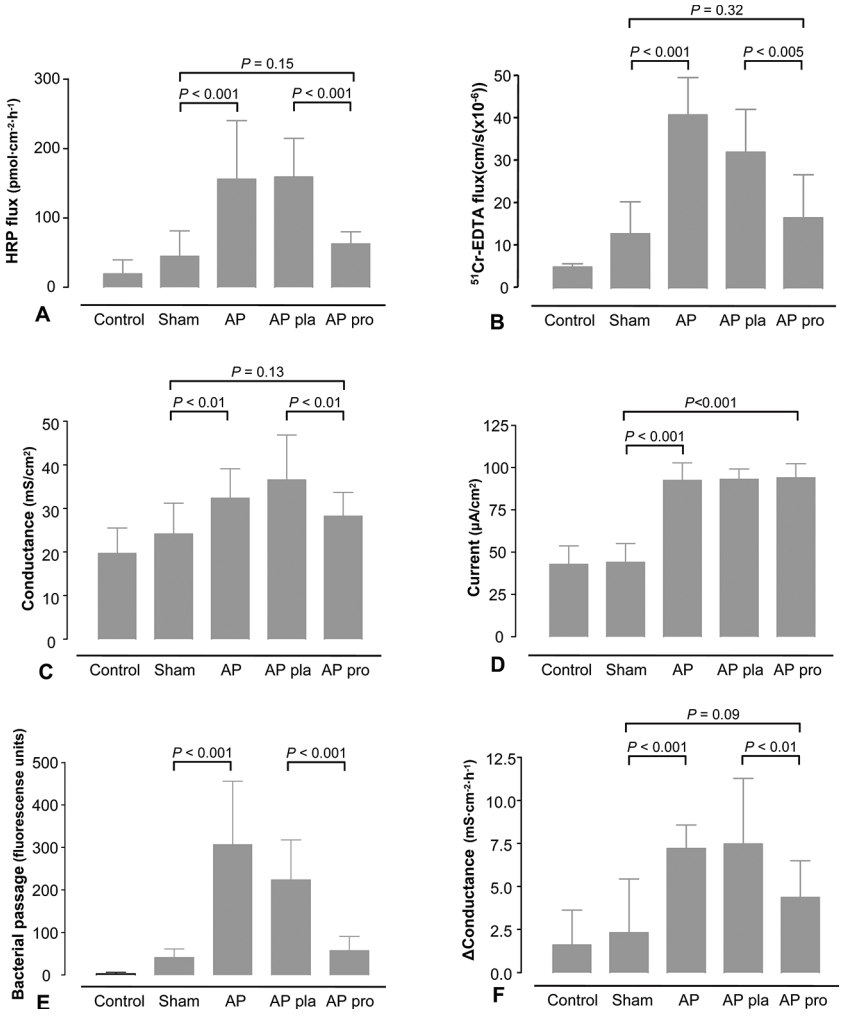


Figure 2. Probiotics prevented acute pancreatitis-induced ileal permeability but not ion secretion. After 5 days of pre-treatment with placebo (pla, n = 12) or probiotics (pro, n = 12), rats were subjected to acute pancreatitis (AP, n = 12), a sham-procedure (n = 12) or not operated (control, n = 5). Ileal segments were mounted in Ussing chambers and (A) horseradish peroxidase (HRP) and (B) 51Cr-EDTA flux were studied for two hours. (C) Baseline conductance, (D) baseline short circuit current (Isc), (E) passage of *Escherichia* coli K12 and (F) elevation of conductance during one hour after challenge with *E. coli* were measured. The graphs show average (±SD). The data were collected from independently acquired sets of 3 tissue segments per rat. Comparisons were performed using ANOVA followed by Tukey's HSD.

strongly correlated with bacterial passage (fig 4C), ^{51}Cr-EDTA flux (r =0.93) and tissue conductance (r= 0.87), which supports the hypothesis that epithelial cell apoptosis disrupts barrier integrity.

Six hours after induction of pancreatitis, intestinal injury (fig 5A) was characterized by villus denudation, lamina propria disintegration and ulceration (fig 5B), resembling intestinal ischemia-reperfusion injury[31], which is associated with ROS release, epithelial apoptosis and TJ disruption.[5,6,8] Therefore, oxidative stress-induced lipid peroxidation was quantified, which was indeed found to be elevated after induction of AP (fig 5C) and also showed a strong positive correlation with barrier dysfunction (^{51}Cr-EDTA flux r = 0.83, bacterial passage r = 0.88).

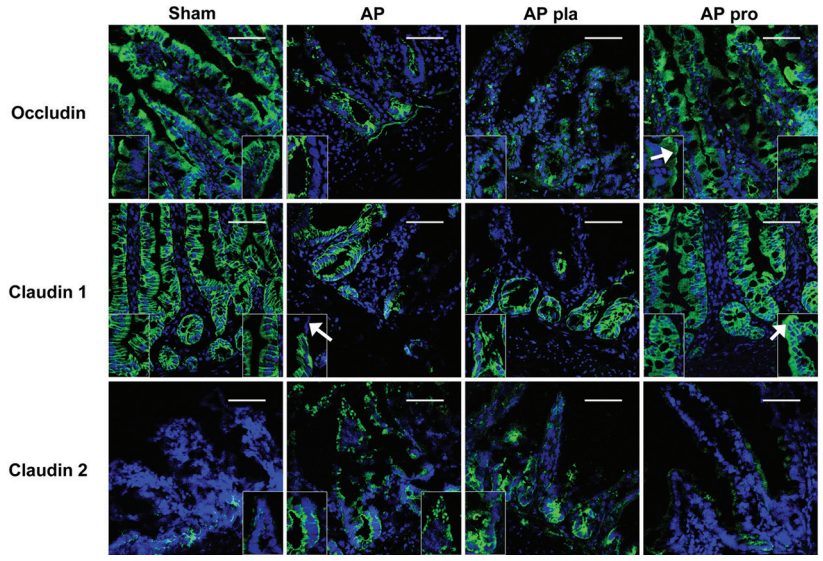


Figure 3. Probiotics prevented disruption of tight junction proteins. After 5 days of pre-treatment with placebo (pla) or probiotics (pro), rats were subjected to acute pancreatitis (AP), or a sham-procedure Ileal sections were stained with occludin, claudin-1 or -2 antibodies (green), counterstained with DAPI (blue) and visualized by confocal laser scanning microscopy. Bar = 500 µm. The higher magnification (100x/1.30) images shown in the insets are typical details of crypts (left) and villi (right). Probiotics prevented the deleterious effects of AP on occludin and caused redistribution of occludin to the apical surface (arrowhead). Acute pancreatitis-induced detachment of epithelial cells seems to be preceded by loss of claudin-1 (arrowhead) and was reduced by probiotic pre-treatment; though probiotics could not prevent the AP-induced formation of aggregates of claudin-1 in the cytosol (arrowhead). Probiotics prevented AP-associated up-regulation of claudin-2 in both crypts and villi (arrowhead). The patterns of staining are typical of that seen in 4 sections of 4 rats per group.

Probiotics prevented acute pancreatitis-induced barrier dysfunction

No rats receiving probiotics showed signs of diarrhea or loss of appetite during the pre-treatment period. Increase in animal weight was similar in all groups (sham-operated 22.3 (1.09) *vs.* AP 21.9 (1.11) *vs.* placebo 20.6 (0.82) *vs.* probiotics 20.5 (0.61)). Five days of pre-treatment with probiotics abolished the deleterious effects of AP on numerous parameters of barrier function. AP-induced increase in ileal permeability to HRP (fig 2A) and ^{51}Cr-EDTA (fig 2B) as well as tissue conductance (fig 2C) was normalized after probiotic pre-treatment. Elevation in tissue conductance after adding *E.coli* K12 was 40% smaller in tissues from probiotic treated rats compared to placebo (fig 2F). In contrast, there were no inhibitory effects of probiotics on APinduced elevation of Isc (fig 2D).

Probiotics also modified the localization of TJ proteins. APassociated partial disruption of the distribution of occludin in crypts and villi was prevented and redistribution to the apical surface was apparent in crypts of probiotic treated animals (fig 3). In both claudin-1 and -2 staining patterns the AP-induced deleterious effects were reduced by probiotic pre-treatment (fig 3). Furthermore, probiotics attenuated AP-induced epithelial cell apoptosis, showing a 70% reduction in apoptotic rate (8.85 (8.60–9.15) *vs.* placebo 31.75 (31.60–32.90) TUNEL$^+$ cells/100 epithelial cells; P<0.001, fig 4A), which was also demonstrated by analysis of mucosal DNA-fragmentation (fig 4B). Histological scoring demonstrated that probiotics ameliorated pancreatitis-induced mucosal damage (1.0 (1.0–1.25) *vs.* placebo 4.0 (4.0-4.0); P<0.001, fig 5A) and normalized mucosal lipid peroxidation (fig 5C).

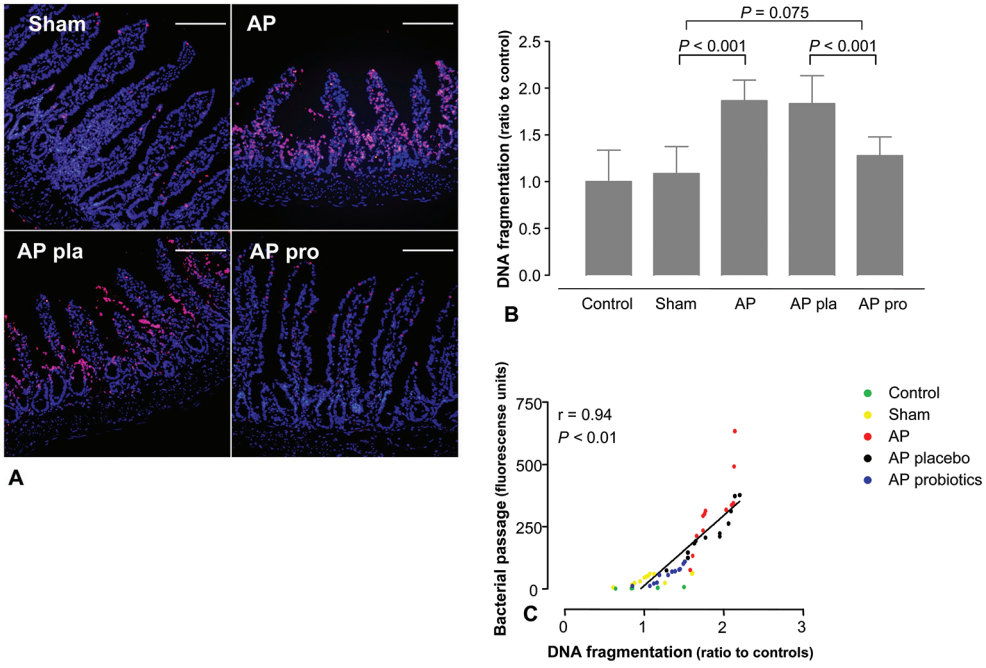
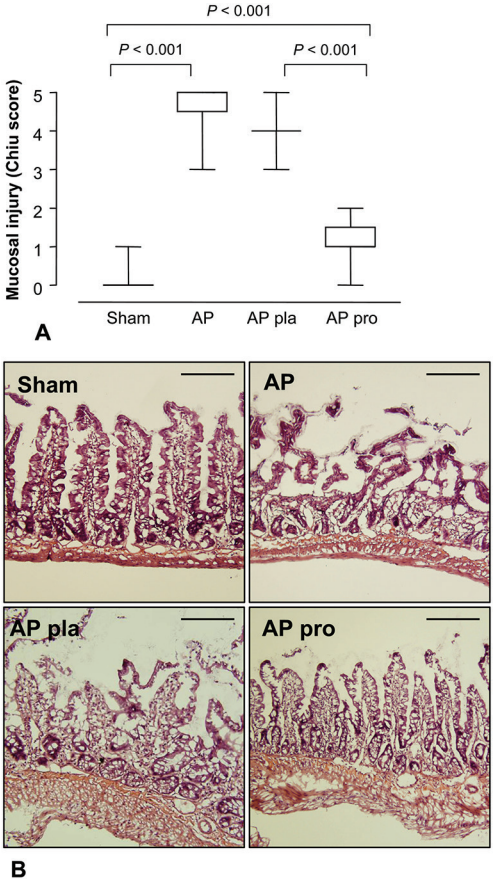


Figure 4. Probiotics reduced pancreatitis-associated intestinal apoptosis. After 5 days of pre-treatment with placebo or probiotics, rats were subjected to acute pancreatitis, or a sham-procedure. (A) Sections of ileum were TUNEL stained. The results shown are typical images from 4 sections of 4 rats per group. Bar = 200 µm. (B) DNAfragmentation (control n = 5, sham n = 12, AP n = 12, AP pla n = 12 and AP pro n = 12). The graph shows the average (±SD). Comparisons were performed using ANOVA followed by Tukey's HSD. (C) Positive correlation between intestinal apoptosis and *Escherichia coli* K12 passage was computed using Spearman's rank correlation coefficients.



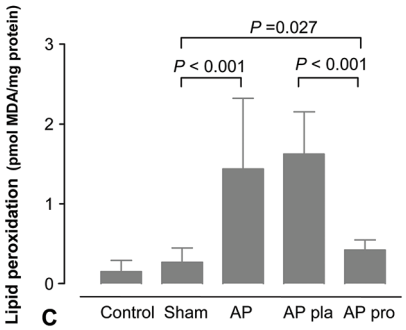


Figure 5. Probiotics attenuated acute pancreatitis-associated mucosal damage. After 5 days of pre-treatment with placebo (pla, n = 12) or probiotics (pro, n = 12), rats were subjected to acute pancreatitis (AP, n = 12), or a sham-procedure (n = 12). (A) Sections of ileum were H&E stained and graded according to Chiu *et al.*[25] The graph shows median (±range). Comparisons were performed using Kruskal-Wallis followed by Mann–Whitney U test. (B) Compared with sham-operated animals (Sham), acute pancreatitis (AP) caused widespread destruction of villi. Placebo treated animals (AP pla) also showed a severe degree of mucosal damage. Probiotic animals (AP pro) showed extensive epithelial lifting, but with intact epithelium. The mucosal damage is typical of that seen in 4 sections from 12 rats per group. Bar = 200 µm. (C) Lipid peroxidation (MDA levels) (control n = 5, sham n = 12, AP n = 12, AP pla n = 12 and AP pro n = 12). The graph shows the average (±SD). Comparisons were performed using ANOVA followed by Tukey's HSD.

Beneficial effect of probiotics by increasing mucosal glutathione

The decline in mucosal lipid peroxidation after probiotic pretreatment may have resulted from either reduced amounts of ROS, or enhanced antioxidative capacity. Therefore, we quantified mucosal oxidized glutathione (GSSG) and GSH in thoroughly rinsed ileal mucosal tissues. Probiotics attenuated AP-induced elevation in GSSG (fig 6A), prevented depletion of mucosal GSH (fig 6B) and normalized the GSH/GSSG ratio (fig 6C). Of note, mucosal GSH/GSSG ratios showed an inverse correlation with DNA-

fragmentation (fig 6D) and mucosal barrier dysfunction (fig 6E, F). Most interestingly, pre-treatment with probiotics induced increased levels of GSH, also in comparison with healthy control animals (fig 6B).

Production of glutathione by the individual probiotic strains

Since mucosal GSH is partially dependent on uptake of dietary GSH[35], we quantified intrabacterial GSH of the separate probiotic strains, as well as GSH levels in medium after 6 and 24 hours of strictly anaerobic cultivation. Only *B. bifidum*, *B. lactis* and *L. acidophilus* contained abundant intracellular GSH (Table 1). GSH in cultivation medium increased over time, except for *Lc. lactis*.

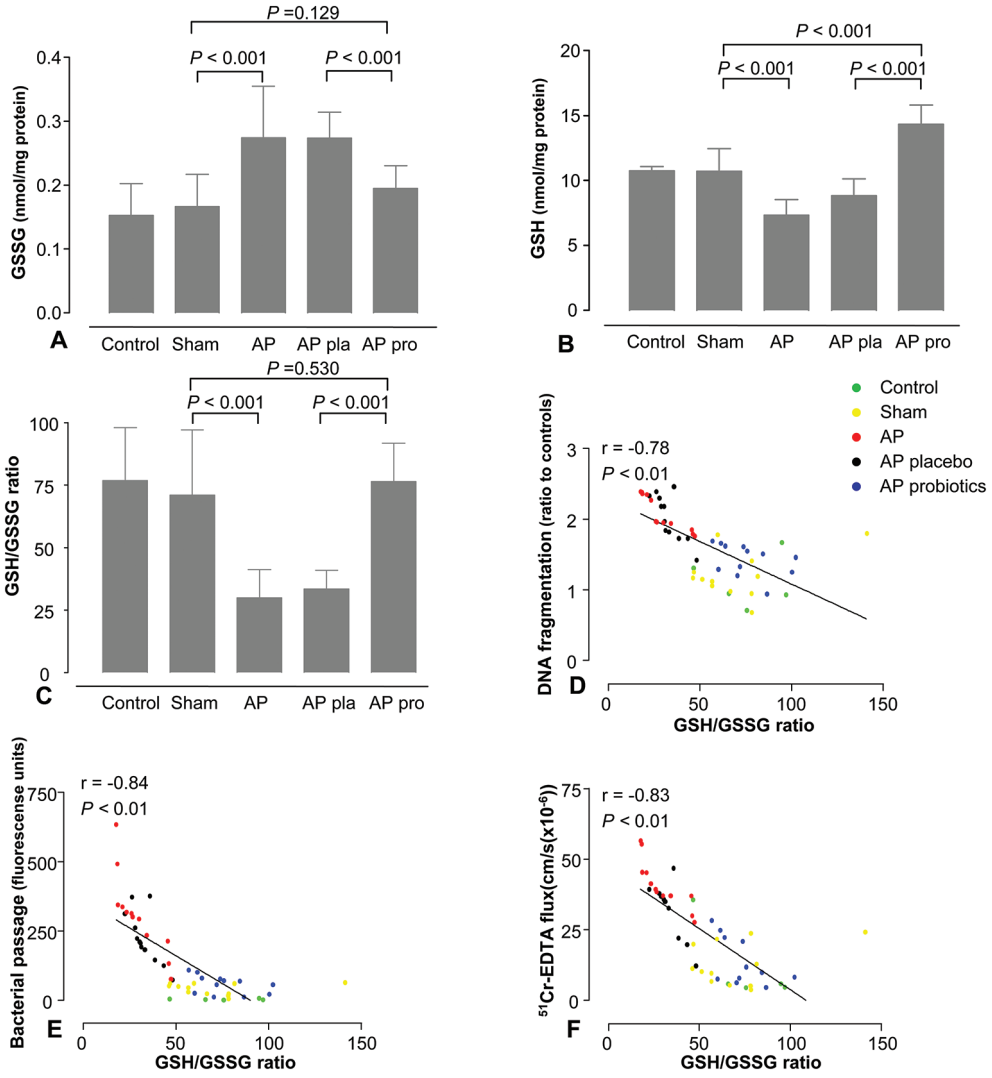


Figure 6. Probiotics enhanced mucosal glutathione levels. After 5 days of pre-treatment with placebo (pla, n = 12) or probiotics (pro, n = 12), rats were subjected to acute pancreatitis (AP, n = 12), a sham-procedure (n = 12) or not operated (control, n = 5). Six hours after induction of the AP or sham-procedure, mucosal (A) oxidized glutathione levels (GSSG), (B) reduced glutathione levels (GSH) and (C) GSH/GSSG ratios were determined. The graphs show average (±SD). Comparisons were performed using ANOVA followed by Tukey's HSD. Correlation analyses revealed an inverse correlation between GSH/GSSG ratio and (D) DNA-fragmentation, (E) ileal permeability to *Escherichia coli* K12 and (F) ^{51}Cr-EDTA flux. Spearman's rank correlation coefficients were computed for correlation analyses.

Table 1. Intracellular GSH contents of the probiotic strains and GSH levels in medium at different time points after start of cultivation.

Bacterial strain	Intracellular GSH (nmol/mg protein)	GSH content in culture medium (nmol/ml supertant)		
		0 hour	6 hours	24 hours
B. bifidum W23	0.37 (0.002)	0.00 (0.000)	0.10 (0.006)	0.51 (0.006)
L. salivarius W24	0.11 (0.002)	0.00 (0.000)	0.08 (0.004)	0.15 (0.006)
B. lactis W52	0.01 (0.002)	0.00 (0.000)	0.00 (0.000)	0.01 (0.008)
L. casei W56	0.09 (0.004)	0.00 (0.000)	0.00 (0.000)	0.01 (0.002)
Lc. lactis W58	0.04 (0.014)	0.00 (0.000)	0.00 (0.000)	0.00 (0.000)
L. acidophilus W70	0.14 (0.005)	0.00 (0.000)	0.04 (0.002)	0.19 (0.002)

Bifidobacterium (B.); *Lactobacillus* (L.); *Lactococcus* (Lc.); glutathione (GSH).
Mean (SD), n = 4 separate experiments.

Local mucosal biosynthesis of glutathione

Mucosal GSH is, besides dietary uptake, also dependent on biosynthesis, which is regulated by availability of cysteine and GCL activity.[36] Mucosal cysteine levels did not differ significantly between the groups (fig 7A). GCL activity, however, was affected by AP and by probiotics pre-treatment. AP *per se* increased GCL activity compared to sham, but the most abundant increase was seen in the probiotics group which showed a 10-fold increase compared to controls (fig 7B).

Because GCL is composed of both a catalytic and modulatory subunit[37], the increase in GCL activity in this study could be due to enhanced expression of either GCLm and/or GCLc. Therefore, quantitative real-time PCR was performed to monitor changes in GCLc and GCLm message abundance. The level of mRNA expression of the reference gene, 18S, was comparable between all groups. When normalized to 18S, GCLc levels in probiotic pretreated rats were 6.78 (0.95) a.u., which was 1.8 fold higher than in placebo treated rats (3.83 (0.49) a.u., fig 7C, D). Similarly, levels of GCLm mRNA after probiotic pre-treatment (10.3 (2.56) a.u) were 2.7 fold higher than average values seen in placebo treated rats (3.88 (0.75) a.u). The increase in GCLm mRNA expression was less pronounced in the AP and placebo groups when compared to sham operated animals (3.07 *vs.* AP 4.68 a.u.; 3.07 *vs.* placebo 3.88 a.u., respectively, fig 7C, E). These data suggest that the enhanced GCL activity after probiotic pre-treatment may be due to increased gene expression in the ileal mucosa.

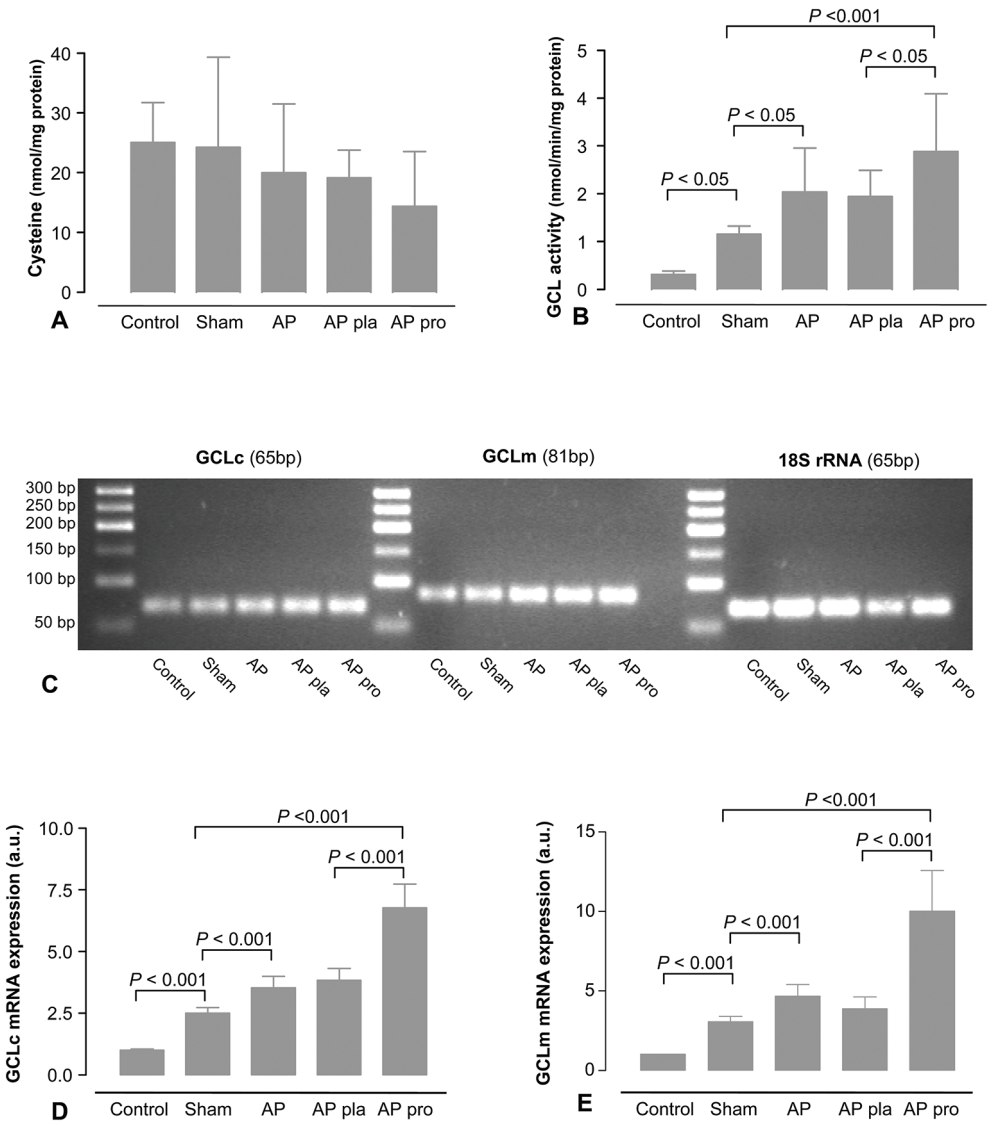


Figure 7. Probiotics have no effect on cysteine availability, but induce glutamate-cysteine-ligase activity. After 5 days of pre-treatment with placebo (pla, n = 12) or probiotics (pro, n = 12), rats were subjected to acute pancreatitis (AP, n = 12), a sham-procedure (n = 12) or not operated (control, n = 5). Six hours after induction of the AP or sham-procedure, tissue cysteine availability (A) and mucosal glutamate-cysteine-ligase (GCL) activity (B) were determined in ileum samples. (C) RT PCR was conducted on ileal mRNA. PCR products of specific primers for the catalytic (GCLc, 65 bp) and the modulatory (GCLm, 81 bp) subunit of GCL and for 18S rRNA (65 bp) as control were identified on 2.5% agarose gel, using a GeneRuler 50 bp DNA Ladder (Fermentas GMBH, St. Leon-Rot, Germany). mRNA expression of (D) GCLc and (E) GCLm were quantified. Data are normalized to 18S rRNA expression and expressed as ratio to control animals. The graphs show average (±SD). All analyses were run in triplicates. Comparisons were performed using ANOVA followed by Tukey's HSD.

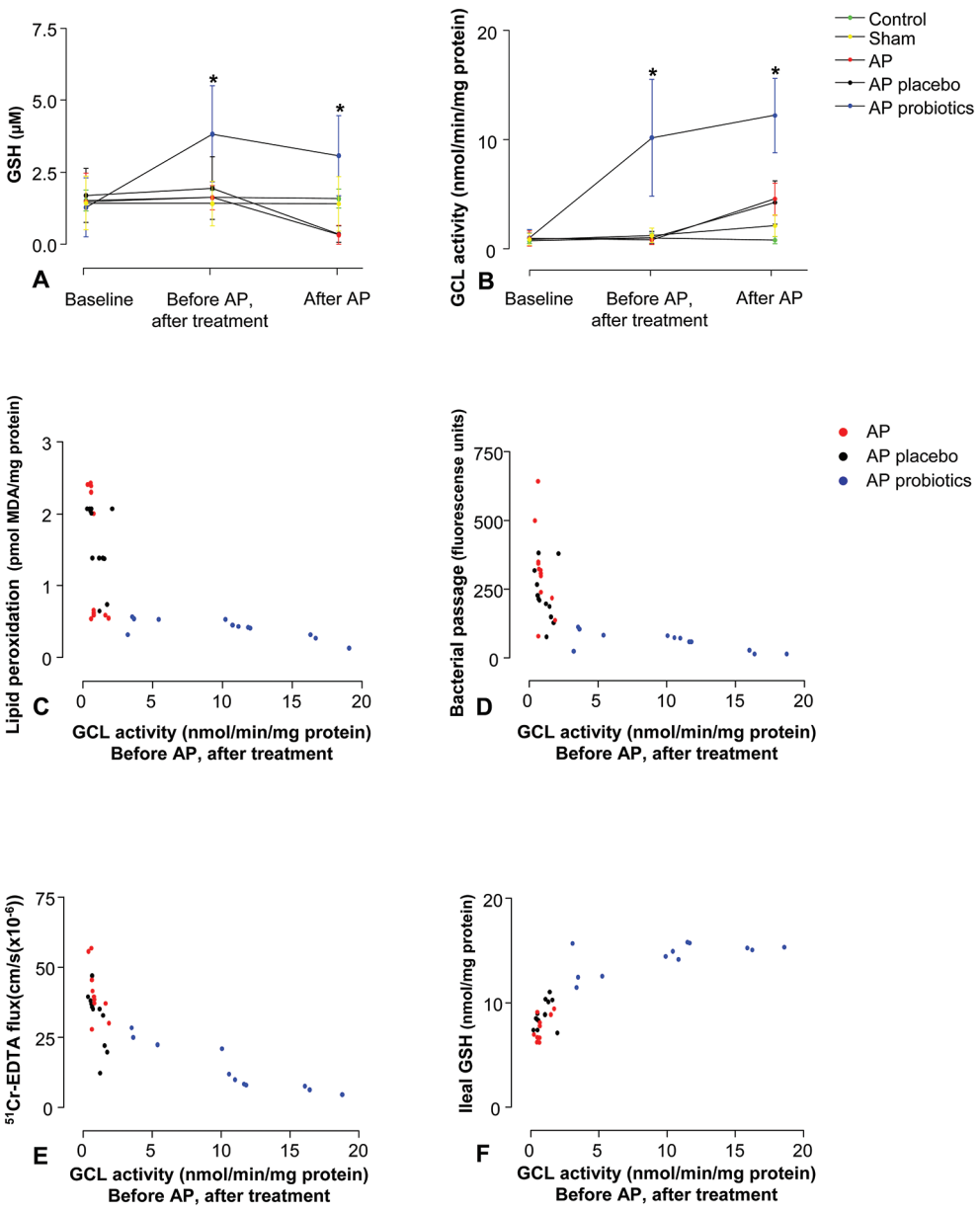


Figure 8. Probiotics induce systemic increase in GSH levels and GCL activity. After 5 days of pre treatment with placebo (pla, n = 12) or probiotics (pro, n = 12), rats were subjected to acute pancreatitis (AP, n = 12), a sham-procedure (n = 12) or not operated (control, n = 5). Whole blood was sampled 1) before treatment, 2) after 5 days of pre-treatment, immediately before induction of AP and 3) six hours after induction of AP or shamprocedure. Time course of plasma GSH levels (A) and GCL activity in red blood cells (B) was monitored. The graphs show average (±SD). All analyses were run in duplicates. Comparisons were performed using ANOVA followed by Tukey's HSD. *P,0.001, probiotics *vs*. placebo. Associations between (C) ileal lipid peroxidation, (D) bacterial passage, (E) ^{51}Cr-EDTA flux, (F) ileal GSH content six hours after induction of AP and GCL activity in red blood cells immediately before subjection to AP.

Probiotics increase systemic glutathione levels

To gain insight into the antioxidative capacity prior to induction of AP, plasma GSH levels before treatment, before induction of AP and at time of termination were determined. In the course of the 5 days of the pre-treatment period, plasma GSH levels showed a 2 fold increase in probiotic pre-treated animals (fig 8A). This was in contrast to rats receiving placebo, in which plasma GSH levels did not differ significantly after 5 days of pre-treatment. This is in keeping with the increase in GCL activity in red blood cells (RBC) after 5 days of pre-treatment with probiotics (fig 8B). Correlation analyses between pancreatitis-induced oxidative damage, as measured by ileal lipid peroxidation and GCL activity immediately before induction of pancreatitis, suggested that GCL activity greater than 5 nmol/min/mg protein was protective against oxidative injury (fig 8C). Furthermore, GCL activity in RBCs immediately before induction of acute pancreatitis correlated inversely with parameters of mucosal barrier dysfunction in animals subjected to AP (bacterial passage: r =-0.80, ^{51}Cr-EDTA: r =-0.86, fig 8D, E). Not surprisingly, GCL activity also correlated positively with ileal GSH levels in animals subjected to AP (r = 0.82, fig 8F).

Discussion

The present study is the first to demonstrate that pre-treatment with multispecies probiotics increases mucosal GSH levels and stimulates GSH biosynthesis in the ileum, resulting in attenuated oxidative mucosal damage. Furthermore, normalization of GSH/GSSG ratios strongly correlated with improved barrier function. Therefore, increased mucosal GSH levels represent a candidate mechanism underlying the protection against barrier dysfunction afforded by pre-treatment with probiotics in experimental AP.

GSH synthesis was up-regulated in probiotic pre-treated rats, as demonstrated by enhanced GCL activity and in creased mRNA expression of both of the GCL subunits, shown herein. GSH plays a pivotal role in maintenance of the redox balance (expressed as GSH/GSSG ratios), preventing oxidative damage[4] and maintaining mucosal barrier, which was reflected by the inverse correlation between mucosal GSH/GSSG ratios and parameters of barrier dysfunction. Two factors directly associate with mucosal GSH: dietary GSH levels[35] and GSH biosynthesis, of which the latter is in turn dependent on cysteine availability and GCL activity.[36] First, it has been reported that certain probiotics contain and release GSH[20,38,39] and Peran *et al.*[20] previously showed increased intestinal GSH following oral administration of *Lactobacillus fermentum* in experimental colitis. Our present in vitro experiments showed strain specific differences in intracellular GSH content within the range previously reported.[38] Moreover, time-dependent GSH release was found during anaerobic cultivation, which

was abundant in *B. bifidum*, *B. lactis* and *L. acidophilus*. Nevertheless, considering an estimated GSH production of 31.0 nmol GSH by the total administered probiotic dose (17.9 nmol intrabacterial GSH (mean 0.13 nmol GSH/mg protein) +13.1 (1.53) nmol GSH secreted in 5 days; calculated from Table 1) compared with an estimated total increase in small intestinal GSH of 1190 nmol (small intestinal length 90 cm, mucosal protein content 2.4 mg/cm (n= 6); pre-treatment yielded increase in ileal GSH of 5.5 nmol/mg protein; placebo 8.8 *vs.* probiotics 14.3 nmol/mg protein, fig 6B), bacterial GSH could only partially account for the rise in ileal GSH content. Consequently, the possibility of local GSH biosynthesis was investigated. The present study did not show significant differences in mucosal cysteine, implying that cysteine availability was not an important discriminating factor. On the other hand, we found enhanced GCL activity and expression of the GCL subunits GCLc and GCLm in probiotic-treated animals leading to a significant increase in GSH contents. Although the contribution from the probiotic bacteria may be higher than calculated because of colonization and expansion, it is conceivable that enhanced CGL activity in the intestinal mucosa was the major factor contributing to the increased ileal GSH content.

Interestingly, correlation analysis between GCL activity and parameters of mucosal barrier failure in animals subjected to AP, suggested the existence of a threshold GCL activity above which mucosal protection against oxidative stress is functional. This may explain that the relatively small increase in GCL activity between the probiotic and the placebo pre-treated groups resulted in considerable protection, whereas the rise in GCL activity between the sham and the AP group did not ameliorate the AP-induced damage. This hypothesis is supported by the correlation between GCL activity and ileal GSH content; the latter was only above a certain threshold of GCL activity able to withstand the deleterious effects of AP.

As previous experimental studies have shown that GCL gene expression is up-regulated both after low dose H_2O_2[17] and after administration of weak inducers of oxidative stress[18], the increase in GCL activity found in the present study could be indicative of cellular stress as a mechanistic factor. Administration of probiotics may have caused a minor oxidative assault, e.g. intracellular accumulation of short-chain fatty acids produced by the bacteria, thereby inducing increased capacity of antioxidant enzymes, preconditioning the mucosa for a major oxidative attack during AP. This hypothesis is further supported by the found increase in systemic GCL activity in the probiotic pre-treated group, which was markedly enhanced already before the induction of AP. At first glance, it may seem contradictory to the current study that the recent placebo-controlled trial by Besselink *et al*.[24], demonstrated increased incidence of bowel ischemia after administration of probiotics in the acute phase of severe AP. However, keeping in mind that enteral probiotics caused low dose oxidative stress, probiotics administered after the onset of AP may act as an extra oxidative burden in an already critically affected redox system[3] thereby, causing increased oxidative stressinduced damage and ischemia.

During critical illness oxidative stress disrupts TJs[5,6], which are crucial in determining epithelial barrier properties[7], as illustrated here by the AP-induced breach in barrier function. Disruption of TJs results in increased permeability to luminal antigens and bacteria that promote release of pro-inflammatory cytokines which further deteriorates mucosal barrier function.[2] This is in keeping with our results that are the first to show AP-induced disruption of the claudin-1 distribution together with up-regulation of claudin-2, which is also the case in inflammatory bowel disease and destabilizes TJs.[7,10,40] Immunostaining revealed that pre-treatment with probiotics maintained TJ integrity with a normal distribution of claudin-1 and -2. The finding of Yasuda and colleagues[9], that AP did not have deleterious effects on occludin, is in contrast with our results. This may, however, be explained by differences in the model of AP used.

Apoptosis is the major mode of cell death during intestinal ischemia/reperfusion[8] and exerts deleterious effects on mucosal barrier function and survival.[9–11,13,14] Yan *et al.*[41] previously showed that soluble proteins produced by *Lactobacillus* strains protect epithelial cells from cytokine-induced apoptosis. Here, we found that probiotics, which induced GSH biosynthesis, normalized AP-induced epithelial cell apoptosis. In addition, we were able to demonstrate a positive correlation between mucosal DNA-fragmentation and barrier dysfunction, providing further evidence that oxidative stress plays an important role in induction of epithelial apoptosis and subsequent loss of barrier function.

Contrary to the effects on permeability, pre-treatment with probiotics showed no effect on ion secretion in our experiment. These findings emphasize the divergent regulation of cellular secretory and barrier functions. It has been reported that epithelia respond rapidly to pathogenic bacteria with altered ion secretion[42], indicating that "flushing out" may be a defense mechanism against the threat of invasion of the mucosa. In this regard, it is perhaps advantageous that probiotics do not inhibit these beneficial adaptive responses to an infectious threat.

In conclusion, the present study is to our knowledge the first to show that pre-treatment with multispecies probiotics, stimulates mucosal GSH biosynthesis and consequently normalizes AP-induced barrier dysfunction and attenuates epithelial cell apoptosis and disruption of TJs in a model of AP. In addition, our data demonstrate strong inverse correlations between mucosal GSH/GSSG ratios and mucosal barrier dysfunction. This further supports the functional relevance of this endogenous antioxidant and gives novel insights into the mechanisms of probiotics. However, as the used compound is a multispecies combination of probiotic strains, it is worth noting that the found effects depend on the combination of the applied bacteria. Additional studies will be necessary to elucidate the effects of each separate strain as well as possible synergistic effects of this specific combination of probiotics.

In addition, the role of oxidative stress has been evaluated in experimental models of acute pancreatitis[43] and it should be emphasized that oxidative stress and excessive

ROS generation are early features in AP and consequently a difficult target for clinical prophylaxis to prevent a severe course of the disease. This has recently been shown in a randomized controlled trial, utilizing intravenous antioxidant (n-acetylcysteine, selenium, vitamin C) therapy, where the results in AP patients were not that encouraging.[44] However, oxidative stress is not only involved in the early stage of AP, but also in the course of the disease and may for that reason be a target for therapy at later stages of the disease. However, as the probiotics used in the current study showed severe adverse effects in intensive care AP patients[24], and since the present effects on GCL activity most likely resulted from a mild oxidative stress, this combination of probiotics is not a defendable treatment option in critically ill patients. Therefore, the appropriate clinical use of multispecies probiotics would be a preventive approach to improve defense against an expected oxidative attack, such as before elective major abdominal surgery[45] or maintenance treatment in IBD and pouchitis.[46,47]

Acknowledgments

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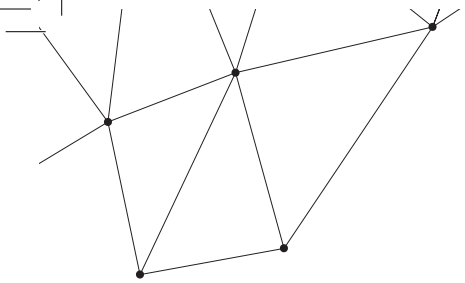

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CHAPTER 10

Farnesoid X Receptor (FXR) activation and FXR genetic variation in inflammatory bowel disease

Rian M. Nijmeijer[1*], Raffaella M. Gadaleta[2,3,4*], Saskia W.C. van Mil[3], Adriaan A. van Bodegraven[5], J. Bart A. Crusius[6], Gerard Dijkstra[7], Daan W. Hommes[8], Dirk J. de Jong[9], Pieter C.F. Stokkers[10], Hein W. Verspaget[8], Rinse K. Weersma[7], C. Janneke van der Woude[11], Janneke M. Stapelbroek[3], Marguerite E.I. Schipper[12], Cisca Wijmenga[13], Karel J. van Erpecum[2], Bas Oldenburg[2], on behalf of the Dutch Initiative on Crohn, Colitis (ICC)

*These authors share first authorship.

[1]Department of Surgery, University Medical Center Utrecht, Utrecht, The Netherlands; [2]Department of Gastroenterology and Hepatology, University Medical Center Utrecht, Utrecht, The Netherlands; [3]Department of Metabolic and Endocrine Diseases, University Medical Center Utrecht and Netherlands Metabolomics Centre, Utrecht, The Netherlands; [4]Laboratory of Lipid Metabolism and Cancer, Consorzio Mario Negri Sud, Santa Maria Imbaro (Ch), Italy; [5]Department of Gastroenterology and Hepatology, VU University Medical Center, Amsterdam, The Netherlands; [6]Laboratory of Immunogenetics, Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands; [7]Department of Gastroenterology and Hepatology, University Medical Center Groningen, Groningen, The Netherlands; [8]Department of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, The Netherlands; [9]Department of Gastroenterology and Hepatology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; [10]Department of Gastroenterology and Hepatology, Amsterdam Medical Center, Amsterdam, The Netherlands; [11]Department of Gastroenterology and Hepatology, Erasmus Medical Center, Rotterdam, The Netherlands; [12]Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands; [13]Department of Genetics, University Medical Center Groningen, Groningen, The Netherlands.

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Abstract

Background: We previously showed that activation of the bile salt nuclear receptor Farnesoid X Receptor (FXR) protects against intestinal inflammation in mice. Reciprocally, these inflammatory mediators may decrease FXR activation. We investigated whether FXR activation is repressed in the ileum and colon of inflammatory bowel disease (IBD) patients in remission. Additionally, we evaluated whether genetic variation in *FXR* is associated with IBD.

Methods: mRNA expression of *FXR* and FXR target gene *SHP* was determined in ileal and colonic biopsies of patients with Crohn's colitis (n = 15) and ulcerative colitis (UC; n = 12), all in clinical remission, and healthy controls (n = 17). Seven common tagging SNPs and two functional SNPs in *FXR* were genotyped in 2355 Dutch IBD patients (1162 Crohn's disease (CD) and 1193 UC) and in 853 healthy controls.

Results: mRNA expression of *SHP* in the ileum is reduced in patients with Crohn's colitis but not in patients with UC compared to controls. mRNA expression of villus marker *Villin* was correlated with *FXR* and *SHP* in healthy controls, a correlation that was weaker in UC patients and absent in CD patients. None of the SNPs was associated with IBD, UC or CD, nor with clinical subgroups of CD.

Conclusions: FXR activation in the ileum is decreased in patients with Crohn's colitis. This may be secondary to altered enterohepatic circulation of bile salts or transrepression by inflammatory signals but does not seem to be caused by the studied SNPs in *FXR*. Increasing FXR activity by synthetic FXR agonists may have benefit in CD patients.

Introduction

Inflammatory bowel disease (IBD) may lead to potentially severe complications and even mortality.[1] Although the exact etiology is unclear, it is thought to result from a combination of mucosal immune system dysregulation, hyperreactivity against the intestinal microbiota, and a compromised intestinal epithelial barrier function in genetically predisposed individuals.[2] Genes associated with IBD highlight key pathogenic mechanisms, including disturbed antibacterial defense (e.g. *NOD2*, *ATG16L1*, cathelicidin, defensins) and barrier function (e.g. *PARD3*, *MAGI2*, myosin IXB).[3-8] In recent genome-wide association studies, the total number of susceptibility loci amounts to 99, but this probably accounts for only 16% of the ulcerative colitis (UC)[9] and 20% of the Crohn's disease (CD)heritability.[10] It has been estimated that future genome-wide association scans will only yield a few more percent of CD and UC heritability. Biological pathway-based analyses or studies focusing on genes involved in established or plausible pathogenetic pathways may be an alternative approach.[11]

The bile salt nuclear Farnesoid X Receptor (NR1H4, nuclear receptor subfamily 1, group H, member 4, alias FXR on chromosome 12q23.1) is a member of the superfamily of nuclear receptors. Nuclear receptors are ligand-activated transcription factors that, in response to lipophilic ligands (e.g. hormones, vitamins and dietary lipids), regulate many aspects of mammalian physiology, including development, reproduction and metabolism.[12,13] *FXR* is mainly expressed in the ileum and liver. Upon activation by bile salts, FXR binds as a heterodimer with Retinoid X Receptor to the FXR responsive elements on the promoters of target genes, such as the small heterodimer partner (SHP). Via this classical route of transactivation, FXR regulates transcription of genes involved in bile salt synthesis, transport and metabolism in the liver and intestine.[14] FXR has also been implicated in immune modulation and barrier function in the intestine.[15,16] We recently reported that pharmacological FXR activation decreases the severity of inflammation and preserves the intestinal barrier integrity in two well-established murine colitis models.[17] As already described for other nuclear receptors, the mechanism by which FXR modulates inflammation is most probably through transrepression of nuclear transcription factor kappa B (NF-kB) signaling. Dysregulated activation of NF-kB has previously been identified as a key factor in the pro-inflammatory response in IBD, resulting in strongly enhanced expression of pro-inflammatory genes such as Tumor Necrosis Factor a or Interleukin-1b and recruitment of an excess of inflammatory cells to the intestinal wall.[18] Notably, we and others previously showed that there is reciprocal repression of FXR and NF-kB *in vitro* and *in vivo*.[19,20]

We therefore investigated *FXR* and FXR target gene mRNA expression in patients with CD and UC in clinical remission. In addition, since FXR acts as a regulator of intestinal inflammation, we hypothesized that polymorphisms in *FXR* might be

associated with IBD and tested this hypothesis in a large Dutch cohort of IBD patients and controls.

Materials and Methods

Patients in mRNA expression study

Seventeen healthy subjects (male/female 7/10; age 55±12.1years), 15 patients with Crohn's colitis (male/female 5/10; age 46±9.8 years) and 12 patients with UC (male/females 4/8; age 44±9.8 years) were enrolled in this study. Montreal classification and medication at the time of endoscopy are shown in Table 1. All IBD patients were in clinical and endoscopic remission without significant histological activity. Patients with significant endoscopic or histological disease activity were excluded. The indication for colonoscopy was screening for cancer or polyps in healthy controls and scheduled dysplasia screening in IBD patients. Biopsies were obtained from the ileum and ascending colon, immediately frozen in liquid nitrogen and subsequently stored at -80°C, until further processing. Written informed consent was obtained from all subjects and the study was approved by the Medical Ethical Committee of the University Medical Centre Utrecht.

mRNA extraction and qRT-PCR analysis

Human biopsies of ileum and ascending colon were homogenized (Omni TH tissue homogenizer, Omni International, Kennesaw, USA) and RNA was isolated using RNeasy Micro kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The quantity, quality and integrity of isolated mRNA were confirmed by absorption measurement and RNA gel electrophoresis. Subsequently, cDNA was generated from 500 ng of total RNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamers (Roche, Basel, Switzerland). qRT-PCR analysis was carried out using SYBR green PCR master mix (Biorad, Veenendaal, The Netherlands) and a MyIQ real time PCR cycler (Biorad). Values were quantified using the comparative threshold cycle method. *FXR* and its target genes are exclusively expressed in the differentiated enterocyte on the top of the villi.[15,17,19] In order to estimate the distribution between villi and crypts in the human biopsies, we determined mRNA expression of Villin and sucrose isomaltase (*SI*), which are both expressed exclusively in differentiated enterocytes in the villi, and of *c-myc* and cyclin D1 (*CCND1*), both expressed only in the crypts. mRNA expression levels of genes of interest were normalized to hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), which was shown to be the most stable reference gene when analyzed with geNorm.[21] Primers are listed in Table S1.

Table 1. Montreal classification and medication of patients of the mRNA expression study.

Characteristic	Healthy subjects	CD patients	UC patients
Number	17	15	12
Male gender (%)	7 (41%)	5 (33%)	4 (33%)
Disease localization (Montreal classification) for CD			
L1: ileum		0	
L2: colon		8 (53%)	
L3: ileocolonic		7 (47%)	
L4: upper disease		2 (13%)	
Disease behavior (Montreal classification for CD			
B1: nonstricturing, nonpenetrating		13 (87%)	
B2: stricturing		1 (7%)	
B3: penetrating		1 (7%)	
P: perianal disease		1 (7%)	
Disease localization (Montreal classification) for UC			
E1: ulcerative proctitis			0
E2: left-sided (distal) UC			7 (58%)
E3: extensive UC (pancolitis)			5 (42%)
Medication			
Steroids		2 (13%)	1 (8%)
Mesalamine		8 (53%)	12 (100%)
Thioguanines		4 (27%)	4 (33%)
Methotrexate		2 (13%)	0
Anti-TNF agents		1 (7%)	0

Patients and controls and the genetic association study

For the genetic association study, a cohort of 2355 Caucasian IBD patients, consisting of 1162 CD patients and 1193 UC patients was used. This is a subset of a cohort previously described by Weersma and colleagues.[22] Patients were recruited from six University Medical Centers in the Netherlands (details in Table S2). All patients had a confirmed diagnosis of CD or UC, fulfilling standard diagnostic criteria according to clinical, endoscopic, radiological and histopathological findings.[23,24], and were phenotyped according to the Montreal classification.[25] All patients had given written informed consent and all DNA samples and data were handled anonymously. The controls consisted of 853 Dutch blood bank donor controls.[4] All control genotypes were in Hardy-Weinberg equilibrium (data not shown, $p>0.05$).

SNP selection and genotyping

Nine tagging single nucleotide polymorphisms (SNPs) to cover the complete *FXR* gene were selected using Haploview 3.32.[26] Additionally, two functional SNPs, -1G>T and 518T>C (rs56163822 and rs61755050), previously described to affect FXR expression

and function[27], respectively, were selected. Two of the tagging SNP assays failed for technical reasons. With the remaining seven tagging SNPs, 89% of the *FXR* gene could be tagged with a genetic variance above 3%. Rs numbers and chromosomal location of the SNPs are shown in Table S3. Genotyping was successful in >98% of all controls, while call rates for all SNPs in patients were >95%, except for rs11110395 (72%) and rs10860603 (91%). From one cohort there was less DNA available so that these SNPs could not be genotyped in that cohort and rs11110395 failed in some cases for technical reasons. Genotyping was performed using TaqMan assays on a TaqMan 7900 HT (Applied Biosystems, Foster City, California, USA). All reported p values are uncorrected unless stated otherwise.

Statistical analysis

Statistical significance in mRNA expression study was determined by the Student's t-test or the non-parametric Mann-Whitney U test as appropriate. Correlation and regression analyses were used to determine the relationships between expression values. Statistical significance for correlation was determined by Spearman's coefficient test. All statistical calculations were performed with GraphPad PRISM software (Graphpad Software, La Jolla, CA, USA). Two-sided p-values <0.05 are considered statistically significant.

Statistical analysis of the genetic association study was performed using 2-tailed x^2 tests of case vs. control allele and haplotype counts for tagging and functional SNPs in Haploview v4.0[26] P-values, odds ratios (OR) and 95% confidence intervals (95% CI) are shown. The Bonferroni method was used to correct for multiple testing. All tables show the uncorrected p values.

Results

mRNA expression of FXR and its target gene SHP

FXR and its target gene *SHP* were expressed both in the ileum and ascending colon of IBD patients in remission and controls. Expression levels of *FXR* and *SHP* were markedly lower in the right colon compared to the ileum (53% and 55% lower in the right colon, respectively). There was no significant difference inileal *FXR* expression between controls, CD and UC patients (Figure 1A). However, ileal expression of *SHP* was 50% lower in CD patients compared to controls (p = 0.039), and 33% lower in UC patients compared to controls (p= 0.21) (Figure 1B). A similar trend, although not significant, was observed in the colon (data not shown). *FXR* and its target genes are exclusively expressed in the differentiated enterocyte in the villi.[15,17,19] We, therefore, also correlated FXR and SHP mRNA expression to *Villin* expression, a marker exclusively expressed in differentiated enterocytes. *Villin* expression was associated with sucrose isomaltase (*SI*, another gene expressed in differentiated enterocytes) expression in

controls, UC and CD patients (Figure 2A–C). *Villin* expression correlated also with FXR expression in healthy controls. However, the correlation was lost in UC and CD patients (Figure 2D–F). In addition, *Villin* expression showed significant correlation with *SHP* expression in healthy controls and UC patients, whereas the correlation was lost in CD patients (Figure 2G–I). Similar results were found for the correlation between *SI* expression and either *FXR* or *SHP* (data not shown). The expression of the crypt markers *c-myc* and *CCND1* were significantly correlated. However, *c-myc* and *CCDN1* did not correlate to *Villin*, *SI*, *FXR* or *SHP* expression in any of the groups (data not shown).

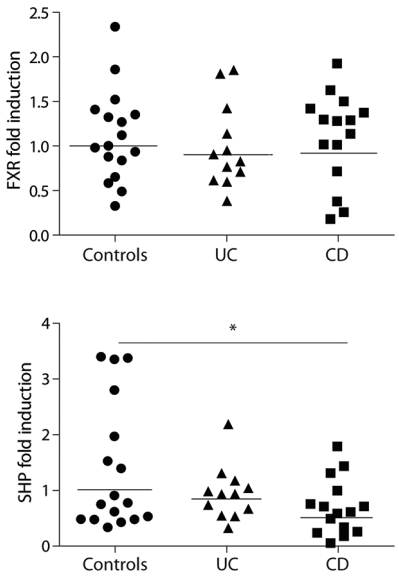


Figure 1. FXR target gene expression is decreased in patients with Crohn's disease.
Scatter plot of mRNA expression of FXR and SHP in the ileal mucosa of healthy controls (circles), ulcerative colitis (triangles) and Crohn's disease patients (squares). Horizontal lines indicate mean values. *p<0.05 compared to healthy controls.

Assessment of FXR genetic variation in IBD patients

A total of 2355 IBD patients and 853 controls were genotyped with seven tagging SNPs and two functional SNPs in *FXR*. None of the functional SNPs was associated with the presence of IBD. One of the tagging SNPs, however, displayed a significant association with IBD (rs12313471, p = 0.03, OR 1.32, 95% CI 1.02–1.71; Table S4). CD (n= 1162) and UC patients (n= 1193) were also separately compared to the 853 healthy controls. The same tagging SNP (rs12313471) was associated with UC (p = 0.049, OR 1.32, 95% CI 1.00–1.76; Table S5). None of the SNPs was associated with CD (Table S6). None of the above described associations remained significant after Bonferroni correction for multiple testing.

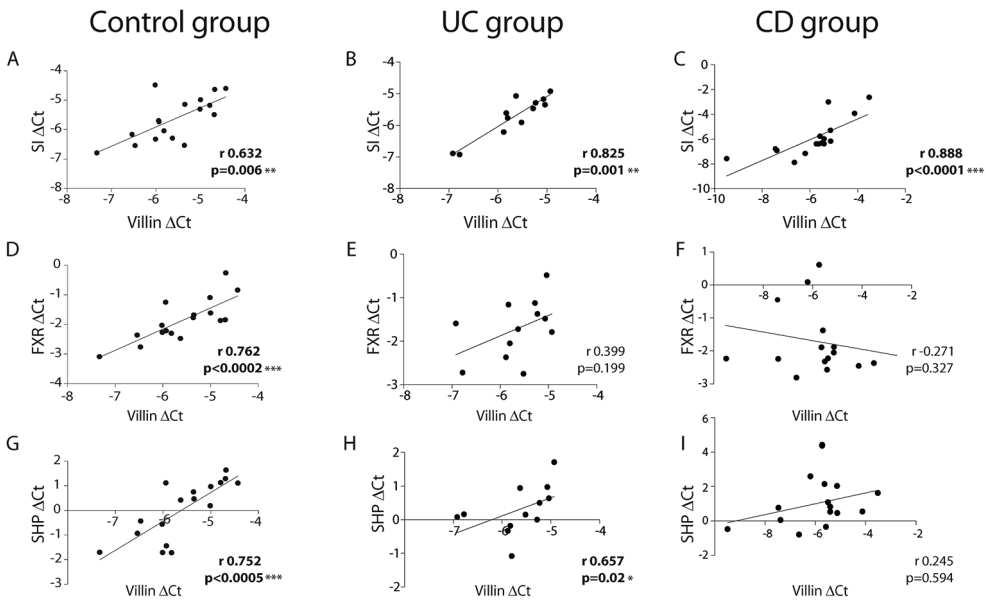


Figure 2. *FXR* and *SHP* correlate with *Villin* in healthy controls but not in Crohn's disease patients.
Ileal mRNA expression of *SI*, *FXR* and *SHP* were related by regression analyses to ileal mRNA expression of differentiation marker *Villin* in healthy controls (A, D, G), ulcerative colitis (B, E, H) and Crohn's disease patients (C, F, I). Spearman's coefficients and p values are shown. Values in bold show statistically significant correlations; *p<0.05; **p<0.01, ***p<0.001.

Subgroup analyses

Phenotypic information on the localization of the disease was present for 1132 of 1162 (97.4%) patients with CD. We analyzed whether polymorphisms of *FXR* were associated with CD location using the Montreal classification [25]. Patients with L1 (terminal ileum location; n= 257), L2 (colonic location; n = 295) and L3 (ileocolonic location; n= 580) were compared to CD patients with other disease locations. Two tagging SNPs displayed a significant association with ileal CD (L1; rs11110390, p = 0.03, OR 1.26, 95% CI 1.02–1.55 and rs4764980, p= 0.03, OR 0.80, 95% CI 0.65–0.98, Table S7). None of the SNPs was associated with colonic CD (L2, Table S8). Two SNPs showed a significant association with ileocolonic CD (L3), namely the functional SNP 518T.C (p =0.015, OR 3.08, 95% CI 1.08–8.83) and one of the tagging SNPs (rs10860603, p= 0.013, OR 1.39, 95% CI 1.07–1.81; Table S9). None of these subgroup analyses, however, remained significant after Bonferroni correction for multiple testing.

Discussion

Although the exact etiology of IBD is not completely understood, several lines of evidence point to an impaired intestinal barrier function and an abnormal immune response in genetically susceptible hosts. Recently, we reported that activation of the nuclear receptor FXR prevents inflammation in animal models of IBD with improvement of colitis symptoms, preservation of the intestinal epithelial barrier function and reduction of goblet cell loss.[17] Furthermore, a negative cross-talk between FXR and the inflammatory response at the intestinal level was demonstrated[19], probably contributing to an attenuated intestinal inflammatory status. In the present study, we showed that ileal mRNA expression of the FXR target gene *SHP* is markedly reduced in Crohn's colitis patients, whereas *FXR* expression remained unchanged. This suggests that FXR activity is decreased in this IBD subtype. Previously published genome-wide association scans in IBD patients did not identify loci containing the *FXR* gene.[9,10] Since these association studies explain only a small part of the genetic contribution in IBD, we took a candidate-gene approach and studied genetic variation in *FXR*. In the present study, none of the functional or common tagging SNPs proved to be significantly associated with CD or UC. Interestingly, the SNP 518T.C, resulting in the amino acid change M173T, showed an association with the ileocolonic phenotype of CD (Montreal L3)[25] (p=0.015, OR 3.08, 95% CI 1.08–8.83). The same allele has previously been shown to be associated with intrahepatic cholestasis of pregnancy[27], and to result in a 60% decrease in transcriptional activity of FXR. Although theM173T was not significantly associated with CD after correction for multiple testing, it may well be that it plays a modifier role in the etiology of CD in conjunction with other genes. In addition, other weak associations of different tagging SNPs with colonic or ileocolonic phenotypes disappeared after correction for multiple testing. Thus, a primary genetic defect underlying the role of FXR in CD could not be substantiated. Since the functional SNP 518T.C has a very low prevalence, the possibility of a type II error cannot be excluded. Moreover, two of the selected tagging SNP assays failed due to technical reasons. Thus it cannot be excluded that some common SNPs tagging in the remaining 11% of the *FXR* gene display an association with IBD.

Also other explanations accounting for the decreased FXR activity in CD should be considered. This includes the possibility that bile salt uptake in the ileum is reduced, for example due to decreased intestinal transit times. Indeed, several studies have shown increased fecal excretion of bile salts in patients with CD in clinical remission.[28–31]

Another mechanism contributing to this phenomenon could be an intrinsically different regulation of bile salt uptake in the ileum in CD patients.[32]

Lastly, reduced FXR target gene expression may be secondary to the reciprocal inhibition of FXR by NF-kB.[19,20] It is well established that a range of pro-inflammatory

cytokines is upregulated in the mucosa of IBD patients in remission, potentially resulting in downregulated FXR activity, leading to the observed reduced *SHP* expression in the current study.[33]

In conclusion, we found that *FXR* expression in the ileum is altered in patients with Crohn's colitis. This could not be explained by the presence of common SNPs in the FXR gene. Treatment with synthetic FXR agonists may overcome the decrease in FXR activation, possibly resulting in an amelioration of ileocolitis in patients with CD.

Acknowledgments

We thank Ellen Willemsen for technical assistance and Dr. Leo Klomp for fruitful discussions. Prof. Peter Siersema is acknowledged for critically reviewing the manuscript.

Supplementary Table 1. qRT-PCR primer list.

FXR F	5'-CTACCAGGATTTCAGACTTTGGAC-3'
FXR R	5'-GAACATAGCTTCAACCGCAGAC-3'
SHP F	5'-AGGGACCATCCTCTTCAACC-3'
SHP R	5'-TTCACACAGCACCCAGTGAG-3'
HRPT F	5'-ATTGTAATGACCAGTCAACAGGG-3'
HRPT R	5'-GCATTGTTTTGCCAGTGTCAA-3'
VILLIN F	5'-AGGGCAAGAGGAACGTGGT-3'
VILLIN R	5'-TCCCCTCGGTTGAAACTCTTC-3'
SI F	5'-GGAGATACACCAGAACAAGTAGTTCAA-3'
SI R	5'-AATCCAAGATTCCAATATGCTGG-3'
c-myc F	5'-CCACCACCAGCAGCGACT-3'
c-myc R	5'-CAGAAACAACATCGATTTCTTCCTC-3'
CCDN1 F	5'-CGTGGCCTCTAAGATGAAGGA-3'
CCDN1 R	5'-CGGTGTAGATGCACAGCTTCT-3'

Supplementary Table 2. Number of patients and hospitals.

Hospital	**Patients**
Academic Medical Centre Amsterdam	439
VU University Medical Centre Amsterdam	647
University Medical Centre Groningen	547
University Medical Centre Leiden	494
University Medical Centre St. Radboud, Nijmegen	148
University Medical Centre Utrecht	80
Total number of patients	**2355**

Supplementary Table 3. RS numbers and chromosomal locations of the SNPs in FXR.

	SNP number	**Chromosomal location on chromosome 12 (dbSNP build 132)**
Tagging SNPs	rs11837065*	100859733
	rs12313471	100864393
	rs11110390	100874901
	rs4764980	100885107
	rs11110395	100888664
	rs17030285*	100929963
	rs11610264	100932375
	rs10860603	100943948
	rs35739	100948515
Functional SNPs	-1G>T#	100887351
	518T>C#	100926058

*The rs11837065 and rs17030285 SNPs failed for technical reasons.

#The rs numbers of the functional SNPs are: rs56163822 (-1G>T) and rs61755050 (518T>C).

Supplementary Table 4. Association of genetic variants in FXR with the entire IBD cohort (patients with Crohn's disease and ulcerative colitis).

		IBD patients			Controls			p value*	OR	95% CI
		Allele counts			Allele counts					
		Minor	Major	MAF	Minor	Major	MAF			
-1G>T	A/C#	125	4461	0.027	36	1588	0.022	0.2674	1.21	0.84-1.76
518T>C	G/A	29	4545	0.006	6	1616	0.004	0.2227	1.52	0.65-3.57
rs12313471	G/A	277	4245	0.061	76	1548	0.047	**0.0317**	1.32	1.02-1.71
rs11110390	T/C	1473	3103	0.322	544	1070	0.337	0.2641	0.93	0.83-1.05
rs4764980	A/G	2271	2261	0.501	778	832	0.517	0.2179	1.07	0.96-1.20
rs11110395	T/G	156	3182	0.047	84	1538	0.052	0.4365	0.89	0.68-1.17
rs11610264	C/T	1343	3141	0.300	458	1160	0.283	0.2138	1.08	0.95-1.23
rs10860603	A/G	549	3687	0.130	214	1398	0.133	0.7492	0.97	0.82-1.15
rs35739	C/T	2033	2441	0.454	712	900	0.442	0.3790	1.05	0.94-1.18

For each table: OR = odds ratio; 95% CI = 95% confidence interval. # Minor allele / major allele; MAF = minor allele frequency. * Two-tailed P values were calculated by $\chi 2$ analysis of allele counts. Significant p values are shown in bold.

Supplementary Table 5. Association of genetic variants in FXR with ulcerative colitis.

		UC patients			Controls			p value*	OR	95% CI
		Allele counts			Allele counts					
		Minor	Major	MAF	Minor	Major	MAF			
-1G>T	A/C#	58	2264	0.025	36	1588	0.022	0.5688	1.12	0.74-1.70
518T>C	G/A	10	2292	0.004	6	1616	0.004	0.7549	1.11	0.42-2.95
rs12313471	G/A	141	2155	0.061	76	1548	0.047	**0.0487**	1.32	1.00-1.76
rs11110390	T/C	719	1605	0.309	544	1070	0.337	0.0673	0.88	0.77-1.01
rs4764980	A/G	1182	1116	0.514	778	832	0.483	0.0554	1.13	1.00-1.29
rs11110395	T/G	58	1340	0.041	84	1538	0.052	0.1824	0.80	0.57-1.12
rs11610264	C/T	705	1585	0.308	458	1160	0.283	0.0949	1.13	0.98-1.29
rs10860603	A/G	283	1769	0.138	214	1398	0.133	0.6507	1.04	0.86-1.26
rs35739	C/T	1053	1227	0.462	712	900	0.442	0.2135	1.08	0.95-1.23

Supplementary Table 6. Association of genetic variants in FXR with Crohn's disease.

		CD patients			Controls			p value*	OR	95% CI
		Allele counts			Allele counts					
		Minor	Major	MAF	Minor	Major	MAF			
-1G>T	A/C#	67	2197	0.030	36	1588	0.022	0.1550	1.33	0.88-2.00
518T>C	G/A	19	2253	0.008	6	1616	0.004	0.0725	2.05	0.84-4.99
rs12313471	G/A	136	2090	0.061	76	1548	0.047	0.0548	1.32	0.99-1.76
rs11110390	T/C	754	1498	0.335	544	1070	0.337	0.8845	1.01	0.88-1.16
rs4764980	A/G	1089	1145	0.487	778	832	0.483	0.7954	1.02	0.89-1.16
rs11110395	T/G	98	1842	0.051	84	1538	0.052	0.8636	1.03	0.72-1.31
rs11610264	C/T	638	1556	0.291	458	1160	0.283	0.6024	1.04	0.90-1.20
rs10860603	A/G	266	1918	0.122	214	1398	0.133	0.3153	0.91	0.75-1.10
rs35739	C/T	980	1214	0.447	712	900	0.442	0.7597	1.02	0.90-1.16

Supplementary Table 7. Association of genetic variants in FXR: subgroup analysis of patients with L1 Crohn's disease vs. Crohn's disease with other disease localization.

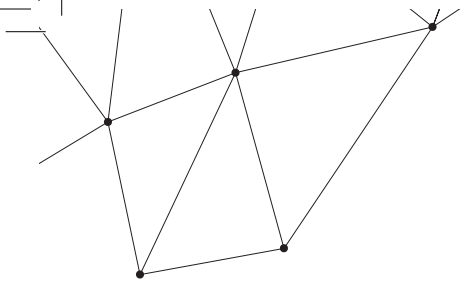
		CD L1 patients			CD patients			p value*	OR	95% CI
		Allele counts			Allele counts					
		Minor	Major	MAF	Minor	Major	MAF			
-1G>T	A/C#	15	483	0.030	49	1657	0.029	0.8701	1.10	0.61-1.96
518T>C	G/A	1	503	0.002	18	1690	0.011	0.0674	0.35	0.07-1.87
rs12313471	G/A	26	462	0.053	105	1575	0.062	0.4517	0.87	0.56-1.34
rs11110390	T/C	187	309	0.377	551	1147	0.324	**0.0294**	1.26	1.02-1.55
rs4764980	A/G	221	279	0.442	834	840	0.498	**0.0273**	0.80	0.65-0.98
rs11110395	T/G	26	400	0.061	71	1393	0.048	0.3021	1.30	0.82-2.06
rs11610264	C/T	138	348	0.284	486	1166	0.294	0.6625	0.95	0.76-1.19
rs10860603	A/G	48	436	0.099	211	1431	0.129	0.0830	0.76	0.54-1.05
rs35739	C/T	211	273	0.436	754	896	0.457	0.4139	0.92	0.75-1.13

Supplementary Table 8. Association of genetic variants in FXR: subgroup analysis of patients with L2 Crohn's disease vs. Crohn's disease with other disease localization.

		CD L2 patients			CD patients			p value*	OR	95% CI
		Allele counts			Allele counts					
		Minor	Major	MAF	Minor	Major	MAF			
-1G>T	A/C#	17	561	0.029	47	1579	0.029	0.9503	1.05	0.60-1.84
518T>C	G/A	3	573	0.005	16	1620	0.010	0.3065	0.66	0.21-2.11
rs12313471	G/A	28	538	0.049	103	1499	0.064	0.2032	0.77	0.51-1.19
rs11110390	T/C	184	388	0.322	554	1068	0.342	0.3870	0.92	0.75-1.12
rs4764980	A/G	275	293	0.484	780	826	0.486	0.9502	0.99	0.82-1.20
rs11110395	T/G	30	472	0.060	67	1321	0.048	0.3174	1.27	0.82-1.98
rs11610264	C/T	173	395	0.305	451	1119	0.287	0.4366	1.09	0.88-1.34
rs10860603	A/G	59	489	0.108	200	1378	0.127	0.2394	0.84	0.62-1.14
rs35739	C/T	258	304	0.459	707	865	0.450	0.7029	1.04	0.86-1.26

Supplementary Table 9. Association of genetic variants in FXR: subgroup analysis of patients with L3 Crohn's disease vs. Crohn's disease with other disease localization.

		CD L3 patients			CD patients			p value*	OR	95% CI
		Allele counts			Allele counts					
		Minor	Major	MAF	Minor	Major	MAF			
-1G>T	A/C#	32	1096	0.028	32	1044	0.030	0.8481	0.95	0.58-1.56
518T>C	G/A	15	1117	0.013	4	1076	0.004	**0.0150**	3.08	1.08-8.83
rs12313471	G/A	77	1037	0.069	54	1000	0.051	0.0806	1.37	0.96-1.95
rs11110390	T/C	367	759	0.326	371	697	0.347	0.2879	0.91	0.76-1.08
rs4764980	A/G	559	547	0.505	496	572	0.464	0.0558	1.18	1.00-1.39
rs11110395	T/G	41	921	0.043	56	872	0.060	0.0808	0.70	0.46-1.05
rs11610264	C/T	313	771	0.289	311	743	0.295	0.7479	0.97	0.80-1.17
rs10860603	A/G	152	942	0.139	107	925	0.104	**0.0130**	1.39	1.07-1.81
rs35739	C/T	496	592	0.456	469	577	0.448	0.7276	1.03	0.87-1.22



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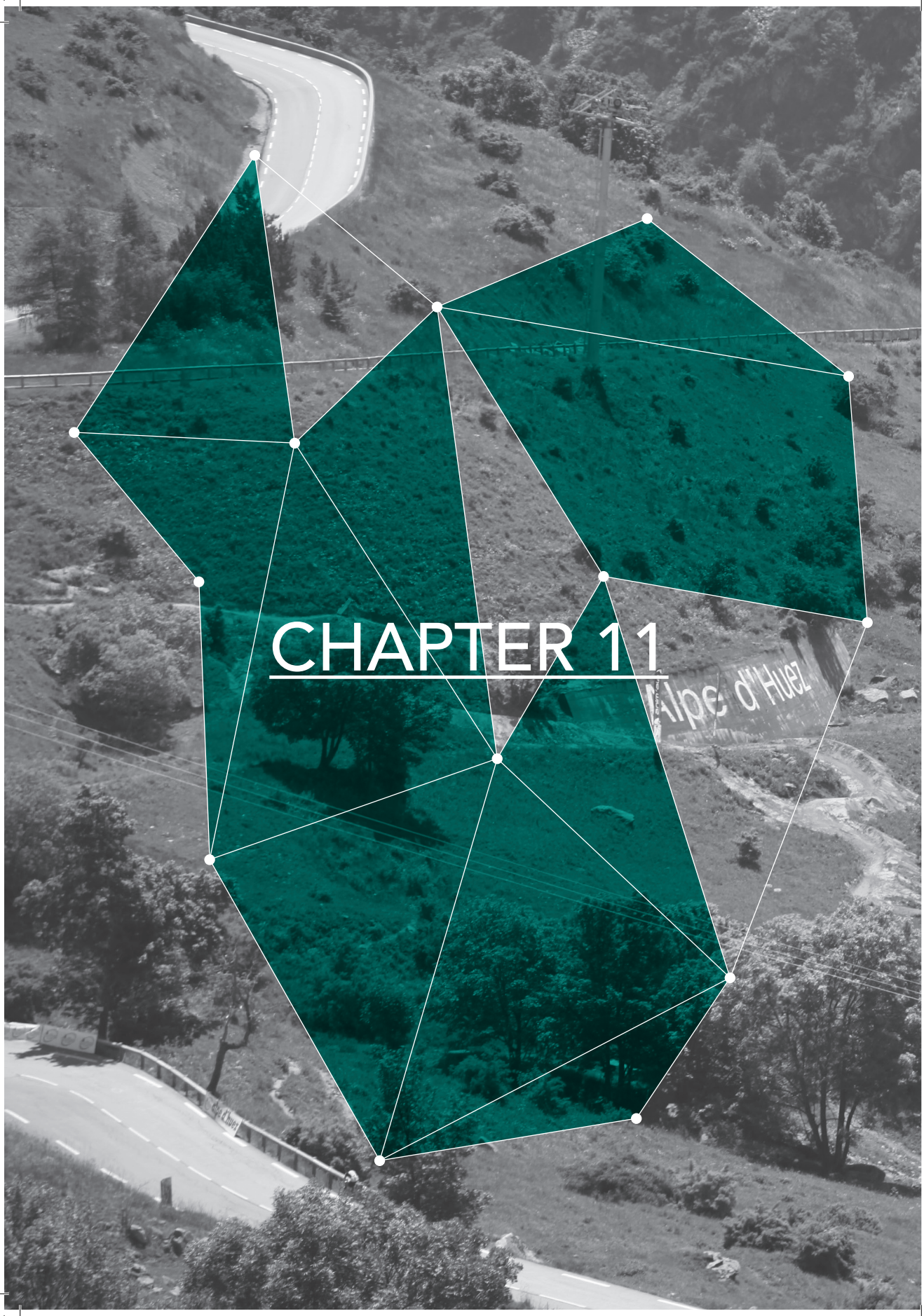


CHAPTER 11

DISCUSSION

The long and winding road

Acute pancreatitis is a disease with a highly variable course.[1] At one end of the spectrum, there are patients who are mildly ill with self-limiting disease, who require only brief hospitalization. At the other end, there are patients who have to be admitted to intensive care units because of multiple organ failure. Even if they do survive the initial acute pancreatitis attack, it is possible that they will still have to remain hospitalized for a long period because of complications that arise over time. When the studies described in this thesis were designed, our first aim was to try to distinguish patients with severe acute pancreatitis from patients with mild acute pancreatitis, preferably on their admission to hospital. We also aimed to find genes that might be associated with the development of infectious complications, and more specifically with infection of (peri-)pancreatic necrosis, since infected necrosis leads to a major increase in morbidity and mortality.[2-4]

The most prevalent causes of acute pancreatitis are gallstones or biliary sludge, and alcohol abuse.[1] Although many people develop cholelithiasis during their lives and many people drink large quantities of alcohol, only a subgroup of these will become patients who develop acute pancreatitis.

Acute pancreatitis can be described as a complex disorder, where not only environmental and lifestyle factors (i.e. gallstones and alcohol) play a role in the development of the disease, but also genetic factors. We and others therefore hypothesized that genes or, more specifically, genetic variants in genes (single nucleotide polymorphisms, SNPs) may play a role in the development of both acute pancreatitis, but also in the course and outcome of the disease.[5]

The studies that I have executed during my PhD research have all been hypothesis-driven: studies in which we searched for candidate genes that fitted the pathogenesis of acute pancreatitis. Whereas genome-wide association studies (GWAS) have been performed in many human diseases over the years, we had neither a patient cohort that was large enough to perform a GWAS, nor the resources to conduct these very

expensive studies. Over the years my research was carried out, many other groups published reports on single gene association studies in acute pancreatitis, most of which were performed in relatively small patient groups and without yielding convincing results. In order to improve the statistical power for these genetic studies, collaborations between research groups formed and several interesting results have now been published, such as the report showing that the SPINK1 N34S variant is associated with acute pancreatitis.[6] Even this collaborative study was, however, performed in a cohort of only 468 patients with acute pancreatitis.

Do genes involved in mucosal barrier integrity determine the course or outcome of acute pancreatitis?

In acute pancreatitis, infectious complications lead to an increase in morbidity and mortality. Intestinal barrier dysfunction, in the form of enterocyte damage and increased intestinal permeability, and subsequent bacterial translocation are thought to give rise to these infectious complications.[7-9] Our group showed that in acute pancreatitis, there is indeed a relationship between intestinal barrier dysfunction and infectious complications such bacteremia and infected necrosis.[10] Epithelial barrier failure may play a role not only in the origin of complications in the disease, but also contribute to the development of acute pancreatitis early on. In experimental acute pancreatitis in mice and rats, tight junction failure is an extremely early event.[11-13] In rats with experimental pancreatitis, it was shown that induction of pancreatitis led to disruption of the cytoskeleton and tight junctions in the intralobular pancreatic ducts and acinar cells. This resulted in a paracellular leak of secretions, including pancreatic enzymes, from acini and duct cells.[12,14]

Two inflammatory diseases of the digestive tract in which intestinal permeability plays a primary pathophysiological role are celiac disease and inflammatory bowel disease. Before the start of our research, genetic variants in the gene Myosin IXB (*MYO9B*), a gene presumably involved in mucosal barrier function, had been shown to be associated with both diseases.[15,16] Genetic variants of two tight junction adaptor genes, *PARD3* and *MAGI2*, were also found to be associated with celiac disease and ulcerative colitis.[17] These findings led us to believe that there might be a common deficit leading to an increase of intestinal permeability in diseases of the digestive tract and we therefore tested genetic variants in all three genes in a Dutch cohort of acute pancreatitis patients (Chapter 2). Contrary to our expectations, both *MYO9B* and *PARD3* were associated with the development of acute pancreatitis, but we did not find any association to the course or outcome of the disease. To validate our findings, we used a German cohort to test the same genetic variants, but could only show association to one variant in *MYO9B*. No association with subgroups of acute pancreatitis patients was found. A meta-analysis of both cohorts combining all the results showed that two variants in *MYO9B* were associated with the development of acute pancreatitis.

An important question in this study, but also in genetic association studies in general[18], is why we could not replicate our results. Several explanations are possible: the initial association study in our Dutch acute pancreatitis patients might show false-positive results. After correction for the number of SNPs tested, however, three SNPs in *MYO9B* and one SNP in *PARD3* were still associated with the disease. Another explanation could be that the findings in the German cohort were false-negative. The German cohort consisted of fewer patients than the Dutch cohort and the patient:control ratio was 1:1 in comparison to 1:4 in the Dutch cohort. A ratio of 1:4 is said to be the gold standard in case-control genetic association studies, as it is a way of achieving higher statistical power in common diseases using SNPs with rather small effect sizes[19], as was the case in this study. A third reason could be that there is heterogeneity between the Dutch and German cohorts. We tested for heterogeneity and found very modest evidence for it in two SNPs, one of which was the SNP in *MAGI2* that was only associated in the German cohort. The other SNP was one of those in *MYO9B*. This finding was not unexpected given the frequency differences for these two SNPs across the Dutch and German controls. In the meta-analysis, these two SNPs did not remain associated.

If we take a closer look at the Dutch and German cohorts, there appear to be several differences between them. One obvious difference is that the percentage of patients with biliary acute pancreatitis in the Dutch cohort is considerably higher than in the German cohort (54% vs. 40%). The percentage of patients who developed acute pancreatitis because of alcohol abuse is also different (19% in the Dutch cohort vs. 28% in the German cohort). Although the pathophysiology of the disease is said to be similar regardless of its etiology[20], we could imagine there would be some differences. After many years of research, the precise way in which biliary obstruction leads to acute pancreatitis still remains unclear.[21] Recently it was suggested that biliary pancreatitis is the result of bile reflux into the pancreas, where bile acids act as ligands for specific G-protein coupled receptors on the acinar cell surface, suggesting that biliary pancreatitis may be a receptor-mediated disease.[22] The mechanism of how alcohol leads to acute pancreatitis appears to be different. Alcohol and one of its metabolites (fatty acid ethyl esters) that are formed within the pancreas appear to promote the inappropriate intracellular activation of pancreatic enzymes, acute inflammation and necrosis of pancreatic tissue.[23] The metabolites also appear to generate aberrant calcium signals leading to acinar cell injury.[21]

To conclude: the results of our study show that genetic variants in the *MYO9B* gene are associated with the development of acute pancreatitis, but not with the course or outcome of the disease. Genetic association studies can never show a causal relationship between a gene and a disease, but it is possible that *MYO9B* does play a role in the (early) pathogenesis of acute pancreatitis.

Do genes involved in the innate immune system play a role in the development, course and outcome of acute pancreatitis?

Our first hypothesis-based genetic association study generated unexpected results in the sense that we found association of *MYO9B* with the development of acute pancreatitis but not with the course and outcome of the disease. Our next genetic association studies focused on genes of the innate immune system, where we hypothesized that the inflammatory response – in other words the severity of the disease and the development of complications – might be associated with these genes. Most of these genes are not only of importance for the immune reaction, but also play an important role in mucosal barrier integrity.

What role does the (innate) immune system play in acute pancreatitis? The innate immune response forms our first line of defense and after being triggered by an initial stimulus, the cells of this system release an enormous amount of pro- and anti-inflammatory mediators, including cytokines, chemokines, and complement-activation products.[24] In the early stages of acute pancreatitis, local inflammation of the pancreas develops, with tissue damage including cell necrosis and apoptosis. These are triggers for the innate immune response, often leading to a generalized pro-inflammatory response (systemic inflammatory response syndrome, SIRS). In response to SIRS, the immune system also generates a compensatory anti-inflammatory response syndrome (CARS) to limit SIRS-mediated damage. A delicate balance, therefore, between SIRS and CARS is highly important for a patient's prognosis and severity of acute pancreatitis appears to depend on this balance.[24,25] During the early phase of acute pancreatitis, patients can develop multiple organ dysfunction resulting in death because of an initial hyperinflammatory state. Recent data show that about 50% of all pancreatitis patients that need intensive care treatment are transferred to the intensive care unit on their first day of hospitalization.[26] Additionally, the incidence of organ failure is greatest on the first day of hospitalization due to acute pancreatitis.[27] Later in the disease, infections can arise because of prolonged immunosuppression, which can also be fatal.[20,24]

Activation of the innate immune system includes activation of several pathways, including the Toll-like receptor (TLR) pathway, the mannose-binding lectin pathway of complement activation and NOD-like receptor pathway. Our first study into genes of the innate immune system included selected SNPs in genes of the TLR pathway (Chapter 3). TLRs are so-called pattern recognition receptors. They are expressed by dendritic cells of the mucosal immune system of the gut, where they are constantly sensing luminal contents and have to discriminate resident luminal bacteria from potentially pathogenic bacteria.[28] TLRs are essential for maintaining an intact intestinal barrier, but they can also induce inflammatory responses to pathogenic bacteria.[28,29] Inappropriate activation of TLRs can lead to tissue damage.

Reports on the TLR pathway in acute pancreatitis are inconsistent. In experimental animal acute pancreatitis, genes and gene products of the TLR

pathway are upregulated and probably play a key role in the immune response to acute pancreatitis[30] and may determine severity and infections. Since there is a lack of information on the role of the TLR pathway in human acute pancreatitis, we performed a candidate-pathway genetic analysis in our Dutch cohort of acute pancreatitis patients, including SNPs of several genes, namely *TLR2*, *TLR4*, *CD14*, *MyD88*, *IRAK4* and *IRAK3* (*IRAK-M*). We did not find any SNPs that were associated with the development, course, or outcome of acute pancreatitis, suggesting that these genes have no major role in the pathogenesis of acute pancreatitis. We also did not find any association to (bacterial) infection of pancreatic necrosis, although others have shown that individuals carrying a genetic variant in TLR4 have a greater risk of developing Gram-negative bacterial infections.[31,32]

The two most important problems in this study were that the selected tagging SNPs had low coverage of the complete common variation within these genes, and that there was a lack of power to detect a real difference in allele frequencies between patients and controls. If, however, there were a genetic variant with a large clinical effect, it would probably have been relatively common in patients, indicating that we would probably have found it in this low-powered study.

After our study on the TLR pathway, we focused on mannose-binding lectin (MBL), which is encoded by the gene *MBL2*. MBL acts as a soluble pattern recognition molecule and is able to recognize invading micro-organisms[33], but also apoptotic and necrotic cells.[34] Recognition leads to initiation of the lectin pathway of the complement system and inflammatory mechanisms.[33] MBL might also function as a regulator of inflammation.[35] For MBL to function properly, it is necessary that it is present in the plasma in its oligomeric structure. Three coding SNPs in exon 1 of *MBL2* interfere with the formation of these higher oligomers, resulting in a decrease of functional MBL levels.[36] Three additional SNPs in the promoter region of *MBL2* also influence plasma MBL.[36] All the variants are usually in linkage equilibrium and seven haplotypes with different MBL expression levels are regularly observed.[37,38] It appears that high levels, but also low levels of MBL can be beneficial in certain pathologic situations. Deficiency of MBL is, on the one hand, associated with an increased risk of infections[37,38], such as invasive pneumococcal disease[39,40], meningococcal disease[41], and with the development of sepsis.[39,42,43] On the other hand, high levels of MBL are sometimes suggested to cause overactivation of the complement system, resulting in hyperinflammation and tissue damage.[44]

The role of MBL in acute pancreatitis is poorly understood, but it is likely that MBL plays a role in the inflammatory response in the early stages of acute pancreatitis, but possibly also in the occurrence of infections during the course of the disease. We thus performed a candidate genetic association study for the genetic variants in *MBL2* that are known to influence MBL plasma levels in our Dutch cohort of acute pancreatitis patients and controls. On a single SNP level, we did not find any association with the development of acute pancreatitis, nor with the course or outcome of the disease.

The LYPA haplotype, which leads to intermediate MBL production[38], was more prevalent among acute pancreatitis patients and increased the risk of developing the disease. There was no association of haplotypes with the disease course or outcome. Deeper exploration of the associated haplotype showed that it was not the 6-SNP haplotype that showed the strongest association with acute pancreatitis, but the 5-SNP haplotype, where rs1800451 had been excluded because of a lack of complete linkage disequilibrium.

Many studies have shown that genetic variants resulting in low MBL plasma levels are associated with predisposition to infections, but also with the development of SIRS and sepsis.[39,42,43,45] We expected to find an association with the development of (bacterial) infection of pancreatic necrosis, but in contrast we found an increased risk for the development of acute pancreatitis. Since pancreatitis has many similarities with a state of sepsis, association with the development of the disease was not completely surprising, but association to this specific haplotype is more difficult to explain. Haplotypes leading to low MBL levels are often found to be associated with infections, but it has been suggested that high functional MBL levels can lead to uncontrolled complement activation, resulting in pro-inflammatory adverse effects and rendering patients more susceptible to the development of SIRS and requiring intensive care.[44] The clinical relevance of association with the LYPA haplotype is currently unclear.

The third innate immunity component that we studied was the nucleotide-binding oligomerization domain protein 2, also known as caspase recruitment domain-containing protein 15 (NOD2/CARD15) encoded by the *NOD2* gene (Chapter 5). NOD2 belongs to the Nod-like receptors (NLRs), is expressed in intestinal epithelial and antigen-presenting cells, and can detect intracellular microorganisms and endogenous non-microbial danger or stress signals thereby promoting their clearance through initiation of pro-inflammatory responses and contributing to mucosal barrier integrity.[46] There are three loss-of-function *NOD2* genetic variants that are hypothesized to lead to impairment of intestinal barrier function and subsequent bacterial translocation.[46] These polymorphisms were the first ones to be associated with Crohn's disease[47,48], and have recently been shown to also be associated with the risk of developing spontaneous bacterial peritonitis in cirrhotic patients with acites.[49,50] Furthermore, although *NOD2* polymorphisms are not a susceptibility factor for sepsis, one of the loss-of-function polymorphisms (p.L1007fs) is associated with a greater risk of sepsis-related mortality.[51]

In our study, we used our two European cohorts (German and Dutch cohorts) and a replication cohort of US patients to study the potential association of the three loss-of-function SNPs with the development and outcome of acute pancreatitis. We observed that the p.R702W allele was, in the combined analysis, associated with a mildly increased risk of developing the disease, but showed a stronger association with acute pancreatitis-related mortality. No association with severity of pancreatitis was found, nor was there any association with either of the other two variants. When only

severe acute pancreatitis patients were entered in the analysis, there was an increase in multiple organ failure and mortality in this group as well. Patients who were homozygous for the variant allele had a higher risk of mortality than those who were heterozygous.

In the European cohorts, there was no association between the *NOD2* variant p.R702W with the development of acute pancreatitis, but there was an association when only the US cohort was analyzed. Meta-analysis showed that a mild association remained. We were unable to find association of a genetic variant with the disease outcome in our other studies, but in this study there did appear to be an association with mortality. The fact that these associations are both with the same genetic variant and that the risk increases when the subgroup analysis was performed, strengthens this finding and implies that this variant is a true causal variant in the progression of acute pancreatitis. No association with infectious complications was found, however, suggesting that acute pancreatitis patients carrying this specific variant are likely to have a compromised immune response rendering them more susceptible to organ failure with an increased associated risk of death.

The question is why only one variant in the *NOD2* gene shows association with the course (multiple organ failure) and outcome (death) of the disease in contrast to all the other genes we studied (Chapters 3 and 4). One reason that probably contributed to this is that there is some redundancy within the innate immune system. It is likely that having only one genetic variant of the genes we studied is not enough to increase susceptibility to acute pancreatitis or to complications from the disease. Individuals harboring combinations of polymorphisms may be more susceptible to complications. An example of this is suggested in patients with spontaneous bacterial peritonitis, where *NOD2* risk alleles have an additive effect with specific variants in the *TLR2* gene, leading to an even higher risk of infection.[50] Instead of an additive effect, polymorphisms of other genes within the innate immune system might also have an overriding effect, such that there is no net effect. Alternatively, SNPs could alter penetrance of other gene variants to modify disease manifestations and affect the disease severity. Combinations of genetic variants are, however, hard to investigate in our patient cohort. In general, cohorts of acute pancreatitis patients are relatively small and performing subgroup analyses reduces the patient numbers considerably, leading to severely decreased analytical power. Furthermore, looking into several combinations of genetic variants also leads to the problem of multiple testing. To overcome this, larger cohorts are needed but these are not yet available. Besides the actual size of the subgroups, they might also be rather heterogeneous. Acute pancreatitis is a disease with wide clinical variation among patients and its classification is subjective, thus leading to groups that cannot be compared adequately.

It is possible that the genes we studied contain rare variants, i.e. genetic variants with frequencies below 1%, which do have large effect sizes. Tagging these variants with common SNPs is difficult but a more thorough analysis could be carried out

by next-generation sequencing. This technique, however, also demands very large sample sizes to ensure sufficient power and the clinical relevance of SNPs that are only present in small subgroups of patients is questionable.

Another problem of genetic association studies performed in acute pancreatitis patients and focusing on genes of the innate immune system is that the results are often inconsistent. Many studies have been carried out by Asian groups, but since different ethnic populations often show variations in allele frequencies, the interpretation and comparison of results is hard.[52]

Although our results did not point to a very strong role of the innate immune system, besides the association seen between *NOD2* and mortality in acute pancreatitis patients, and although studies so far have led to inconsistent results, it is most likely that the innate immune system is closely involved in the development and course of acute pancreatitis. Even if there is no genetic basis for this involvement, it is likely that the immune system will be involved on a molecular or cellular level, or via cross-talk with the microbiota.

Do genetic polymorphisms of genes involved in the renin-angiotensin system contribute to the development and progression of acute pancreatitis?

Although the precise pathogenesis of acute pancreatitis is now being unraveled, part of the early pathogenesis remains unclear. No causal treatment is currently available for acute pancreatitis and its management is largely supportive. A better understanding of the molecular processes that lead to acute pancreatitis might, in the end, lead to new therapeutic strategies. One way of detecting genes or gene products with a potentially causal role in the pathogenesis of a disease is through genetic association studies. Our next aim was to study the genetic variants of the renin-angiotensin system (RAS) and vitamin D, which is a negative regulator of RAS, in patients with acute pancreatitis, to determine if they have a role in the development, course or outcome of the disease.

RAS is known as an actor in intravascular homeostasis, where it tightly controls and regulates extracellular fluid volume and blood pressure.[53] Within the pancreas, local RAS can stimulate increases in pancreatic enzyme secretion.[54,55] Furthermore, local RAS has also been implicated in the initiation and propagation of acute pancreatitis. In experimental acute pancreatitis, increased expression of RAS components led to proinflammatory effects and inhibition of RAS resulted in attenuated expression of proinflammatory molecules and less pancreatic damage.[56-58]

Eight SNPs from RAS and vitamin D were selected on the basis of known effects and studied in a cohort of Dutch, German and British acute pancreatitis patients. The minor allele of the renin SNP rs5707 appeared to be associated to the development of acute pancreatitis, to infection of pancreatic necrosis, and to mortality. When all the cohorts were combined, these results were replicated. Additionally, the ACE I allele (rs4646994) appeared to be associated with acute pancreatitis due to alcohol abuse in

the combined cohorts. No associations with biliary acute pancreatitis were found.

In this study we used three cohorts to obtain a larger number of patients to overcome power issues. The associations that were found, however, could not withstand correction for multiple testing and it is therefore questionable whether the reported associations represent true causal variants. The association of the renin SNP with the development of pancreatitis, infection of pancreatic necrosis, and mortality was only found in the Dutch cohort and could not be replicated in the other two cohorts. A combined analysis still led to significant results, but since the Dutch cohort was the largest cohort, it may well have driven this finding. Again, the lack of replication could possibly have been caused by heterogeneity between cohorts, which appears to be most evident if we look at the proportion of patients that had alcoholic or biliary acute pancreatitis. In the Dutch cohort, 19% of patients had pancreatitis due to alcohol abuse and 54% due to biliary disease, whereas in the German cohort these percentages were 27% and 39%, respectively. The UK cohort had too few patients to draw a fair comparison.

The question whether RAS and vitamin D are involved in the pathogenesis of acute pancreatitis cannot, therefore, be answered at this time. Further genetic association studies in larger cohorts will have to be performed in order to be able to draw firm conclusions.

Do FXR and the enterokine FGF19 play an important role in the pathogenesis of acute pancreatitis?

Early on in the course of the PhD work leading to this thesis, we had hypothesized that the farnesoid X receptor (FXR), a member of the nuclear receptor family, might be involved in acute pancreatitis, as it had been shown to maintain intestinal barrier function and prevent bacterial translocation in bile duct ligation in mice.[59] In order to study a potential role of FXR thoroughly, we decided to study the influence of Fxr on the severity of experimental acute pancreatitis in mice and to look for association of genetic variants in the *FXR* gene in acute pancreatitis patients (Chapter 7).

FXR is mainly expressed in the ileum and liver and plays a major role in the regulation of bile acid homeostasis. FXR can induce enterokine fibroblast growth factor 19 (FGF19, mouse ortholog Fgf15), resulting in negative feedback regulation of hepatic bile salt neosynthesis and, in mice, gallbladder refilling at the end of the postprandial phase.[60] More recently, both FXR and FGF19 have been suggested to play a role in inflammation. FXR has anti-inflammatory effects, which have been shown in murine models for colitis[61] and in hepatic inflammation in mice.[62] Both FXR and FGF19 probably regulate inflammation by inhibition of NF-κB.[62-64]

While we did find signs of altered Fxr activity in mice with experimental acute pancreatitis (as shown by lowered ileal expression of Fgf15), we did not find any differences in the severity of acute pancreatitis when we compared wild-type and mice deficient for *Fxr*. This led us to conclude that *Fxr* is not a major player in

experimental acute pancreatitis. In human acute pancreatitis, we found lower serum levels of FGF19 than in healthy controls, suggesting that activity of FXR in patients is also downregulated. A genetic association study in which we used nine tagging SNPs covering the complete *FXR* gene and two additional functional SNPs did not show any association with the development, course, outcome or etiology of acute pancreatitis. On the basis of these negative results, we concluded that FXR does not play a major pathogenic role in acute pancreatitis.

In contrast to acute pancreatitis, a role for FXR in inflammatory bowel disease has been more firmly established. Although the precise etiology of inflammatory bowel disease is also unclear, it is thought to result from a combination of dysregulation of the mucosal immune system, an exaggerated immune response to microbiota, and a compromised intestinal barrier function in genetically predisposed individuals.[65] Genes that have been shown to be associated with inflammatory bowel disease have generated insight into key pathogenic mechanisms, such as disturbed antibacterial defense (e.g. *NOD2*)[47,48] and involvement of mucosal barrier function (*MYO9B*).[16] Genome-wide association studies have so far found 71 confirmed disease susceptibility loci for Crohn's disease[66] and 47 loci for ulcerative colitis.[67]

Previous studies had shown that pharmacological FXR activation decreases inflammation and preserves the mucosal barrier integrity in murine colitis.[61] In addition, reciprocal repression of FXR and NFκB had been observed *in vitro* and *in vivo*.[62,68] We decided to study *FXR* and FXR target gene mRNA expression in patients with Crohn's disease and ulcerative colitis who were in clinical remission (Chapter 10). Additionally, because the susceptibility loci found in genome-wide association studies in inflammatory bowel disease only explain part of the heritability of that disease, and because we hypothesized that FXR might act as a regulator of intestinal inflammation, we decided to perform a candidate-gene study to search for association of genetic variants in *FXR* with inflammatory bowel disease. mRNA expression analysis suggested that FXR activity is decreased in patients with Crohn's disease, but not in patients with ulcerative colitis. We found that *FXR* genetic variants were not associated with either Crohn's disease or ulcerative colitis.

Is Fgf21 capable of reducing severity of acute pancreatitis? And is it possible to extrapolate results from experimental animal models to human acute pancreatitis?
Many experimental animal models for acute pancreatitis have been developed over the years to improve our understanding of the pathogenesis of pancreatitis and to develop new therapeutic strategies. One of the molecules that was tested in a mouse model and that was shown to protect acini from damage caused by pancreatitis is fibroblast growth factor 21 (FGF21).[69] Like FGF19, FGF21 is a member of the family of atypical fibroblast growth factors (FGFs), which differ from other FGFs because of their systemic, hormone-like effects.[70] FGF21 is secreted mainly by the liver, but also by other tissues including pancreas.[71,72] In cerulein-induced mouse experimental

pancreatitis, FGF21 expression was shown to be transiently upregulated in pancreatic tissue and appeared to protect the pancreas against damage from pancreatitis.[69] Our objective was to address the role of FGF21 in human acute pancreatitis (Chapter 8). We determined FGF21 plasma levels during the course of the disease and noticed, besides a considerable variation between patients, a marked elevation of FGF21 which was most clear on days three and four after onset of symptoms. We also determined FGF21 plasma levels in patients with elevated C-reactive protein (CRP) or elevated pancreatic lipase, irrespective of the underlying cause, and found elevated levels in those patients as well, indicating that FGF21 may not be specific for acute pancreatitis, but is increased in a number of inflammatory conditions. In addition, we genotyped four presumed functional SNPs in a Dutch cohort of acute pancreatitis patients and controls, but we found no association with FGF21. Finally, we studied mRNA expression of *Fgf21* and *Ddit3*, a general stress marker, in the livers of mice with cerulein-induced acute pancreatitis 24 hours after induction of the disease and found no effect. Johnson *et al.*[69] already showed that expression of *Fgf21* was only temporarily increased, indicating that this time point may have been too late. We concluded that FGF21 may play a role in general stress, but is not specific to acute pancreatitis.

An important issue in experimental animal studies, such as our own studies (Chapter 7) and the FGF21 study[69], is whether these experimental animal models of acute pancreatitis are a good way to study its pathogenesis and potential treatments. Rodent models are most widely used, but the question is whether these models are truly representative for human pancreatitis.[73] The model that we used in our studies (Chapter 7) is the so-called secretagogue hyperstimulation model. In this model, high concentrations of a molecular ortholog of the hormone cholecystokinin (cerulein) are administered and induce a mild and reversible form of pancreatitis in both rats and mice, although in mice more acinar necrosis is formed.[73,74] This supramaximal stimulation of the pancreas with an exogenous agent may not be the best model to study biliary or alcoholic acute pancreatitis, although pancreatitis due to high concentrations of secretagogue has been described, for instance after a scorpion sting or ingestion of certain kinds of insecticides.[75] The secretagogue model of acute pancreatitis has many advantages, since it is easy to use, is non-invasive and needs no surgery, can be easily reproduced, can be applied in knock-out animals and can be used to test potentially therapeutic substances. It is, however, a mild model with little mortality and few infectious complications, so that models that generate more severe acute pancreatitis may be more suitable.[75,76]

For *FGF21*, which shows 75% sequence homology at the amino acid level between humans and mice[71], it is clear that not all the data from experimental animal studies can be directly extrapolated to humans. The question whether FGF21 can reduce severity in human acute pancreatitis cannot be answered yet, but on the basis of our data it can be hypothesized that FGF21 is more likely to play a role as a

biomarker in states of general stress than a particular role in pancreatitis.

The difficulties of extrapolating results from experimental animal studies to pancreatitis in humans are widely acknowledged. In Chapter 9, we describe a study in which pretreatment with a probiotic mixture led to attenuation of damage in rats that later had experimental acute pancreatitis. After thorough research, this probiotic mixture was also given to patients with predicted severe acute pancreatitis, as a means to prevent infectious complications. This trial, however, showed that probiotic prophylaxis not only did not reduce the risk of infections, but was associated with an increased risk of mortality.[77] In our rat study, we found that in rats that had received probiotics, mucosal levels of the antioxidant glutathione were strongly upregulated because of increased biosynthesis.[13] This suggested that this was the mechanism behind improvement of intestinal barrier function in those rats. In another clinical trial, acute pancreatitis patients received antioxidants, but they had no beneficial results.[78] This demonstrates that it may be possible to strengthen a rat's endogenous defense system or its mucosal barrier function by increasing biosynthesis of glutathione using probiotics as pretreatment before induction of experimental pancreatitis, but that treating a patient with acute pancreatitis is of a completely different dimension. In another study later executed, our group has shown that treatment of rats with experimental acute pancreatitis using probiotics did not have an effect on intestinal barrier impairment.[79] Probiotics are still in use in surgical patients. A recent meta-analysis demonstrates that probiotics are safe and reduce the rate of postoperative sepsis when used in the setting of elective major abdominal surgery.[80]

Is there a future for genetic testing in acute pancreatitis?

The single gene association studies that we have performed in this PhD research have not led to a change in the management of acute pancreatitis, although they do add to our knowledge on the disease pathogenesis. The questions now are whether we should continue to do single genetic association studies, or set up large collaborations in order to perform GWAS, or should we not be focusing on genetic aspects at all?

Since I started my PhD research in 2007, much has changed in the field of genetics: new high-throughput technologies have been developed and the cost of genotyping and sequencing has dropped considerably. GWAS have detected many associations between common polymorphisms and complex diseases, such as inflammatory bowel disease, celiac disease, and diabetes mellitus. These associated variants have, however, very modest effect sizes with odds ratios lying between 1.1-1.4. To be able to detect these associated variants, large patient cohorts are necessary to reach meaningful statistical power.[81] Two necessities for such studies are research groups that are willing to cooperate and sufficient funding resources. In acute pancreatitis, however, our study in Chapter 5, with 941 patients, is the largest patient cohort studied so far. This number is still far from ideal for a hypothesis-free GWAS. In theory, it should be possible to collect DNA and clinical data on a much larger patient

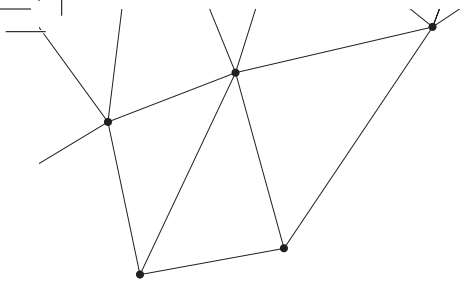
cohort, but the prerequisite is that both blood samples and data are collected in a structured manner during hospitalization of a patient, that there are resources for DNA isolation and storage, and that there is funding to collaborate in and supervise such a study. This is not yet the case in our Dutch Pancreatitis Study Group.

The next step after finding associated variants is identifying the functional implications of the associated gene in the disease studied. The study of *in vivo* effects of genes with only small effect sizes is a challenge, which is made even more complicated when one considers that associated variants do not necessarily work in isolation. Variants can have cumulative effects, but can sometimes also compensate for each other. Finally, associated variants are often located in non-coding regions of genes and apart from the hypothesis that these variants will have a function in regulating genes, there is only limited information on the biological significance of these variants.[81] New strategies include next-generation sequencing, which makes it possible to study human variation in more detail, and the study of rare variants with a frequency of <1%.[81] But there are downsides to these new techniques as well: sequencing results are sometimes hard to interpret and large cohorts are necessary to study rare variants. This is clearly a bridge too far in the study of acute pancreatitis at the moment.

Another option to study genetic association besides hypothesis-free studies might be to shift our focus somewhat. There are several causes of acute pancreatitis and the early pathogenesisis, therefore, variable. It may be wiser to focus on one distinct cause when trying to find associated genes. Between 5-25% of the adult Western population have gallstones[82], but only a small percentage of these patients will develop acute pancreatitis. There may be genes associated with the development of biliary acute pancreatitis. In a clinical setting, these genetic variants could be used to determine which patients need a cholecystectomy to prevent future episodes of acute pancreatitis.

Medical treatment and theory have evolved from expert-based medicine to evidence-based medicine, which is based upon interpreting data from large clinical trials. This means that patients are currently treated based on average outcomes from studies.[81] The new and developing approach of personalized medicine aims to treat patients on the basis of as many personal features as possible. In the future, individual genome sequences will become part of personalized medicine, although it will take time before personal sequence data will be helpful in clinical decision-making. At the start of this research, I hoped to find genetic variants associated with the risk of severe acute pancreatitis and its complications, such as infection of pancreatic necrosis. I now think it is questionable whether we could use those variants in a clinical setting for risk stratification of patients, since there are probably very many genes involved. But future research in this area will undoubtedly help to decipher the processes in acute pancreatitis pathogenesis that are not yet well understood and may lead to improved treatment for this serious disease.

The absence of a causal treatment of acute pancreatitis and of an adequate prognostic scoring method for a patient admitted with acute pancreatitis probably makes the effort that would be needed to perform genetic association studies on large cohorts rather questionable. If, however, a clinically relevant genetic association is detected in the future, patients at risk, like gallstone patients, could represent a subgroup for genetic screening and their DNA profile may be helpful in deciding whether they need a cholecystectomy.



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CHAPTER 12

SUMMARY

Acute pancreatitis is the leading cause of acute hospitalization for gastrointestinal diseases. In recent years, a multitude of clinical trials have been performed in patients with acute pancreatitis, some of which have led to significant changes in managing the disease. The majority of the trials performed have focused on ways to prevent infectious complications, because these are commonly accepted as the major cause of death for patients with this disease. The following options have been explored with this goal in mind: prophylactic use of antibiotics, probiotics, antioxidants, and early enteral feeding.

Other studies have concentrated on timing and type of intervention, once infection of the (peri-)pancreatic necrosis has been established. These studies have shown the benefits of delayed intervention in the case of infection of pancreatic necrosis and of minimal invasive surgery instead of open laparotomy, and have led to changes in practice guidelines. Many of these trials have been executed by the Dutch Pancreatitis Study Group.

Although it is obvious that these clinical trials have led to great steps forward for all patients, including those with severe and complicated acute pancreatitis, they have not been able to provide more insight into the early pathogenesis of acute pancreatitis or ways to prevent or treat the disease at an early stage. There is no causal treatment for acute pancreatitis, and treatment as such consists of supportive measures and intervention in the case of any complications that may occur. To make things even more difficult, on hospital admission it is not clear which patients will need aggressive support and which will be able to leave the hospital after several days without any intervention at all. There are several known risk factors for severe pancreatitis, including age, comorbid illnesses, a history of chronic alcohol consumption, and obesity. To preselect patients who will develop the severe form of the disease, a number of clinical scoring systems have been developed in the last three decades to estimate the risk during the early stages of the disease.

The aim of the research described in this thesis was to gain more insight into some of the determinants of acute pancreatitis, including the risk of developing the severe form of acute pancreatitis or complications during the disease.

In Part 1, five genetic association studies are described with a focus on genes involved in intestinal barrier function, the innate immune system and the renin-angiotensin-system. In Part 2, the hypotheses that FXR, a nuclear receptor involved in bile salt metabolism, in conjunction with one of its target genes FGF19, and FGF21, an atypical fibroblast growth factor that functions as a hormone in the digestive tract, are involved in the pathogenesis of acute pancreatitis were explored using both experimental animal studies and human genetic association studies. In Part 3, first the impact of pretreatment with probiotics on intestinal barrier function in rats with acute pancreatitis was explored to further unravel the pathophysio¬logy and to try and understand the effects of probiotics at the level of the small bowel mucosa. Finally, the role of FXR in inflammatory bowel disease is further explored.

The first study to be published from this PhD research was Chapter 9 (in Part 3 of this thesis), which had the mucosal barrier function as its subject, which was, at that time, the focus of our research group. Rats were subjected to experimental acute pancreatitis after pretreatment with probiotics. Acute pancreatitis led to clear intestinal barrier dysfunction, as shown by increased transepithelial bacterial passage of *E. coli*, disruption of tight junctions, and epithelial cell apoptosis in the intestine. Rats that received probiotic pretreatment showed a decrease of intestinal permeability to *E. coli*, reduced disruption of tight junctions, and attenuated epithelial cell apoptosis. Because the intestinal damage that was found in rats with experimental acute pancreatitis resembled the damage caused by ischemia-reperfusion injury of the intestine, oxidative stress parameters were studied. Oxidative stress-induced lipid peroxidation was elevated after induction of acute pancreatitis, but was not increased in the rats that had received probiotic pretreatment. It was noteworthy that these pretreated rats had increased levels of glutathione, even in comparison with sham-operated rats: this was the result of an increased ileal biosynthesis in the rats that had received probiotics.

In Chapters 2 to 6, we report our genetic association studies. The first of these studies elaborated on the mucosal barrier function. We explored genetic variants in Myosin IXB (*MYO9B*) and two tight junction adaptor genes (*PARD3* and *MAGI2*) in Dutch and German patients with acute pancreatitis. In the Dutch population, both *MYO9B* and *PARD3* were associated with the development of acute pancreatitis, whereas in the German population only one variant in *MYO9B* and one variant in *MAGI2* appeared to be associated. A meta-analysis on the results of the whole cohort, combining both Dutch and German patients, was performed and showed that two variants in *MYO9B* remained associated with acute pancreatitis. There was no association of genetic variants in these genes with the complications of the disease, such as the development of severe acute pancreatitis, infectious complications

including infection of (peri-)pancreatic necrosis, or death. Neither was any association found with a biliary cause of disease.

Chapters 3, 4 and 5 describe genetic association studies of genes involved in the innate immune system, with acute pancreatitis and the course and outcome of the disease. We first analyzed whether selected single nucleotide polymorphisms (SNPs, both tagging and functional SNPs) in genes of the Toll-like receptor (TLR) pathway, namely *TLR-2*, *TLR-4*, *CD14*, *MyD88*, *IRAK3* and *IRAK4*, were associated in patients with acute pancreatitis. We did not find any association of these genetic variants with either development of the disease, or with its course or outcome. Secondly, we showed that one of the haplotypes of the gene encoding Mannose-binding Lectin (MBL, gene *MBL2*) was associated with susceptibility to acute pancreatitis (Chapter 4). Again, no clear association on single SNP level was found and there was no association with the course or outcome of the disease. In Chapter 5, we show that a *NOD2/CARD15* loss-of-function variant p.R702W in a combined cohort of Dutch, German and US patients and controls was associated with an almost three-fold higher risk of dying from acute pancreatitis. Additionally, the risk of mortality increased with the number of affected alleles: the odds ratio to die from acute pancreatitis was 2.5 for heterozygous patients and 9.0 for patients homozygous for the p.R702W carriers.

In Chapter 6, we used a combined cohort of Dutch, German and British acute pancreatitis patients and controls to investigate genetic variants of the renin-angiotensin system and vitamin D for association with the disease. We show that one SNP in the renin gene (rs5707) was associated with development of acute pancreatitis, with infection of pancreatic necrosis, and with mortality due to acute pancreatitis in the Dutch cohort. These results were also found when all three cohorts were combined. Additionally, the ACE I allele that results in lower ACE activity (rs4646994) was associated in the combined cohort with alcohol-related acute pancreatitis.

Part 2 of this thesis reports studies on two proteins we hypothesized were involved in the pathogenesis of acute pancreatitis. In Chapter 7, we focused on the nuclear receptor FXR and the enterokine FGF19 (mouse ortholog Fgf15). To gain insight into the potential role of Fxr in acute pancreatitis, we induced acute pancreatitis in mice and studied the ileal expression of *Fxr* and its target genes including *Fgf15* after 24 and 72 hours. We saw that the expression of Fxr remained the same during acute pancreatitis, but that expression of *Fgf15* was decreased after 24 hours and normalized again after 72 hours. This change was paralleled by a change in ileal permeability, as was measured using Ussing chamber experiments. To study a pathogenic role of Fxr, we used mice lacking *Fxr* ($Fxr^{-/-}$ mice). We did not find any difference in severity of the disease between wild-type and $Fxr^{-/-}$ mice, indicating that in mouse, experimental pancreatitis Fxr does not play a major pathogenic role. In patients with acute pancreatitis, we first investigated serum levels of FGF19, which were downregulated in comparison to healthy controls. A genetic association study using nine tagging SNPs to cover the complete *FXR* gene and two functional SNPs in

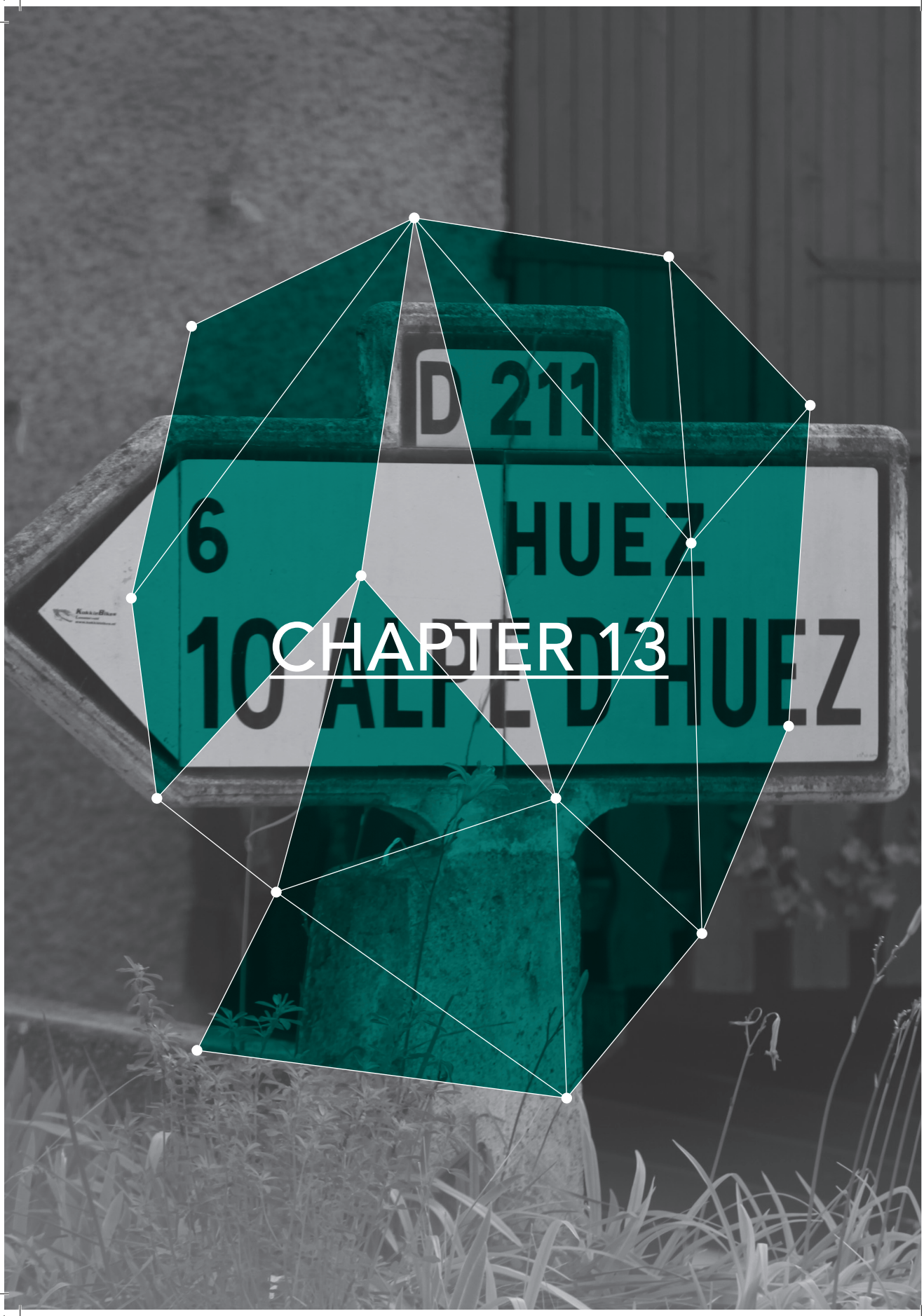
FXR did not show any association with the development, course, outcome or a biliary cause of acute pancreatitis. We concluded that *FXR* is not a major determinant in acute pancreatitis, neither in its cause nor in the disease course.

In Chapter 7, we focused on fibroblast growth factor 21 (FGF21), an atypical member of the FGF family functioning as a hormone. It is a stress-induced hepatokine affecting glucose and lipid homeostasis and can also reduce the severity of experimental acute pancreatitis in mice. We aimed to address the role of FGF21 in human and mouse acute pancreatitis. As an initial step, we determined FGF21 plasma levels in patients with acute pancreatitis. FGF21 levels were highly variable, but also clearly elevated in patients with acute pancreatitis, which was most clear at days three and four after onset of symptoms. Elevation of FGF21 was, however, not specific for acute pancreatitis, since elevated FGF21 levels were also observed in patients with elevated CRP or elevated pancreatic lipase, irrespective of the underlying cause. We also studied genetic variation in the *FGF21* locus using three SNPs with predicted loss of FGF21 function and one promoter variant with predicted impairment of stress-induction of FGF21. However, we found no association of these SNPs with acute pancreatitis.

Chapter 10 in Part 3 reports a study on FXR in inflammatory bowel disease (IBD). Pharmacological activation of FXR leads to decreased severity of inflammation and preservation of intestinal barrier integrity in murine models of colitis. FXR modulates inflammation most probably through transrepression of nuclear transcription factor kappa B (NF-κB) signaling. First we studied *FXR* and FXR target gene expression in patients with Crohn's disease and ulcerative colitis in remission. While *FXR* expression remained unchanged in both patients and controls, expression of the FXR target gene *SHP* was markedly reduced in patients with Crohn's disease, which was not the case in patients with ulcerative colitis. This suggests that FXR activity is decreased in patients with Crohn's disease. Secondly, since FXR acts as a regulator of intestinal inflammation, we hypothesized that SNPs in *FXR* might be associated with IBD. Using seven tagging SNPs and two functional SNPs in *FXR*, we found however that none of these SNPs was associated with the presence of IBD, or with Crohn's disease or ulcerative colitis separately. There was also no association with any subtypes of both diseases based on the location of inflammation.

To summarize:

- Distortion of the mucosal barrier function is an important phenomenon in acute pancreatitis, as it seems to allow intestinal bacteria to invade the patient's bloodstream, lymph nodes and, after that, also the lungs, liver and pancreas.
- The hypothesis: "*The more extensive the mucosal damage, the higher the risk of bacterial invasion and of bacterial infection of (peri-)pancreatic necrosis*" underlies most of the experiments described in this thesis.
- Both the genetic association studies and the experimental animal studies conducted to broaden our understanding of the pathogenesis for severe acute pancreatitis gave mostly negative results. We are thus unable to provide further clues on how to prevent patients with acute pancreatitis from developing the severe form of this disease.



CHAPTER 13

Nederlandse samenvatting

Bij ziekten van de tractus digestivus, het maagdarmkanaal, vormt acute pancreatitis, de acute ontsteking van de alvleesklier, de belangrijkste reden voor urgente opname in het ziekenhuis. In de laatste jaren zijn verschillende klinische studies verricht bij patiënten met acute pancreatitis die deels hebben gezorgd voor veranderingen in de zorg voor patiënten met deze ziekte. De meeste studies hebben zich gericht op het voorkomen van infectieuze complicaties, omdat deze complicaties worden gezien als de voornaamste doodsoorzaak in deze patiëntengroep. De behandelingen die onderzocht zijn, waren het profylactische gebruik van antibiotica, probiotica, antioxidanten en vroege enterale voeding, sondevoeding toegediend rechtstreeks in de darm.

Andere klinische trials waren gericht op de timing en het type interventie bij patiënten bij wie bacteriële infectie van (peri-)pancreatische necrose was vastgesteld. Deze studies tonen aan dat het belangrijk is om interventies bij geïnfecteerde pancreas necrose uit te stellen en dat een zogenaamde getrapte of gefaseerde behandeling, waar bij eerst een drain wordt geplaatst en het effect wordt afgewacht alvorens tot operatie te besluiten, de voorkeur moet krijgen boven het primair operatief verwijderen van het dode alvleesklierweefsel door middel van het openen van de buik, de laparotomie. Hierdoor zijn de richtlijnen voor de behandeling van acute pancreatitis wereldwijd veranderd. Veel van deze klinisch belangrijke studies zijn uitgevoerd door de Pancreatitis Werkgroep Nederland.

Deze klinische trials hebben geleid tot een betere behandeling voor patiënten met acute pancreatitis, in het bijzonder bij patiënten met een ernstig en gecompliceerd verloop van de ziekte. Helaas is het vooralsnog niet mogelijk gebleken meer inzicht te verkrijgen in de vroege fase van de ontstaanswijze van acute pancreatitis. Manieren om deze ziekte te voorkomen of te behandelen in een vroegtijdig stadium zijn ook nog niet gevonden. Er bestaat op dit moment dan ook geen, op het ontstaan van de ziekte gerichte behandeling voor acute pancreatitis. Bij het optreden van complicaties, zoals de bacteriële infectie van pancreas necrose, wordt gebruik gemaakt van

interventies zoals antibiotica, het plaatsen van drains voor het afvloeien van necrose en een operatie wanneer er onvoldoende drainage plaatsvindt. Een complicerende factor is dat het bij opname in het ziekenhuis niet duidelijk is of een patiënt een milde ziekte zal gaan doormaken met een snel en voorspoedig herstel zonder noodzaak voor een interventie of dat er sprake zal zijn van een gecompliceerd beloop waarvoor intensieve zorg nodig is. Van grote invloed op het krijgen en op het verloop van ernstige pancreatitis, zijn factoren als leeftijd, comorbiditeit, chronisch alcohol gebruik en obesitas. Er bestaan verschillende klinische score systemen, waarmee geprobeerd wordt om in een vroegtijdig stadium een ernstig verloop van acute pancreatitis te voorspellen. Geen van alle voldoen aan de verwachtingen.

Het onderzoek dat is beschreven in dit proefschrift was erop gericht meer inzicht te krijgen in enkele determinanten van acute pancreatitis, zoals het risico op een ernstig verloop van acute pancreatitis en het ontstaan van complicaties.

In Deel 1 van dit proefschrift worden vijf genetische associatiestudies beschreven, die gericht zijn op enkele genen betrokken bij de intestinale barrière, het aangeboren immuunsysteem en het renine-angiotensine-systeem. In Deel 2 stond de hypothese centraal dat FXR, een galzout receptor betrokken bij de stofwisseling van galzouten, samen met FGF19, een van de doelgenen van FXR, en FGF21, een groeifactor die functioneert als hormoon in het maagdarmkanaal, betrokken zijn bij de pathogenese van acute pancreatitis. Om dit uit te zoeken werd gebruik gemaakt van zowel studies in proefdieren als genetische associatiestudies bij de mens. In Deel 3 werd het effect van voorbehandeling met probiotica op de darmbarrière getest in ratten met acute pancreatitis. Tenslotte werd de rol van FXR in inflammatoire darmziekten (de ziekte van Crohn en colitis ulcerosa) verder onderzocht.

Als eerste van alle in dit proefschrift beschreven studies werd Hoofdstuk 9 gepubliceerd. Het onderwerp van deze studie was de mucosale barrière functie (de darmbarrière functie), op dat moment tevens de focus van onze onderzoeksgroep. Voor deze studie werd bij ratten, nadat zij voorbehandeld waren met probiotica, kunstmatig acute pancreatitis opgewekt. Acute pancreatitis leidde bij ratten die geen probiotica hadden gekregen tot een verslechtering van de intestinale barrière functie. Dit werd aangetoond doordat er meer bacteriën (de zogenaamde *E. coli*, een 'gewone' darmbacterie, ook aanwezig bij de mens) het kapotte slijmvlies van de darm konden passeren. Dit werd veroorzaakt , doordat de "tight junctions", de verbindingen tussen de cellen van het darmslijmvlies, beschadigd waren, en doordat er apoptose, geprogrammeerde celdood, werd waargenomen in de darm. In ratten, die voorbehandeling met probiotica hadden ontvangen, werd minder passage van *E. coli* gezien, minder schade aan de tight junctions en minder epitheliale apoptose. De darmschade die werd gevonden bij ratten met experimentele acute pancreatitis leek op de schade die wordt gezien na ischemie-reperfusie schade aan de darm. Ischemie-reperfusie schade is de beschadiging die optreedt wanneer, na tijdelijke onderbreking van de bloedstroom (en dus zuurstof gebrek en daardoor celbeschadiging) naar de

darm, de bloedstroom wordt hersteld. Daarom werden parameters voor oxidatieve stress, een situatie van zuurstoftekort, bestudeerd. Bij oxidatieve stress komen vrije radicalen vrij en in de bloedbaan. De schade die door vrije radicalen werd veroorzaakt, was verhoogd bij ratten met experimentele acute pancreatitis die geen probiotica hadden gekregen. Opvallend was dat ratten, die wel voorbehandeld waren met probiotica, verhoogde waarden van glutathion hadden, zelfs wanneer zij werden vergeleken met ratten die een operatie hadden ondergaan waarbij geen acute pancreatitis veroorzaakt werd. De oorsprong voor deze verhoogde glutathion waarden was een verhoogde aanmaak in het ileum bij ratten die probiotica hadden gekregen.

In Hoofdstukken 2 tot en met 6 worden genetische associatiestudies beschreven. De eerste van deze studies was gericht op de mucosale barrière functie. We onderzochten de aanwezigheid van genetische varianten in het Myosin IXB gen (*MYO9B*) en twee tight junction adaptor genen (*PARD3* en *MAGI2*) in Nederlandse en Duitse patiënten met acute pancreatitis. In de Nederlandse populatie waren *MYO9B* en *PARD3* geassocieerd met het ontstaan van acute pancreatitis, terwijl in het Duitse cohort slechts een variant in *MYO9B* en een in *MAGI2* geassocieerd leken te zijn. Een meta-analyse van de resultaten van het volledige cohort, waarbij de gegevens van Nederlandse en Duitse patiënten gecombineerd werden, liet zien dat twee genetische varianten in het *MYO9B* gen geassocieerd bleven met de ontwikkeling van acute pancreatitis. Geen van de geteste genetische varianten was geassocieerd met het ontstaan van complicaties van acute pancreatitis, zoals infectieuze complicaties, infectie van (peri-) pancreatische necrose specifiek of overlijden ten gevolge van de ziekte. Ook waren de genetische varianten niet geassocieerd met galstenen als oorzaak van de acute pancreatitis.

In Hoofdstukken 3, 4 en 5 worden wederom genetische associatiestudies beschreven. De onderzochte genen zijn betrokken bij het aangeboren immuunsysteem. Allereerst onderzochten we of genetische varianten in genen uit de Toll-like receptor (TLR) cascade, te weten *TLR-2*, *TLR-4*, *CD14*, *MyD88*, *IRAK3* en *IRAK4*, geassocieerd zijn met het ontstaan van acute pancreatitis en met de complicaties van de ziekte. In ons cohort van patiënten met acute pancreatitis waren deze genetische varianten niet geassocieerd met de ontwikkeling, het verloop en de uitkomst van acute pancreatitis. Daarna toonden we aan dat een van de haplotypen, gevormd door de functionele varianten in het Mannose-binding Lectin (MBL, gecodeerd door het gen *MBL2*), geassocieerd is met het ontstaan van acute pancreatitis (Hoofdstuk 4). Wanneer genetische varianten afzonderlijk werden bestudeerd, werd geen associatie gevonden met ontstaan, verloop en afloop van de ziekte. In Hoofdstuk 5 laten we aan de hand van een cohort van Nederlandse, Duitse en Amerikaanse patiënten en controles zien dat een van de varianten in *NOD2/CARD15*, p.R702W, die resulteert in verlies van functie van het eiwit, geassocieerd is met een bijna drievoudige toename van het risico op overlijden aan acute pancreatitis. Het overlijdensrisico hing samen met het aantal aangedane allelen: de odds ratio

om te overlijden aan acute pancreatitis was 2.5 in heterozygote patiënten en 9.0 in homozygote p.R702W dragers.

In Hoofdstuk 6 gebruikten we de gegevens van een cohort van Nederlandse, Duitse en Engelse acute pancreatitis patiënten en controles om te onderzoeken of genetische varianten in genen van het renine-angiotensine systeem en vitamine D geassocieerd zijn met de ziekte. We laten in dit hoofdstuk zien dat een genetische variant in het renine gen (rs5707) geassocieerd is met het ontstaan van acute pancreatitis, met infectie van pancreas necrose en met mortaliteit ten gevolge van de ziekte in het Nederlandse cohort. Wanneer de resultaten van alle cohorten gecombineerd werden, werd hetzelfde resultaat gevonden. Aanvullend laten we zien dat het ACE I allel dat resulteert in een lagere ACE activiteit (rs4646994) geassocieerd is met acute pancreatitis ten gevolge van overmatig alcohol gebruik in het gecombineerde cohort.

In Deel 2 van dit proefschrift worden twee studies over eiwitten beschreven waarvan we de hypothese hadden dat deze betrokken zouden zijn in de pathogenese van acute pancreatitis. In Hoofdstuk 7 was het onderwerp de nucleaire galzout receptor FXR en het intestinale hormoon FGF19 (ortholoog in muizen is Fgf15). Om de potentiële rol van Fxr in acute pancreatitis te bestuderen, maakten we gebruik van een muizenmodel van acute pancreatitis. In muizen werd acute pancreatitis veroorzaakt door het toedienen van injecties met een cholecystokinine analoog, waarna de mRNA expressie in het ileum van *Fxr* en de doelgenen, inclusief *Fgf15*, werd bestudeerd. De expressie van *Fxr* bleek gelijk tijdens acute pancreatitis. De expressie van *Fgf15* echter was verminderd 24 uur na inductie van acute pancreatitis en normaliseerde na 72 uur. Deze verandering werd geflankeerd door een verandering in ileum permeabiliteit gemeten bij Ussing kamer experimenten. Om een rol in de pathogenese van acute pancreatitis te onderzoeken, maakten we gebruik van muizen zonder het *Fxr* gen ($Fxr^{-/-}$ ofwel Fxr knock-out muizen). Bij het vergelijken van muizen met het *Fxr* gen met $Fxr^{-/-}$ muizen zagen we dat er tussen deze twee groepen muizen geen verschil was in de ernst van de ziekte. Dit toont aan dat *Fxr* in experimentele pancreatitis bij muizen geen belangrijke rol speelt in de pathogenese van acute pancreatitis. In patiënten met acute pancreatitis onderzochten we de plasmawaarden van FGF19. We lieten zien dat FGF19 in patiënten met acute pancreatitis verlaagd was in vergelijking met gezonde personen. Een genetische associatiestudie waarin elf genetische varianten in het *FXR* gen werden bestudeerd, liet geen associatie zien met het ontstaan, het verloop, de afloop of een biliaire oorzaak van acute pancreatitis. Wij concludeerden dat *FXR* geen belangrijke determinant is in acute pancreatitis: noch in het ontstaan, noch in het verloop van de ziekte.

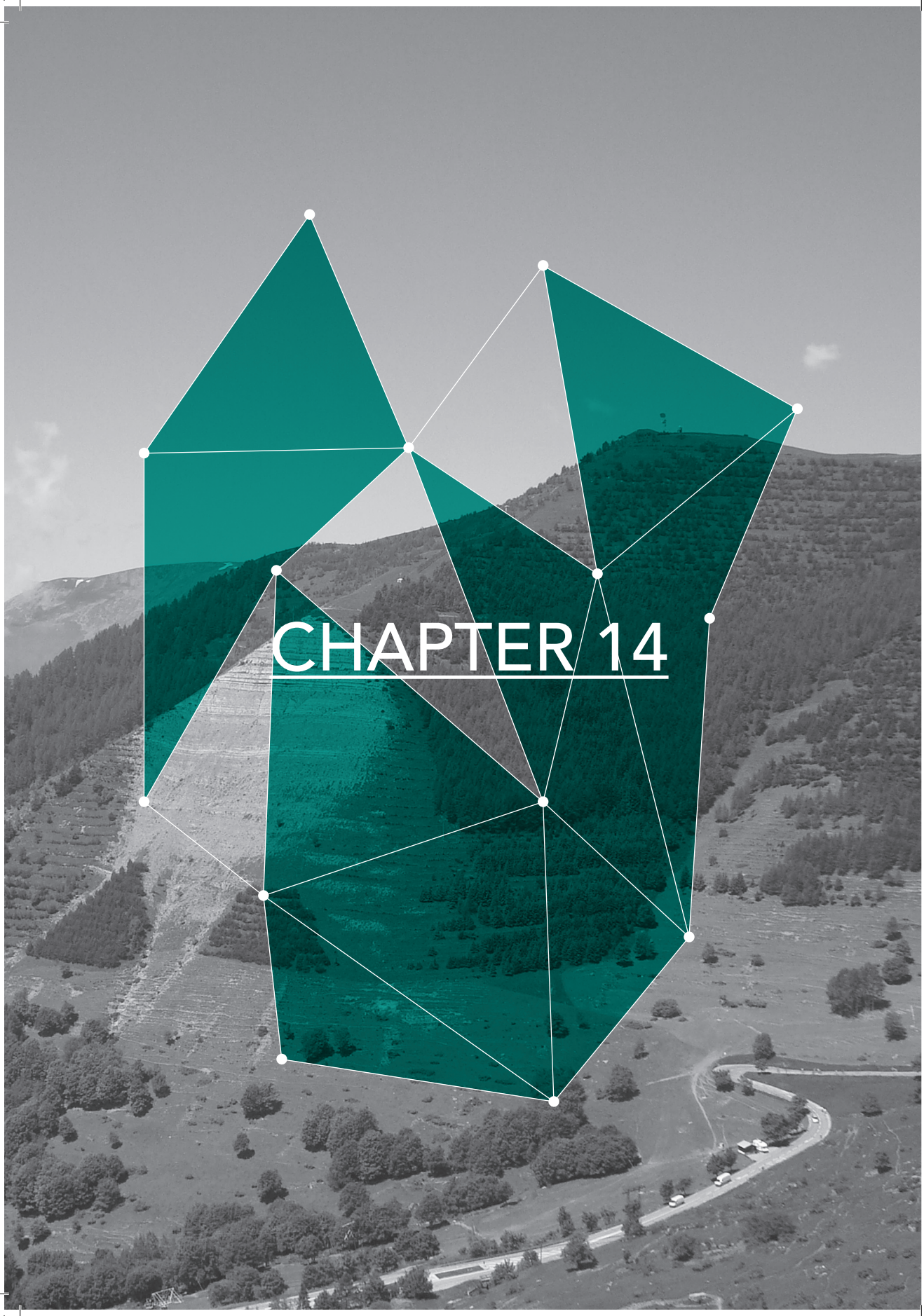
In Hoofdstuk 7 was het onderwerp van studie fibroblast groeifactor 21 (FGF21), een atypisch lid van de FGF familie, dat functioneert als een hormoon. FGF21 wordt vrijgegeven uit de lever bij stress en beïnvloedt het glucose en lipiden metabolisme en is ook in staat de ernst van experimentele acute pancreatitis in muizen te beïnvloeden.

Ons doel was om de rol van FGF21 te onderzoeken in acute pancreatitis in de mens en in de muis. We zagen dat FGF21 waarden in het bloed zeer variabel, maar ook duidelijk verhoogd waren in patiënten met acute pancreatitis. Dit was het meest duidelijk op dagen drie en vier na het ontstaan van symptomen. Verhoging van de FGF21 waarden bleek echter niet specifiek te zijn voor de ziekte acute pancreatitis. We zagen dat het FGF21 ook verhoogd was bij patiënten met een verhoogd CRP of verhoogd pancreas lipase, ongeacht de onderliggende oorzaak. Daarnaast bestudeerden we genetische variatie binnen het *FGF21* locus, waarbij we keken naar vier genetische varianten waarvan voorspeld is dat ze zorgen voor verminderde FGF21 functie. We vonden echter geen associatie van deze varianten met acute pancreatitis.

In Hoofdstuk 10 wordt een studie over FXR bij zogenaamde inflammatoire darmziekten (IBD) gepresenteerd. Het farmacologisch activeren van FXR leidt tot vermindering van de ernst van ontsteking en behoud van de darmbarrière-integriteit in muis modellen van colitis, ontsteking van de darm. FXR beïnvloedt het ontstekingsproces waarschijnlijk door het onderdrukken van de signalering van de nucleaire transcriptiefactor kappa B (NF-κB). We bestudeerden eerst de mRNA expressie van *FXR* en FXR doelgenen in patiënten met de ziekte van Crohn en colitis ulcerosa in remissie. Terwijl de expressie van *FXR* onveranderd was in zowel patiënten als de gezonde controlegroep, verminderde de expressie van *SHP*, een doelgen van FXR, in patiënten met de ziekte van Crohn. Dit was niet het geval in patiënten met colitis ulcerosa. Dit wekt de indruk dat de FXR activiteit is verminderd in patiënten met de ziekte van Crohn. Daarnaast hebben we gekeken of genetische varianten in *FXR* geassocieerd zijn met IBD. Gebruik makend van negen genetische varianten in *FXR*, zagen we dat geen van deze varianten geassocieerd is met de aanwezigheid van IBD en ook niet met de ziekte van Crohn of colitis ulcerosa apart. Bij analyse van subgroepen van patiënten, gebaseerd op locatie van inflammatie in de darm, werden ook geen associaties gevonden.

Samenvattend:

- Verstoring van de mucosale barrière functie is een belangrijk fenomeen in acute pancreatitis, aangezien dit bijdraagt aan de translocatie van bacteriën uit de darm naar het bloed van een patiënt.
- De hypothese: "*Hoe uitgebreider de mucosale schade, des te groter het risico op bacteriële invasie en bacteriële infectie van (peri-) pancreatische necrose*" ligt aan de basis van het merendeel van de experimenten beschreven in dit proefschrift.
- Zowel de genetische associatiestudies als de experimentele dierstudies uitgevoerd om het begrip van de pathogenese van ernstige acute pancreatitis te vergroten, resulteerden grotendeels in negatieve resultaten. Het is dus op basis van deze experimenten niet mogelijk geweest om nieuwe aanknopingspunten te vinden om bij patiënten de ernstige vorm van acute pancreatitis te voorkomen.


CHAPTER 14

Dankwoord

Hoe sterk is de eenzame fietser…

Mijn promotie markeert voor mij het einde van de tijd waarin ik heb geleerd wetenschappelijk onderzoek te doen: een tijd van veelvuldig vallen en telkens ook weer opstaan. Het begon allemaal in 2001, toen ik voor mijn opleiding biologie voor het eerst in een laboratorium werkte, en eindigt vandaag, 10 april 2014, in het Academiegebouw in Utrecht. Mijn promotieonderzoek, gestart in 2007, vormt het zwaartepunt in deze periode. Parallel hieraan nam wielrennen een grote plaats in mijn leven in. Jaarlijks vertrok ik in de zomer naar Le Bourg d'Oisans, het dorp aan de voet van Alpe d'Huez. Verschillende keren nam ik deel aan La Marmotte, een cyclosportieve tocht die wordt verreden over een afstand van 174km over vier Alpen cols. Afgelopen zomer nam ik opnieuw deel aan deze tocht. Het gevecht dat ik op zondag 6 juli 2013 met mezelf en de elementen aanging, heeft veel weg van de jaren waarin ik bezig was met het onderzoek voor mijn proefschrift. Dat ik geen eenzame fietser was in de afgelopen jaren moge duidelijk zijn. Er zijn veel mensen die ik dankbaar ben voor hun hulp tijdens mijn promotie onderzoek. Ik hoop dat ik hieronder niemand vergeet, maar als het zo is: vergeef het mij.

Terug naar La Marmotte. De eerste col die je tegenkomt is de Col du Glandon. Dit is een beklimming van ruim 24 km met 1150 te overbruggen hoogtemeters, eindigend op 1924m hoogte. Deze col vormt de basis voor de prestatie van de dag. De basis voor mijn proefschrift werd samen met een aantal voor mij heel belangrijke mensen gelegd.

Prof. dr. H.G. Gooszen, geachte promotor. De dag van ons eerste contact kan ik me nog goed herinneren. Ik was coassistent Chirurgie in het ziekenhuis in Apeldoorn. Door de opleider was ik uitgenodigd een operatie bij te wonen. Een professor uit Utrecht zou met hem een Belsey Mark IV-operatie uitvoeren. Die professor was u. Tijdens de operatie nodigde u mij uit om te komen praten over de mogelijkheden om onderzoek te doen binnen de Chirurgie in Utrecht. Ik koos voor uw onderzoeksgroep vanwege het grote enthousiasme dat u en de andere onderzoekers lieten zien.
U gaf mij in de afgelopen jaren alle ruimte om dit onderzoek te doen, hoewel het onderwerp toch een flink eind van de kliniek verwijderd is. Met grote belangstelling volgde u mijn onderzoek en u wist steevast de vinger op de zere plek te leggen wanneer mijn resultaten in de groep besproken werden. U toonde niet alleen interesse in mijn werk, maar ook in de mens achter het onderzoek. We hebben fijne gesprekken gevoerd en u was een belangrijke steun in moeilijke tijden. U gaf mij de mogelijkheid om zelf mijn weg te zoeken als het even niet liep zoals ik vond dat het

moest lopen. Ik heb groot respect voor u gekregen en beschouw het als een voorrecht met u samengewerkt te hebben. Daar heb ik veel van geleerd.

Prof. dr. C. Wijmenga, geachte promotor, beste Cisca. Vanaf het begin van mijn onderzoeksperiode werkte ik als gast in het laboratorium van de afdeling Medische Genetica in Utrecht onder jouw supervisie. Hoewel je in de eerste jaren van mijn onderzoeksperiode naar Groningen verhuisde om daar te gaan werken, bleef je altijd heel betrokken en was altijd bereikbaar voor alle vragen, groot of klein. Ik ben buitengewoon onder de indruk van je snelheid van reageren. Soms was er al binnen een uur reactie op resultaten, ja zelfs op manuscripten. Ik heb ons contact als zeer bijzonder ervaren: welke promovendus woont er nu bijna een half jaar in huis bij haar promotor… De goede gesprekken tijdens het eten met jou en Marten en de prettige omgang zorgden ervoor dat ik me thuis voelde.

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Dr. F.G. Schaap, beste Frank. Via Karel kwamen wij met elkaar in contact. Vanuit Amsterdam hielp jij met de FXR experimenten. Aansluitend hebben we samen een subsidie in de wacht gesleept die het mogelijk maakte om een genetische associatiestudie met als onderwerp FGF21 te doen. Je bent van alle mensen met wie ik heb samen gewerkt de meest "basale" onderzoeker en ik vind het jammer dat ik niet meer met je heb samen kunnen werken. Ik denk dat ik nog veel van je kan leren. Misschien in de toekomst?

Prof. dr. G.T. Rijkers, beste Ger. Toen ik in 2007 met mijn onderzoek begon vormde jij met Harro de basale tegenpool tegenover al het klinische geweld in onze groep. Door

jouw connecties in Nieuwegein kon ik al snel ook in dat laboratorium aan het werk. In het begin alleen voor DNA isolatie, maar gaandeweg kwam ik vaker naar Nieuwegein voor ELISAs en multiplex cytokine assays. Voor mij was het jammer dat je helemaal naar Middelburg naar de Roosevelt Academy vertrok, maar prachtig dat je nu prof bent!

Prof. dr. M.A. Boermeester, beste Marja. Omdat jij coördinator bent van de genetische studies binnen de Pancreatitis Werkgroep Nederland lag het voor de hand dat wij met elkaar zouden samenwerken. Dat samenwerking op afstand niet altijd gemakkelijk is, hebben we samen ervaren, maar niettemin hebben we samen een aantal mooie hoofdstukken geschreven. Voor de manier waarop jij je werk in de kliniek als chirurg combineert met het uitvoeren en begeleiden van veel wetenschappelijk onderzoek, waardoor je nu professor bent, heb ik groot respect.

Ik wil ook graag de MD/PhD commissie van het Universitair Medisch Centrum Utrecht bedanken voor het Alexandre Suerman Stipendium dat zij mij hebben toegekend. U hebt het mogelijk gemaakt dat ik dit onderzoek kon verrichten.

Ook de leden van de beoordelingscommissie, prof. dr. L.P.H. Leenen, prof. dr. P.D. Siersema, prof. dr. M.J.M. Bonten, prof. dr. M.J. Bruno en prof. dr. J.P.H. Drenth, ben ik zeer dankbaar voor hun tijd en interesse in mijn proefschrift.

In La Marmotte komt er na de lange, maar ook snelle en deels gevaarlijke afdaling van de Col du Glandon een stuk door de vallei. Als je alleen aan het ploeteren bent, is die weg lang en vooral met tegenwind zwaar. Dan volgt de Col du Télégraphe, een col van een kleine 12km, 856 hoogtemeters en een gemiddeld stijgingspercentage van 7,3%. In mijn hoofd altijd een gemakkelijke klim, maar de laatste keer heb ik vreselijk afgezien. Gelukkig was er een ploeg medestanders die je op deze moeilijke momenten tot onontbeerlijke steun is.

Er zijn binnen het onderzoek heel veel mensen van wie ik heb geleerd en met wie ik mooie momenten heb meegemaakt. Marc Besselink, jij bent een van mijn illustere voorgangers en was al bijna gepromoveerd toen ik begon met mijn onderzoek. Indrukwekkend hoeveel jij voor elkaar krijgt. Hjalmar van Santvoort, ik begon als jouw student met mijn project dat later uitgroeide tot mijn eigen promotie onderzoek. Net als bij Marc heb ik grote bewondering voor de wijze waarop jij je werk in de kliniek weet te combineren met het onderzoek, zeker nu ik zelf ook in de kliniek bezig ben. Harro Timmerman, het was gezellig in het dierenlab en af en toe op de fiets! Femke Lutgendorff, dank voor de fijne samenwerking waarbij je mij de kans gaf mee te werken aan hoofdstuk 9 in dit proefschrift, juist een belangrijk onderdeel in jouw eigen proefschrift. Usama Ahmed Ali, wij begonnen samen met ons

promotieonderzoek en hebben een kamer gedeeld, erg gezellig! Heel bijzonder vond ik het dat je ons, je collega's, uitnodigde voor je huwelijk in Libië. Een unieke ervaring! Olaf Bakker, ook wij begonnen bijna tegelijk aan ons onderzoek. Dank voor de fijne samenwerking en leuk dat ik kon bijdragen aan de PENGUIN studie. Sandra van Brunschot, Stefan Bouwense, en Yama Issa, ik heb niet veel met jullie samen gewerkt doordat jullie in Nijmegen zaten en ik in Utrecht. Bijzonder was het om samen met jullie bij de bruiloft van Usama te zijn. Mark van Baal en Nicolien Schepers, dank voor de gezelligheid, vooral ook in Berlijn bij de laatste UEGW! David da Costa, Janneke van Grinsven en Bob Hollemans, jullie zijn de jongste onderzoekers in onze groep. Succes met jullie onderzoek!
Een belangrijke rol in onze onderzoeksgroep spelen de research-verpleegkundigen. Ik heb vooral met Vera Zeguers en Anneke Roeterdink te maken gehad. Vera, met jou heb ik veel boven de vriezer gehangen om ingevroren materiaal op te sporen, wat ondanks de kou altijd gezellig was. En samen met Anneke hebben we vaak koffie gedronken, dank voor jullie luisterend oor. Marianne van Leeuwerden, als secretaresse van Louis wist je in Utrecht altijd van alles te regelen voor mij. Willem Renooij, dank voor de goede gesprekken. Martin de Smet, ik kan me nog een zeer onsmakelijke klus herinneren van het overgieten van … Dank voor je hulp! André Verheem, jij was van levensbelang voor alle experimenten in het dierenlab! Buiten het feit dat ik die experimenten in mijn eentje nooit voor elkaar had gekregen, was het ook altijd heel gezellig. En ik heb kennis gemaakt met veel nieuwe muziek. Alfons Kroese en Jakub Rychter, dank voor jullie hulp bij de Ussing kamerexperimenten.

Op veel verschillende locaties heb ik experimenten uitgevoerd. Ik heb zo met veel mensen samengewerkt. Onder hen ben jij, Sasha Zhernakova, één van de belangrijkste personen. Jij hebt mij de fijne kneepjes bijgebracht van de genetische associatiestudies, zowel van het praktische werk als van de analyses. Zeker voor mijn eerste studies was jij buitengewoon belangrijk. Het was voor mij een eer dat ik je paranimf mocht zijn bij jouw promotie.

I would also like to thank my colleagues from abroad with whom I had the honour to collaborate during these years. You made it possible, by combining our patient cohorts, to increase the numbers which resulted in three very nice studies, two of which have been published by now. Prof. M.M. Lerch, prof. J. Mayerle and dr. F.U. Weiss, it was a great pleasure for me to collaborate with you on chapter 2 and chapter 5 described in this thesis. I vividly remember the visit to Greifswald by private plane with our Dutch research group. It was also a pleasure to join forces with you, James Skipworth and Steven Olde Damink, on the study described in chapter 6 of this thesis. I hope we will meet again in the future!

Als beginnend onderzoeker was ik te gast in het lab van de Medische Genetica in Utrecht. Daar heb ik de hulp van veel mensen mogen ervaren. Carolien de Kovel, dank voor je hulp met de Cochran-Mantel-Haenszel analyses van hoofdstuk 2. Karen Duran, ook veel dank aan jou: van jou heb ik het sequencen geleerd.

Raffaella Gadaleta, samen hebben we gewerkt aan hoofdstuk 10 van dit proefschrift. Een stuk dat bizar snel werd geaccepteerd voor publicatie. Een mooi resultaat van onze samenwerking. Bas Oldenburg, dank voor je begeleiding bij dit werk. José ter Linde, dank voor je hulp bij het RNA isoleren, PCR-en, enzovoort. Romy Verbeek, Pauline Bus en Fiona van Schaik, jullie waren gezellige kamergenootjes! Marguerite Schipper, veel dank voor je hulp bij het scoren van de pancreas histologie, geweldig dat je er zelfs voor terugkwam op je vrije dag!

Ook in het laboratorium van de Medische Microbiologie in Nieuwegein mocht ik als gast experimenten uitvoeren. Ben de Jong, fijn dat je altijd tijd had om mij te helpen met protocollen die voor mij nieuw waren, maar voor jou gesneden koek.

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Na de Col du Télégraphe heb je nauwelijks tijd om uit te rusten. De afdaling tot aan de start van de Col du Galibier is slechts 3.4km. Afgelopen zomer was de beklimming van de Télégraphe een ware beproeving. Tegelijk wist ik dat het ergste nog moest komen. De Galibier is een prachtige klim, maar onderweg zie je daar vaak maar weinig van. Je klimt over een afstand van 18.1km, 1245 hoogtemeters. De eerste 9km

zijn qua stijgingspercentage redelijk goed te doen, maar bleken dit keer een enorme bakoven. De laatste 9km zijn erg steil. Gelukkig kom je dan in koudere luchtlagen, zodat het AFZIEN weer verandert in afzien.
Als het dan echt zwaar is, als de uitdaging het grootst is, zijn er altijd mensen die voor je klaarstaan.
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Hans en Paulien, Els en Jan, Fieke en Leon, Hetty en Alex, ook jullie leven met mij mee en staan altijd voor mij klaar. Dank dat jullie er zijn!

Na de Col du Galibier is er de lange afdaling terug naar Le Bourg d'Oisans, een kleine vijftig kilometer die in hoog tempo afgelegd kunnen worden. Dan wacht er nog een laatste, maar grote uitdaging: de Alpe d'Huez. 21 Bochten bergop over 13.8km, 1061 hoogtemeters met een gemiddeld stijgingspercentage van 7.9%. Hoewel dit

in mijn ogen bij lange na niet de mooiste beklimming van de dag is, voelt deze berg altijd als een soort thuiskomen. Niet vreemd na al die jaren. Na een dag waarop je al zo'n 160km in de hitte hebt afgelegd, waarop je jezelf al ontelbare keren bent tegengekomen, dan zijn sommige mensen er altijd. Jaar in, jaar uit in de Alpen, maar ook dag in, dag uit op alle andere dagen van het jaar.

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Curriculum Vitae

Rian M. Nijmeijer (14 januari 1980, Rolde) behaalde in 1998 haar gymnasium diploma aan het Stedelijk Gymnasium Arnhem. Zij behoorde tot de eerste lichting studenten van het University College te Utrecht waar zij in 2001 cum laude haar Bachelor's degree of Science verwierf. In het kader van haar doctoraal examen in de Biologie liep zij stage in het Hubrecht Instituut voor Ontwikkelingsbiologie en Stamcelonderzoek te Utrecht bij prof. dr. C.L. Mummery en in het Erasmus Medisch Centrum te Rotterdam bij dr. (inmiddels prof.) W.L. de Laat en prof. dr. F. Grosveld. In 2003 maakte zij deel uit van wederom de eerste lichting studenten die werd toegelaten tot SUMMA (Selective Utrecht Medical Master), de vierjarige Geneeskunde opleiding te Utrecht. Het laatste jaar bestond uit een keuze co-schap Chirurgie en een wetenschappelijke stage onder supervisie van prof. dr. H.G. Gooszen en prof. dr. L.M.A. Akkermans van de afdeling Heelkunde en prof. dr. Wijmenga van de afdeling Medische Genetica in het Universitair Medisch Centrum Utrecht. Voor het verslag van deze stage, tevens de basis van Hoofdstuk 2 uit dit proefschrift, werd haar in 2008 de Talma Eykman Prijs Geneeskunde toegekend. In oktober 2007, na het behalen van haar artsexamen, startte zij met het onderzoek dat heeft geresulteerd in dit proefschrift onder begeleiding van de promotoren prof. dr. H.G. Gooszen en prof. dr. C. Wijmenga en copromotoren dr. K.J. van Erpecum en dr. F.G. Schaap. Voor haar onderzoek ontving zij in 2007 het Alexandre Suerman Stipendium van de Raad van Bestuur van het Universitair Medisch Centrum Utrecht. Het laatste deel van het onderzoek voerde zij uit op de afdeling Genetica van het Universitair Medisch Centrum Groningen. Gedurende haar promotietraject presenteerde Rian haar onderzoek op diverse (inter-) nationale congressen. Voor Hoofdstuk 8 kreeg zij een Gastrostart subsidie van de Nederlandse Vereniging voor Gastroenterologie. In april 2012 begon zij in het kader van haar opleiding tot Maag-, Darm-, en Leverarts met de vooropleiding Interne Geneeskunde in het Rijnstate Ziekenhuis te Arnhem onder supervisie van dr. E.J.M. Mattijssen. In juni 2014 zal zij haar opleiding voortzetten bij de Maag-, Darm-, en Leverziekten in het Rijnstate Ziekenhuis bij dr. P.J. Wahab. De laatste twee jaar van de opleiding zullen plaatsvinden in het Universitair Medisch Centrum St. Radboud te Nijmegen onder begeleiding van prof. dr. J.P.H. Drenth.

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Review committee

Prof. dr. L.P.H. Leenen
Department of Surgery
University Medical Center Utrecht
Utrecht

Prof. dr. P.D. Siersema
Department of Gastroenterology and Hepatology
University Medical Center Utrecht
Utrecht

Prof. dr. M.J.M. Bonten
Department of Medical Microbiology
University Medical Center Utrecht
Utrecht

Prof. dr. M.J. Bruno
Department of Gastroenterology and Hepatology
Erasmus Medical Center
Rotterdam

Prof. dr. J.P.H. Drenth
Department of Gastroenterology and Hepatology
Radboud University Medical Center
Nijmegen