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Karimi, O.

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General discussion, summary and future perspectives

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Introduction

In this thesis studies of different aspects of inflammatory and infectious processes are described to understand the role of immune response, genetic susceptibility, environmental triggers, and luminal microbial antigens in diseases of the urogenital and gastrointestinal tracts. It is mainly the combination of these factors that determines the manifestations and course of chronic inflammatory diseases. Analysis of these different factors will help to gain insight in the susceptibility to and severity of the diseases studied.

In the first part of the thesis we investigated the role of immunogenetics in the susceptibility to and severity of *Chlamydia trachomatis* (Ct) infections of the female urogenital tract and in the second part the investigation focused on the role of immunogenetics in the chronic inflammatory bowel diseases, ulcerative colitis and Crohn's disease. The third part reviewed the role of probiotics as immune modulators in gastrointestinal diseases especially inflammatory bowel diseases and their extraintestinal manifestations.

The background of the approach taken in this thesis is that mucosal surfaces are continuously exposed to both pathogens and beneficial commensal microbes. Chronic inflammation and infection play an imperative role in the pathogenesis of diseases affecting the gastrointestinal and urogenital mucosa. During continuous immunological stimulation, a certain homeostatic balance between tolerance and immunity to non-self antigens is required, representing a unique challenge for the mucosal immune system. Microbial associations with the host may be beneficial or damaging, based on the role and identity of the participants. Our immune system determines whether a signal comes from the microbe-associated molecular pattern (MAMP) or indicates "danger" —host molecules released by damaged host cells. Apart, the MAMPs or damage-triggered danger signals are not able to induce optimal regulation of the host immunity, but together they seem sufficient to provide effective defence and homeostasis in the gastrointestinal tract.[1] Recently epithelial cells were found to play a key role in maintaining the mucosal immune homeostasis by discriminating pathogen from commensal microbes and their influence on antigen presenting cells and lymphocytes. Epithelial cells express different immune response genes like the MHC class I and II, chemokines, cytokines and prostaglandins needed for the activation of different regulatory pathways. Their function is critical in the initiation, regulation and resolution of the innate and adaptive immune response.

Part I

The first part of the thesis focuses on candidate genes involved in the immunity of *Ct* infections of the female urogenital tract. Epidemiology, diagnostic procedures and treatment of Ct infections, the most prevalent sexually transmitted bacterial infections in the Netherlands, have been described in **Chapter 1**.

The crucial first step in fighting Ct infections in the female genital tract is the recognition of the pathogen. Aberrant recognition could lead to persistent infection, upper genital tract progression and late complications including pelvic inflammatory disease and tubal pathology eventually leading to subfertility and infertility.

In this respect, screening plays a role of immense importance that could be measured by patient and societal indicators and.[2] On the other hand, the "methodological inadequacies" in the trials carried out to date make the benefit of screening uncertain.[3]

Oakeshot *et al.* showed in their POPI trial that most cases of pelvic inflammatory disease occurred in women who tested negative for chlamydia at baseline, probably because infection occurred after screening in women at high risk of infection. They conclude that in order to prevent pelvic inflammatory disease in this category of patients, screening should take place more than once a year to reach a the cost benefit and prevention of long-term sequelae.[4-6]

In case of late complications like tubal pathology the causative microorganism, *Chlamydia trachomatis*, is often not detectable by the presence its DNA since the infection is already cleared in the past. Chlamydia IgG antibody tests are then first-line recommended diagnosing tests to assess the presence of Ct infection in the past. Estimation of the tests used as Chlamydia IgG antibody test, showed that the micro-immunofluorescence test had a better accuracy than the immunofluorescence test and the enzyme-linked immunosorbent assay tests.[7]

Chapter 2 gives an overview of the immunogenetics of susceptibility markers for both ocular and sexually transmitted Ct infections. Polymorphisms in genes involved in the (innate) immune system resulting in aberrant gene function or expression play an important role in the susceptibility to and severity of disease.

Toll-like receptors

TLR2, TLR4, and TLR9 are specialized pattern-recognition receptors strongly implicated in antibacterial host defence in Ct infections and host genetic factors contribute to pathogenesis, course and severity of this infection. The recognition of *Chlamydia* LPS-signalling, and the development of murine tubal pathology have established the importance of TLR2 in the host inflammatory response to *Chlamydia* infection in several studies.[8-10] TLR-mediated signalling has recently been shown to be promoted by MHC class II molecules.[11]

Chapters 3 and **4** describe our findings of a significant trend for *TLR2* haplotype 1 (-16934 T / +2477 G) in the protection against development of symptoms and subsequent development of tubal pathology.

Murine studies have shown a role for TLR4 in *Chlamydial* infections, and recent studies in human indicate also a role for *TLR4* variation in *Chlamydia* infections.[12] Here, we observed an increasing trend for the development of tubal pathology in women positive for both Ct DNA and Ct IgG carrying *TLR4* +896 *G (rs4986790).[12]

Ct-positive patients carrying the genotype *TLR9* -1237 CC (rs5743836) are less frequently symptomatic when compared with the combined CT and TT genotypes (p=0.056). Haplotype analysis showed a decreasing trend in the development of symptoms for *TLR9* haplotype 3 (-1237 C (rs5743836)/ +2848 A (rs352140)). We have previously shown a decreasing trend in the carriage of haplotype 4 (-1237 C / +2848 G) in Ct- positive women without symptoms, with symptoms, and with tubal pathology.[13] Since both haplotypes contain the *TLR9* -1237 C allele, we hypothesise that this allele has a role in the protection against the course of *Chlamydia* infections. Lange *et al.* found a moderate increased promoter activity associated with CC genotype that might be explained by forming a NF-κB binding site due to a change to the C allele which has been shown in *in silico* analysis.[14] *In silico* analysis implicates that this SNP causes a putative NF-κB binding site that might be of relevance for the pathogenesis.[15,16] Moreover, with luciferase reporter constructs El-Omar *et al.* showed that the difference in transcriptional activation between WT and variant constructs under basal conditions was not statistically significant, but TNF-α, LPS, and CpG induced promoter transcriptional activity was significantly higher in cells transfected with the TLR9 -1237 C allele. With a comparable, novel *in silico* regulatory SNP detection method[17] we found a predicted significantly increased binding of NF-κB, avian reticuloendotheliosis viral oncogene homolog A (RelA), and signal transducer and activator of transcription (STAT3) to the *TLR9* -1237 C variant. According to these predictions, the *TLR9* -1237 C allele creates an increased affinity for NF-κB which in its turn increases the transcriptional activity of the gene[15,16], leading to enhanced production of cytokines and chemokines, which provides a plausible mechanistic explanation for our observed decrease of symptoms in Ct infected patients.

Our study shows that TLR2, TLR4, and TLR9 may influence the clinical course of *Chlamydia* infections. Haplotypes protect against a more severe clinical course of infection. Logistic regression modelling of the effect of *TLR4* SNPs, *TLR2* haplotypes, and *TLR9* haplotypes showed significant effects of specific combinations of *TLR2* and *TLR9* on Ct infections. Recently, mice lacking TLR2, TLR4, and TLR9 were found less susceptible to Salmonella infections than were mice lacking only TLR2 and 4 or TLR 4 and 9.[18]

Chemokine receptor CXCR5

Following infection, the chemokine receptor CXCR5 is necessary for formation of tissue lymphoid aggregates at the site of infection. Disruption of this chemokine pathway alters the immune response. We showed the importance of CXCR5 for Ct infections (**Chapter 5**). We observed a statistically significant protective effect of *CXCR5* +9086*C in Ct infections and the development of tubal pathology.

CXCR5 and its ligand CXCL13 are important during development of lymphoid tissue. In mice, lack of CXCR5 causes significantly increased cytokine production by NKT cells during *Chlamydia muridarum* (MoPn) infection compared to wildtype controls. It is possible that activated NKT cells are cytotoxic to *Chlamydial* infected epithelial cells in the genital tract. A new finding that has not been described before is that of *Chlamydia* directly activating populations of NKT cells. Since *Chlamydiae* do not make sphingolipids (Antigens that activate NKT cells). This implies that the *chlamydial* membrane incorporates a self Ag of which there is an autoimmune reaction. Most important, using an intracervical inoculation method it had been demonstrated that chemokines and cytokines essential for induction of both the innate response and adaptive response are produced in the early stages of the first developmental cycle of *Chlamydiae*[19], although they did not measure CXCR5. We found that CXCR5 has the ability to suppress cytokine function and regulate the function of NKT cells. A protective effect of the *CXCR5* +9086 T>C polymorphism and the haplotypes containing its mutant allele was found on the development of primary *Ct* infection and late complications of this disease defined as tubal pathology. Different members of the chemokine family have been studied in relation to infection with *Chlamydiae*. CCR5 and CXCR3 are expressed on Th1 and dendritic cells[20], and present in the immune response of the genital tract and clearance of infection. *Chlamydia* promotes migration of dendritic cells and activation and recruitment of T cells. CCR5-related inflammatory responses seem crucial for the development of tubal factor infertility following *Ct* infection.[21] CCL5 supports the induction of Th1 cytokines, and its suppression results in delayed clearance of *C. muridarum* infections. Thus, *Chlamydial* immunity seems partly mediated by Th1 immune responses driven by CCL5.[22] Expression of CCR7 by *Chlamydia*-pulsed DC may prolong maturation of the dendritic cells following adoptive transfer.[23] CXCR2 seems to have no effect on the disease course in lower genital tract infection caused by *C. muridarum* in mice. However, CXCR2-/- mice show delayed ascending infection into the upper genital tract.[24] Recently, Olive *et al.* have shown that CXCR3 or CCR5 deficient mice are unable to clear *Chlamydia* infections, and that this inability is due to impaired homing of CD4+ T-cells to the genital tract.[25] This indicates a pivotal role of CXCR3 and CCR5 in clearing *Chlamydia* infections and implies a potential role for other chemokines and chemokine receptors. CXCR4, CXCR7 and CCR5 function as co-receptors for virus entry in human immunodeficiency virus (HIV) -1 infection.[26-29]

An altered expression of CXCR5/CXCL13 may cause B-cell dysfunction during HIV-1 infection.[30] Moreover, HIV changes susceptibility to Ct infection, suggesting multiple routes for CXCR5 to affect Ct susceptibility.

Future perspectives

Three important steps have to be made to translate these findings into patient management: (1) the study has to be confirmed and SNPs in other genes have to be added in a genetic trait to obtain synergy in the prediction of susceptibility or protection to CT infection, (2) this defined and confirmed genetic trait has to be added to potentially strengthen current clinical prediction rules on complication rates after CT infection with tubal pathology, and (3) implement strategies to promote a faster path for genetic knowledge from bench to bedside. The various stakeholders in public health play a key role in translating the implications of genomics such as deriving from molecular epidemiology and host-pathogen genomics. This knowledge will not only enable clinical interventions but also health promotion messages and disease prevention programs to be targeted at susceptible individuals as well as subgroups of the population based on their genomic profile (personalized healthcare).[31,32] The field involved in this translation is called Public Health Genomics which has as major task ''the responsible and effective translation of genome-based knowledge and technologies into public policy and health services for the benefit of population health'' (Bellagio statement, 2005: see www.graphint.org for details).

Two potential clinical applications of genetic traits can be foreseen: 1) the subfertility risk profiling is currently done only by CT IgG serology testing using a positive CT IgG to triage women to undergo invasive laparoscopy (only 55-60% will have tubal pathology making them eligible for IVF), while CT negative IgG serology triages women to try to get pregnant for another year (while 20% has actually tubal pathology). These high percentages of misdiagnosis can potentially be reduced by adding genetic profiles to the current serology to better triage women, and 2) CT vaccine programmes, if successful, need clinical trials to define populations at risk, otherwise very large populations need to be enrolled. If a genetic risk trait can be determined one could potentially better define populations at risk and populations not at risk to reduce clinical trial size.

Part II

The second part of the thesis comprises the role of genes involved in the immune regulation of inflammatory bowel diseases and their extraintestinal manifestations. Gene polymorphisms could have an influence on the susceptibility and severity of the disease. JAK2, F13A1, PPAR-γ and those involved in the autophagic process have been studied.

Janus kinase 2 (JAK2)

In **Chapter 6** we studied the presence of the Janus kinase 2 (*JAK2*) V617F somatic gain-of-function mutation (rs77375493) in genomic DNA isolated from peripheral blood of patients with inflammatory bowel disease (IBD) who had suffered episodes of thrombosis.
Despite the use of a sensitive quantitative genotyping technique we were unable to detect the common mutation found on this gene. This suggests that other mechanisms than the acquisition of this mutation or other genes and environmental factors play an important role in the pathogenesis of thrombosis in IBD albeit the small number of cases with splanchnic vein thrombosis (SVT) in our series, which is mainly associated with the *JAK2* V617F mutation[33] could also be an explanation of this finding as it is discussed in the following paragraph.
Recent studies in patients with Philadelphia-negative myeloproliferative neoplasms (MPN) have demonstrated that the mutation is not acquired randomly but is preferentially acquired in *cis* on an inherited common *JAK2* haplotype designated '46/1' or 'GGCC' present in approximately 50% of healthy individuals (34-36). The germline *JAK2* 46/1 haplotype has been associated with the development of *JAK2* V617F-positive as well as *JAK2* V617F-negative MPNs carrying mutations across *JAK2* exon 12 or the *MPL* gene.[37,38] Whether the minor C allele of rs12343867 tagging this haplotype is an MPN phenotype modifier has been studied in relation to complications such as thrombosis. No differences were found in the occurrence of vascular complications (venous and arterial thrombosis, haemorrhage) according to 46/1 genotypes.[39,40] Unexpectedly, in a retrospective study from Napoli, Italy the CC genotype of rs12343867 occurred more frequently than noncarriers of allele C in portal–mesenteric venous thrombosis (PVT) patients with SVT without the *JAK2* V617F mutation suggesting the *JAK2* 46/1 haplotype is an independent risk factor for SVT.[41] This study has been criticized by Smalberg *et al*. since the control group was not in Hardy-Weinberg equilibrium and the frequency of the CC genotype (2.8%) differed significantly from other European control populations (6.0%).[42] Smalberg *et al*. studying a representative sample of 199 newly diagnosed patients with SVT from multiple European countries and unrelated age and sex matched controls, observed a clear association between the 46/1 haplotype and the development of *JAK2* V617F-positive SVT. In addition, this study found an association between haplotype 46/1 and SVT in *JAK2* V617F-negative SVT patients with MPNs, whereas no association was observed in *JAK2* V617F-negative SVT patients without MPNs.[42]
Another allele that tags the *JAK2* 46/1 haplotype, C allele of rs10758669, has shown a clear association in GWAS and meta-GWAS with both CD[43,44] and UC.[45-47] The association of *JAK2* and of genes such as *IL23R*, *STAT3*, *IL12B* emerging from these and other studies implicated the pathogenesis of the IL-23/Th17 signalling pathway, which promotes inflammation in the adaptive immune response, in IBD.[48] Carrying either of the IBD-associated susceptibility alleles (i.e. the 46/1 haplotype) in *JAK2* and in *STAT3* led to a significantly enhanced

susceptibility to DNA damage, as estimated by single cell gel electrophoresis assays in peripheral blood leukocytes from CD patients. This suggested that this enhanced genomic instability could be at least partly responsible for both the enhanced colonic mutagenesis associated with CD, and also the state of chronic inflammation.[49]

Blood coagulation factor Factor XIII and its gene F13A1

Patients with IBD have an increased risk of venous thromboembolisms and recurrent thrombotic disease compared with the general population. Plasma coagulation factor XIII activity has been reported to be decreased in IBD. Since two common non-synonymous polymorphisms in the gene encoding coagulation factor XIII, A1 polypeptide *F13A1* Val34Leu (rs5985) and *F13A1* Pro564Leu (rs5982) are possibly associated with thrombotic diseases, it was important to know whether these polymorphisms may be responsible for the increased risk of thrombotic disease in IBD (**Chapter 7**).

The *Val34Leu* polymorphism within AP-FXIII is located critically nearby the thrombin activation site and seems to protect moderately against myocardial infarction, deep venous thrombosis and ischemic stroke.[50] This protective effect has been attributed to structural characteristics of the bound AP-FXIII, influencing the affinity of thrombin to the bound AP-FXIII. In the study reported in this thesis, we found a significant association between UC, CD and IBD and carriage of allele 34Leu but no association with the Pro564Leu polymorphism. In previous small studies no differences in genotype distributions were found[51,52], and a somewhat larger study failed to reveal any difference in allele frequencies.[53] A slightly greater prevalence of *F13A1* Leu34 homozygozity in CD vs. controls has been found in a more recent population based study.[54] In another small study, Koutroubakis *et al*. compared the prevalence of Val34Leu in IBD patients with vascular complications and non-IBD thrombotic patients, and found no significant differences.[55] Moreover, meta-analyses of GWAS studies on 3,291 UC patients and 12,607 controls[47] and 4,586 CD patients and 12,119 controls[44] found no evidence for the involvement of common *F13A1* SNPs including *F13A1* Val34Leu in UC and CD susceptibility. Altogether, the evidence for involvement of both *F13A1* SNPs in IBD susceptibility is weak at most. Consequently, it cannot be excluded that the findings of a positive association of 34Leu carriers with IBD in our study results from a type II error. We did not observe statistically significant differences between the studied polymorphisms and the extent of UC nor between the subdivisions of the Vienna classification in CD patients. A slight trend was observed for a decreasing carriage of the 564Leu/Leu genotype in penetrating CD. In UC the absence of associations of the polymorphisms with the disease sub phenotypes, maximum disease extent and need for colectomy for medically refractory disease, and in CD with the Vienna classification, are not completely unexpected. No successful replications of genotype – phenotype associations have been reported, with the

exception of the association between *NOD2* genotypes and structuring/penetrating, ileal CD, and the association between extensive and severe UC and the *HLA-DRB1**0103 haplotype.[56] Indeed, it has been suggested that despite the potential promise of genetic markers in prediction of disease course, they may never fully predict evolution of disease, because of incomplete penetrance, their modest to low frequency and the role of other environmental factors. The place of genetic markers in prediction of disease outcome is dependent on their crosstalk with other molecular markers, clinical data and environmental factors.[57] Although considerable efforts in DNA sequencing in patients with atherothrombotic diseases has been performed it cannot be excluded that other *F13A1* genetic variation (rare variants with high penetrance, insertion-deletions, inversion, translocations or copy number polymorphisms) will be discovered in future next-generation deep sequencing studies.

As stated in the discussion of our results, genetic studies in IBD patients with an increased risk to develop venous thromboembolic disorders might follow the recommendations recently made by Muszbek *et al*. for the study of *F13A1* genetic variation in ischemic stroke: i.e., well-designed prospective studies with interaction of the polymorphisms with possible modifying factors, most importantly with fibrinogen level, should be investigated.[50] This will improve our understanding of the mechanisms of pathogenesis of thrombotic complications in IBD.

Peroxisome proliferator-activated receptor gamma (PPAR-γ)

The peroxisome proliferator-activated receptor gamma (PPAR-γ) on the membrane of the cell nucleus is a transcription factor which regulates numerous genes involved in lipid and lipoprotein metabolism, for glucose homeostasis, and down regulates the inflammatory pathways. Impaired expression of PPAR-γ in colonic epithelial cells in UC and increased expression in hypertrophic mesenteric adipose tissue in Crohn's disease have been reported. Recently Yamamoto-Furusho et al.,[58] showed that PPAR-γ mRNA expression was decreased in rectal mucosa from patients with active UC compared to UC patients in remission and also found that PPAR-γ gene expression correlated negatively with endoscopic activity. In **Chapter 8** we explored the association of PPAR-γ polymorphisms Pro12Ala (rs1801282) and C161T (rs3856806) with IBD and with UC and CD subphenotypes in Chinese and Dutch populations. In Chinese UC patients carriers of allele T of the C161T polymorphism were more common than in controls (37.7% vs. 25.5%, OR 1.77, 95%CI 1.18–2.68, P< 0.007) and Ala carriers of the Pro12Ala polymorphism associated with more extensive disease (P< 0.002). No associations were found with CD. In the Dutch population we found no associations with UC, CD or IBD for the studied C161T polymorphism. This extends the results of a previous study in Dutch IBD patients with absence of associations of 8 PPAR-γ polymorphisms (including Pro12Ala).[59] The ethnic difference could be responsible

for the observed difference in associations with the C161T polymorphism in UC patients. Alternatively, this polymorphism is merely a genetic marker associated with Chinese UC and the causative variant allele is in strong linkage disequilibrium with the PPAR-γ 161T allele in the Chinese but not the Dutch population. In addition, conflicting results might be a result of different clinical pathologies, possibly resulting from the interaction of genetic (modifier) genes with varying environmental factors, for example, commensal intestinal flora.

Rosiglitazone a member of the thiazolidinedione class of drugs is a selective ligand of PPAR-γ, therefore by binding this transcription factor it diminishes NF-κB levels. The recent findings confirmed the role of the PPAR-γ gene as a potential therapeutic target in UC, as suggested in a previous study that demonstrated the efficacy of rosiglitazone (thiazolidinedione agonist for PPAR-γ) in patients with mild to moderate UC activity.[60] Unfortunately, the European Medicines Agency recommended on 23 September 2010 that Avandia be suspended from the European market because its "risks outweigh its benefits".

However, other effects may suggest that new pharmacological approaches are possible. For example, PPAR-γ regulates transcription of target genes by the formation of heterodimers with the retinoid X receptor (RXR) and their subsequent binding to PPAR response elements (PPRE) in the promoter regions of target genes.

Recent experimental studies suggest that one of possible mechanism by which PPAR-γ may prevent onset of IBD is by PPAR-γ-mediated differentiation of M1 macrophages into M2 macrophages in the presence of anti-inflammatory cytokines IL-4, IL-13, IL-10, and TGF-β[61] or through PPAR-γ-induced cathepsin L and reduction of beclin 1 and LC3 protein levels may decrease autophagy[62], a process that is discussed in the next paragraph.

Autophagy

Autophagy has long been recognized as an evolutionarily conserved process by which cytoplasmic organelles can be degraded in response to starvation. More recently, it also has been implicated in the engulfment and degradation of intracellular pathogens (xenophagy).[63] In **chapter 9** we describe the link between autophagy and innate and adaptive immunity. We discussed the associations with Crohn's disease (CD) of polymorphisms in the *autophagy related 16-like 1* gene (*ATG16L1*) and the *immunity-related GTPase family, M* locus (*IRGM*) that came in an unbiased way from GWAS and the identification of a common 20.1-kb copy number variation (CNV) deletion in the region upstream of *IRGM*.[64] More recently, GWAS meta-analyses and replication studies confirmed that the *ATG16L1* rs2241880 ("T300A") and noncoding *IRGM* polymorphisms are important low-penetrant markers for CD susceptibility.[44,65,66] Such studies performed in ulcerative colitis (UC) suggested associations with SNPs in these genes are much weaker (and often not significant at the genome-wide level).[47,65,66] One of these studies in UC detected an association wjth *DAP1*

(encoding "death-associated protein").[47] DAP1 is a negative regulator of autophagy and a substrate of mammalian target of rapamycin (mTOR).[67] A single-Centre study from Leuven, Belgium studying tagging SNPs in 12 selected autophagy genes associated the common rs12303764 (with unknown function) in the gene encoding ULK1, an mTOR substrate and positive mediator in autophagy initiation, with CD. No evidence for epistasis with the 3 main CD-associated *NOD2* variants was apparent.[68]

Patients with defects in the intracellular bacteria sensor NOD2 have lower α-defensin expression by Paneth cells[69] and similarly, the *ATG16L1* CD-risk variant impairs exocytosis of Paneth cell secretory granules, thereby inhibiting antimicrobial protein release.[70]

NOD2 also directly interacts with autophagy, and NOD2 might even physically interact with ATG16L1. Thus, autophagy appears to be the converging theme of two of the strongest genetic risk factors of CD.[71,72] These multiple genetic lesions that target different levels of immune control of the microbiota might lead to impaired clearance of bacteria inducing an inflammatory reaction in the mucosa leading to the clinical features of CD.

The associations observed between CD and noncoding *IRGM* polymorphisms in individuals of European descent might result from the "silent" common exonic synonymous SNP rs10065172 in perfect linkage disequilibrium with the 20.1 kb deletion.[73] MicroRNAs of the miRNA-196 family have been shown to be overexpressed in intestinal epithelia of CD patients and downregulate only the protective (C) variant allele. This leads to loss of the regulation of IRGM expression that affects the efficacy of autophagy.[73] We previously put forward that autophagy may play an important role in the mechanisms involved in the aetiopathogenesis of *Chlamydia*l infections.

With regard to *Chlamydia*, the invading bacterium is not being targeted for lysosomal degradation and partially antagonizes the host autophagic process. *Chlamydia*l inclusion bodies are juxtaposed to LC3-positive structures. Al-Younes *et al.* postulated that *Chlamydia*e utilize nutrients derived from autophagy to promote intracellular growth.[74] More recently, they describe that the depletion of LC3 in autophagy-deficient cells reduces *Chlamydia*l propagation.[75]

Future perspectives

GWAS, meta-GWAS and next-generation sequencing technologies have already provided insight into the genetic susceptibility in IBD. It is clear that the future of the immunogenetics in IBD is next-generation GWAS and sequencing. GWAS already identified several genomic regions for IBD-risk factors. These have deepened our insight into several genetic features of UC and CD. The multigenic contributions were found to overlap in both diseases, and different emerging clusters of risk loci imply that disease processes possibly consist of diverse pathology.

As a next-generation sequencing platform, the 1000 genome project is the first to sequence genomes of a large number of people, and thereby provides a widespread resource on human genetic variation. This widespread analysis of genetic diversity in the population will improve our knowledge of rare SNPs, insertion or deletion mutations and copy number variations[76] and provide us with an overview of genetic diversity that represents the general population. Perhaps future studies will explain the missing heredity to explain the genetics of Crohn's disease and ulcerative colitis. Another approach is the results of the first step in sequencing the Crohn's disease-associated exome from Momozawa *et al*. These authors identify low frequency coding variants conferring protection against inflammatory bowel disease in *IL23R*. However, they conclude that rare coding variants in positional candidates do not make a large contribution to inherited predisposition to Crohn's disease.[77] Further developments with genome, targeted capture and sequencing methods have been efficiently used to identify causative variants for rare Mendelian disorders.[39,78,79] Therefore, targeted sequencing of possible candidate genes may identify rare variants that contribute to common diseases but it is too early to make any firm future perspectives of the application of this technology to the understanding of these diseases.

Part III

Probiotics as immunoregulators of intestinal inflammation

It has recently been stated that the gut microbiota is the next frontier in understanding human health and development of biotherapeutics. Therefore, we have concentrated in this thesis (**Chapters 10**, **11** and **12**) and in our publications on the subject in trying to understand the role of probiotics may play in modulating the gut microbiota and in controlling inflammation. The human gastrointestinal tract harbours about 500 to 1000 different bacterial species, with an amount of 10^{14} microorganisms.[80,81] Host genetics, the immune system, and environmental factors significantly influence the composition of the microbiota.[82] The microbiota form an organ system that is essential for nutrient acquisition and fermentation in the colon, metabolism of indigestible compounds and carbohydrate uptake in the small intestine, development of intestinal architecture and the immune system and defence against colonization by pathogens.[83,84]

The importance of the multiple beneficial funtions of the human gut microbiota has been elegantly summarized by Prakash *et al.*.[85] The gut microbiota has multiple metabolic functions such as production of vitamins, amino acid biosynthesis, bile-acid bio-transformation, fermentation of non-digestible substrates, and production of short chain fatty acids (SCFA). These SCFA are important for the absorption of salt and water and as an important energy source. This in turn has important implications for other functions such as structural and

histological functions. The gut microbiota play a role in antimicrobial secretion and by competing for intestinal sites and nutrients has not only for protective functions such as innate and adaptive immunity activation but also for the development of the immune system. The gut microbiota, through these multiple actions regulates several epithelial functions: mucus production in goblet cells, defensin release from Paneth cells and tight junction protein synthesis in normal epithelial cells. Balance in intestinal immunity is partly achieved by development of opposing arms of the adaptive immune response since through this balance there is regulation of the inflammatory cytokine production. The mucosal route is a port of entry of exogenous pathogenic infections, and is together with the internal environment protected by the innate and adaptive immune system. The intestinal immune system responds with a strong inflammatory reaction against invading pathogens while it inhibits such reaction to commensal flora. Disruption of this subtle balance under specific disease conditions like IBD due to lack of tolerance to antigens of the microflora may cause inflammation.[86,87] Evidence from human and animal studies indicates that IBD results from failing tolerance of the intestinal microbiota.

Considerable progress has been made in the study of the role in microbiota on human health in IBD, although difference has been observed in the intestinal microbiota in CD and UC compared to healthy controls.[88,89] The normal intestinal epithelium provides a relatively impermeable barrier to luminal bacteria, bacterial products and dietary antigens. Impairment of the epithelial barrier function is an important feature of CD, where an increased exposure to foreign antigens seems to increase intestinal permeability and antigenic penetration.

To maintain intestinal homeostasis the immune system must develop adequate tolerance of intestinal microorganisms. This is partly possible by development of T-helper-1 (Th1), Th2, or Th17 cells[90], and induction of $CD4^+CD25^+FOXP3^+$regulatory T cells[91] producing anti-inflammatory cytokines like interleukin-10.[92] *Bacteroides fragilis*, a commensal seems to inhibit intestinal inflammation by supporting the development of inducible T_{Reg} cells.[93,94]

Commensal bacteria has a role in altering the progression of disease.[95] From this point of view, antibiotic use is an environmental factor that can very rapidly change the continuum of bacterial species in the gastrointestinal tract and cause disease. In a recent study, the risk of antibiotic use in IBD this risk was confined to development of CD, most strongly CD in childhood.[96]

CD-associated mutations in genes involved in bacterial sensing like the *NOD2* and *IL23R*, strengthen the link between microbiota and inflammation in IBD. Deficient NOD2 function causes defective regulation of defensin expression, impaired bactericidal capacity and inappropriate balance of microbiota in early infancy.[97]

Dysbiosis has been repeatedly observed in patients with IBD, whereas few commensals, like *Helicobacter*, *Clostridium* and *Enterococcus* species may have a pathogenic potential. In the healthy population these commensals cause no inflammation. Adherent and invasive

Escherichia coli or Norovirus trigger disease onset or flare ups.[98,99] A long unsettled dispute around Mycobacterium avium subspecies paratuberculosis as an important environmental factor in the development of IBD is still going on[100,101] and a decrease of *Faecalibacterium prausnitzii* may predict relapse of Crohn's disease after surgery. Based on these observations Sokol *et al*., proposed this bacteria as a candidate probiotics for the management of CD.[102]

Modulation of the microflora could affect its metabolic and immune modulatory functions. Several open studies described that probiotics seem to exert beneficial effects in a variety of disease conditions ranging from IBD to IBS. Thus, in a rospective pilot study of 12 women with diarrhoea-predominant IBS the patients who received oral mesalazine (1.5 g b.d.) for 4 weeks followed by a 4-week washout phase Faecal bacteria decreased by 46% on mesalazine treatment ($P = 0.014$), but returned to baseline during washout (103). More interestingly, this study also showed that *Firmicutes* and *Bacteroidetes* represented 95% of identified phylotypes, with a trend towards an increase in the proportion of Firmicutes at week 4 in symptomatic responders compared with nonresponders. This means that beside the anti-inflammatory and PPAR-γ effects of mesalazine the effect of gut microbiota requires further attention.

Many individual or combinations (like VSL#3) of bacterial species have been shown to ameliorate disease symptoms in IBD, both in humans and in mouse models.[104] However, despite expectations and hopes, double-blind placebo controlled studies have shown no evidence yet for the efficacy of pre-, syn- and probiotics.[105-107]

Use of probiotics in critically ill patients reported serious adverse events and helped to identify risk factors in which probiotic use is contraindicated such as the immune compromised state; impaired intestinal barrier function like in cases with multi organ failure and severe acute pancreatitis; and central venous catheter. Sanders *et al.* considered several factors with regard to the safety of probiotics: 1) Record isolation history and taxonomic classification of the candidate probiotic. 2) Manufacturing controls that eliminate contamination (including cross-contamination between batches) of the probiotic with microbes or other substances. 3) Absence of association of the probiotic with infectivity or toxicity, assessed at the strain level. 4) Absence of transferable antibiotic resistance genes. 5) Physiological status of the consuming population. Special consideration must be made for use in vulnerable populations, including newborn infants and the critically ill. 6) Dose administered. 7) Method of administration (oral or otherwise). 8) Absences of allergenic material (for example, dairy proteins) for products targeted for allergic populations.[108]

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Future perspectives

The field of probiotics and symbiotics will advance with projects such as the "Metagenomics of the Human Intestinal Tract (MetaHIT)" a project financed by the European Commission under the 7th FP program and that participates in the International Human Microbiome Consortium. The project studies the associations between the genes of human intestinal microbiota and human health and disease. Thus immunogenetics will help in the development of novel diagnostic and prognostic tools, based human genes, supporting preventive and personalized medicine. In addition, an even more complete understanding of the global human biology may be achieved with improvement of human health as a result.
Recent studies have shown that the composition of the gut microbiota of ulcerative colitis patients differs from that of healthy individuals but also from that of Crohn's disease patients but it is not clear yet whether this is the cause of the disease or whether these changes follow the disturbance of the intestinal milieu. The answer is also more complex that this but an interesting new insight is coming from the experimental field. Elinav *et al*., showed that dysfunction of the NLRP6 inflammasome (which regulates the production of IL-1β, IL-18, and IL-33) causes a colitogenic gut microbiota to develop. The colitogenic microbiota, dominated by Prevotellaceae, is super-activator of the mucosal immune system and induces local overproduction of CCL5 (RANTES) leading to attraction of T lymphocytes and granulocytes to the site of inflammation.

Learning from infection, IBD and immunoregulation

Understanding the different pathways involved in immunogenetic diseases, together with communication networks that control these pathways may provide not only a new insight in prevention and/or fighting infections but also in the control of chronic inflammatory diseases. In this thesis, emphasis has been given to the role of genes involved in the regulation of the control of a common infection, genes that regulate the chronic intestinal inflammation of UC and CD and probiotics as possible environmental regulatory elements that may intervene in the control of infection and inflammation.

A crucial step in advancing our understanding of immunogenetics of diseases, is defining the functional alteration responsible for association of functional alleles with disease.

Immunogenetics in clinical practice

Recent advances in immunogenetics have facilitated the search for rare variants that will be of help to find the probable causal gene, to identify, and to understand disease-relevant pathways and possible domains important for protein function. Genetically susceptible

individuals appear to have genomes with an appropriate collection of susceptibility alleles that modulate their immune system. This leads to a predisposition to inflammatory and infectious diseases when they are exposed to specific environmental triggers. Signaling of antigens and LPS through TLR or NLR pathways result in the secretion of pro-inflammatory pathways. Almost all TLRs activate the NFκB the adaptor MyD88 followed by the IRAK family of protein kinases. The TLR pathway plays a key role in multiple pathogenic processes and their targeting or their signals are of growing interest. They seem to play an important role in the pathogenesis and control of sepsis[109] when downregulated. Upregulation of TLRs however has been linked to other autoimmune diseases and immune abnormalities in HIV[110,111] and several cancers.[112-114]

The TLR-NOD2 signalling and the Th17 cell differentiation appear to predict susceptibility to CD. Autophagy is responsible for the elimination of unwanted pathogenic microorganisms and organelles entering the cytoplasm through mechanisms involving TLRs and NOD2 signalling.

Identification of clinically relevant parameters for different diseases will help to implement immunogenetics for clinical practice, and thereby see what's on the dark side of the moon. Genetic studies have already been used to foresee sensitivity to for example IBD therapies like 6-mercaptopurine and may also be of use to predict the sensitivity to biological therapeutics. Furthermore, the human intestinal microbiota is, with its dynamics interaction with the human host an important site for modulation of the immune system. In this respect probiotics and prebiotics may play a key role in the management and prevention of disease. However, not all probiotic could be used with the same aim and more information on particular strains with their functional spectrum is needed and not in all categories of patients as we were taught by the bad experience of the use of pro- and prebiotics in critically ill pancreatitis patients.[115,116]

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