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**Influence of cytokine gene polymorphisms
and of the *Helicobacter pylori* outer membrane
protein Hp0638 on bacterial pathogenesis**

Anar Dossumbekova

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2. Univ.-Prof. Dr. G. A. Häcker

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Abbreviations

Ab	Antibody
AD-PCR	Allelic Discriminating TaqMan PCR
AG	Atrophic Gastritis
AP-1	Activating Protein-1
BabA	Blood Group Antigen-binding Adhesin A
BCA-1	B-cellattracting chemokine
BHI	Brain Heart Infusion
bp	Base Pairs
BSA	Bovine Serum Albumin
<i>cagA</i>	Cytotoxin Associated Gene A
cagPAI	Cag Pathogenicity Island
C _T	Threshold Cycle
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotid Trisphosphat
EDTA	EthylenDiaminTetraAcetate
ELISA	Enzyme- Linked Immunoabsorbent Assay
ENA-78	Epithelial Neutrophil Activating Peptide
FCS	Fetal Calf Serum
FRET	Förster Resonance Energy Transfer
G1-G3	Grade of granulocyte Infiltration
GFP	Green Fluorescent Protein
GITC	Guanidine Isothiocyanate
<i>H. pylori</i>	<i>Helicobacter pylori</i>
HeLa	Henrietta Lacks cell line
HLA	Human Leukocyte Antigen
HRP	Horseradish Peroxidase
ICAM-1	Intercellular adhesion molecule 1
IFN- γ	Interferon Gamma
Ig	Immunoglobulin
IL	Interleukin

<i>IL-1B</i>	IL-1 β encoding Gene
IL-1RA	Interleukin-1 Receptor Antagonist
<i>IL-1RN</i>	IL-1RA encoding Gen
IM	Intestinal Metaplasia
IPTG	Isopropyl β -D-1-Thiogalactopiranoside
IRF-1	Interferon Regulatory Factor
ISRE	Interferon-stimulated responsive element
KC	Keratinocyte-derived protein Chemokine
kDA	Kilo Dalton
KO	Knockout
L1-L3	Grade of lymphocyte Infiltration
LD	Linkage Disequilibrium
Le ^b	Lewis B
LPS	Lipopolysacharide
MALT	Mucosa-associated lymphoid tissue
MCP-1	Monocyte Chemoattractant Protein 1
MIP-1 α	Macrophage Inflammatory Protein-1 α
MOI	Multiplicity of Infection
MPO	Myeloperoxidase
mRNA	Matrix Ribonucleic acid
NF-kB	Nuclear factor kB
OD	Optical density
OipA	Outer inflammatory protein A
OMP	Outer membrane proteins
OR	Odds Ratio
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PVDF	Polyvinylidene Difluoride
RANTES	Regulated on Activation Normal T Expressed and Secreted
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RPM	Revolutions Per Minute
RT	Reverse Transcription

RT-PCR	Reverse Transcription PCR
SDS-PAGE	Sodiumdodecylsulfate-Polyacrylamid-Gelelektrophorese
SLC	Surrogate Light Chain
SNP	Single Nucleotide Polymorphism
STAT1	Signal Transducer and Activator of Transcription 1
Tab.	Table
TAE	Tris-Acetat-EDTA
TBS	Tris Buffered Saline
TEMED	Tetramethylethylenediamine
Th1 response	T – Helper 1
TMB	Tetramethylbenzidine
TNF- α	Tumor Necrosis Factor Alpha
TRIS	Tris-Hydroxymethyl-Aminomethan
Ure	Urease
UV	Ultra Violet
VacA	Vacuolating Cytotoxin
WC-Agar	Wilkins Chalgren Agar

1 Introduction

1.1 The history of *H. pylori*

Until the recent explosion of interest in *H. pylori*, studies of bacteria in the stomach were usually conducted on the sidelines of medical research. Despite the fringe of these investigations, research on the presence of bacteria in the stomach has a long and rich history. In 1893, Bizzozero reported the presence of spirochetal bacteria in canine gastric tissue; similar findings were reported by Salomon in 1896 (19). In 1906, Balfour described spirochetes in gastric ulcers of monkeys and dogs and, in the same year, Krienitz reported similar-appearing organisms in human gastric carcinoma tissue. In the mid-1970s, Steer et al refocused attention on the presence of spiral bacteria in the stomach and their association with inflammation (195). Unfortunately they were unable to grow the organism in the laboratory. Robin Warren, a pathologist from Perth, Australia, made the critical observation that these organisms looked like *Campylobacter*. Methods to grow *C. jejuni* in the laboratory had only recently been developed, and Barry Marshall was able to cultivate the bacterium now known as *H. pylori* (82). In a 1983 letter to Lancet, Warren and Marshall proposed a link between *H. pylori* infection and peptic ulcer disease (139). Marshall subsequently inoculated himself with a culture broth containing more than 1 billion organisms, to address whether the bacterium was a pathogen or a commensal that colonizes inflamed gastric mucosa. He developed acute gastritis within 8 days, confirming that *H. pylori* causes gastritis (137). Self-inoculation was also done by Morris, further confirming that *H. pylori* is a pathogen (150). By the time *H. pylori* was isolated, gastritis had been a target of investigation for more than 50 years. Gastritis was already associated with peptic ulcer disease, gastric lymphoma. Thus the cause of these diseases has been discovered.

1.2 The characterisation of *Helicobacter pylori* organism

H. pylori is a gram-negative bacterium measuring 0.6 - 3.5 microns (165). Initial isolation conditions require an enriched medium, an atmosphere with reduced oxygen, ~10% CO₂, and an optimum temperature of 37°. Growth is slow with small (~1mm), smooth, translucent colonies appearing after 3 days (165). The spiral *H. pylori* organism was initially named *Campylobacter pyloridis* because of structural similarities to other

Campylobacter species (139). However, pyloridis is an incorrect Latin term and, in 1987, the species name was changed to pylori. Finally, in 1989, when it became clear that *H. pylori* was not a member of the genus *Campylobacter*, the genus name was changed to *Helicobacter* to reflect its distinct functional and enzymatic properties (83).

1.3 *H. pylori* associated diseases

1.3.1 Gastritis

The self-inoculation experiment by Marshall and Morris provided evidence that *H. pylori* infection causes gastritis (137) (150). Subsequent studies in which antimicrobial therapy resulted in healing of gastritis extended the hypothesis that eradication of the infection might cure or even prevent a number of gastroduodenal diseases associated with gastritis (228) (138). Understanding the outlines of the epidemiology of *H. pylori* infection was relatively straightforward once the connection was made between *H. pylori* and gastritis. The wealth of existing data on gastritis and its associated conditions, natural history, and epidemiology then could be transferred to *H. pylori* infection (30). A number of studies rapidly confirmed that such a transfer was appropriate. Infection with *H. pylori* is universally associated with histopathological findings of mucosal inflammation (ie, gastritis) (55). The severity of the gastritis may vary in different regions of the stomach and is typically most severe in the non-acid-secreting portion of the stomach. Normal gastric mucosa is essentially devoid of inflammatory cells. *H. pylori* infection leads to infiltration of stomach with polymorphonuclear cells (active inflammation) and with mononuclear inflammatory cells (chronic inflammation), resulting in a pattern of active-chronic gastritis. Interestingly, even though *H. pylori* is found throughout the stomach, the inflammation is often very mild, superficial, or even absent in the gastric corpus. The natural history of gastritis is extension of the inflamed area from the antrum into the corpus, resulting in a reduction in acid secretion and eventually loss of parietal cells and development of atrophy.

1.3.2 Peptic ulcer disease

H. pylori infection is closely linked to a peptic disease. Approximately 90% - 95% of duodenal ulcers and 70% -75% of gastric ulcers are attributable to infection with *H. pylori* (39). Use of non-steroidal anti-inflammatory drugs (NSAID) is an increasing cause of peptic ulcer and, as the frequency of *H. pylori* infection declines, it will

become the most common cause. It is now estimated that the lifetime risk for developing peptic ulcer disease in *H. pylori*-infected individuals is ~ 10% (166).

The strong link between *H. pylori* and peptic ulcer, and the observation that cure of the infection results in cure of the ulcer disease, provided strong evidence that *H. pylori* is etiologic in the pathogenesis of peptic ulcer disease. Compelling evidence that *H. pylori* is a cause of recurrent ulcers comes from the observation that the recurrence rate of duodenal ulcers is markedly reduced after successful treatment of *H. pylori* infection (144) (178). Moreover, iatrogenic infection of patients in which a natural *H. pylori* infection has been previously cured by antimicrobial treatment can lead to ulcer recurrence (123). Thus, by several rules of evidence, *H. pylori* infection is accepted as a cause of ulceration.

A recent unifying hypothesis relates increased duodenal acid load, *H. pylori*, and duodenal ulcer disease (84). The critical factor is that lowering the pH in the duodenum below the pKa for bile acids would remove the bile acids that normally inhibit growth of *H. pylori*. The abnormalities in gastroduodenal physiology associated with *H. pylori* infection all increase duodenal acid load. Other factors that may also increase the duodenal acid load are smoking and stress. The corollary is that reducing the duodenal acid load with antisecretory drugs or antacids not only would help accelerate ulcer healing, but also would make the environment in the duodenal bulb inhospitable for growth of *H. pylori*.

An apparent paradox is that two *H. pylori*-associated diseases appear to be mutually exclusive. The presence or history of duodenal ulcer disease protects against development of gastric cancer. The very low risk of gastric cancer in patients with duodenal ulcer has been confirmed in recent epidemiologic studies evaluating the risk of *H. pylori* infection for the development of gastric carcinoma (92).

1.3.3 Gastric cancer

The association between gastritis and gastric cancer was recognized decades before the discovery of *H. pylori* as a cause of gastritis. The link to *H. pylori* infection was confirmed after the discovery of the pathogenic role of the organism in gastritis (85). Although the incidence of gastric adenocarcinoma has steadily declined for the past 50 years, gastric cancer was the most common cancer in the early part of the 20th century and remains the second most frequent cancer related death in the world (170) (197). In 1994, the World Health Organization's (WHO) International Agency for Research on Cancer classified *H. pylori* as a definite carcinogen (in the WHO classification system for putative environmental and biologic carcinogen) (64) (72). This classification was

largely based on the epidemiologic links and not on a specific pathway. Gastric cancer is typically separated histologically into two types: an intestinal form strongly related to *H. pylori* and the diffuse form where the association is positive but weaker (168). The incidence of gastric cancer is consistent with a high incidence of *H. pylori* infection as the cause of the precursor lesions, chronic atrophic gastritis (34). Several case-controlled studies have shown that *H. pylori* seropositivity is associated with increased risk of gastric cancer (2,1 – 16,7-fold greater than in seronegative persons) (16; 72; 91; 99; 114; 119; 145; 168; 190; 191; 212). Prospective studies have demonstrated that the longer the time interval between *H. pylori* detection and gastric diagnosis, the higher the risk of developing cancer. In the EUROGAST Study Group, *H. pylori* increased the risk of gastric cancer by a factor of 6. A recent prospective study from Japan illustrates that in follow-up of approximately one decade, no gastric cancer developed in patients without *H. pylori* or patients who were eradicated (207).

Cancer development in the course of *H. pylori* infection is typically multifactorial. Acute gastritis is the initial lesion, progressing in some patients to multifocal atrophic gastritis. The sequence of events leading to the intestinal type of gastric cancer as a consequence of *H. pylori* infection includes the development of superficial gastritis which then can progress to atrophy, intestinal metaplasia, dysplasia, and finally invasive carcinoma (35). The extent of intestinal metaplasia seems to be a crucial predictor of cancer, but it may simply be a marker for the presence of low acid secretion or the extent of atrophy (124). The risk to the population can be increased by behaviour that promotes cancer (eg, cigarette smoking or a high-salt diet) or decreased by consumption of fresh fruits and vegetables (102). The highest rates of gastric carcinoma are found in populations that have an accelerated acquisition of chronic atrophic gastritis, so that patients with severe multifocal atrophic gastritis have over a 90-fold greater risk of developing adenocarcinoma than those with normal mucosa (192).

Although chronic inflammation is a sufficient cause for progressive damage and loss of cellular elements, other explanations also are possible, including one based on molecular mimicry (7) (155). The molecular mimicry involved mucin glycoproteins, and recent studies have shown that *H. pylori* expresses O-side chain polysaccharides with blood group determinants, including Le^x and Le^y (8). Molecular mimicry leads to the synthesis of autoantibodies and following development of atrophy (7) (8).

H. pylori plays a crucial role in the pathogenesis of primary gastric B-cell lymphoma (MALT Lymphoma) (230) (231). The association between gastric MALT lymphoma and chronic gastritis preceded the discovery of *H. pylori*, and the association has been confirmed by demonstration of strong epidemiologic link between *H. pylori* and gastric MALT lymphoma (167) (31). The normal stomach lacks organized lymphoid tissue, but

after infection with *H. pylori*, lymphoid tissue is believed to be caused by persistent antigen stimulation from by-products of chronic *H. pylori* infection (194). In 70-80% of cases regression of MALT Lymphoma is observed after eradication of *H. pylori* (216).

1.4 Immune response against *H. pylori*

1.4.1 Nonspecific immune response

In addition to the inflammation caused by lymphocytes, tissue damage can be caused by activated neutrophils, macrophages, or even mast cells. A monocyte and macrophage response can be seen in infected gastric mucosa, particularly in children, whereas in adults, polymorphonuclear cells are also present in the inflammatory infiltrate (10). The presence of cells from both the acute and chronic aspects of the inflammatory response can be explained by the stimulation of neutrophil chemokines by other cytokines produced by mononuclear cells (227). A classic example is IL-8. *H. pylori* induces the secretion of IL-8 from gastric epithelial cells (45). Contact of epithelial cells with *H. pylori* causes nuclear translocation of NF- κ B within 30 min, followed by increased IL-8 mRNA (1h) and protein synthesis (4h) (111). It has been further shown that *H. pylori* a functional type-IV secretion system, which is encoded by genes of the *cag* pathogenicity island, is necessary for activation of NF- κ B and upregulation of IL-8 message in epithelial cells (79). This chemokine is particularly specific for neutrophils and likely accounts for the “active” component that is mixed with the infiltrate associated with chronic gastritis. Activated neutrophils can easily damage gastric epithelium. For example, neutrophils can migrate across the epithelium and in so doing disrupt epithelial-cell permeability (130). In addition, secretion of mediators such as histamine (48), proteases (210), adenosine (131), or H₂O₂ (17) from neutrophils or mast cells can also modify the barrier and/or ion transport function of epithelial cells. Oxidative metabolites from neutrophils can also induce cellular damage, including apoptosis and DNA injury (36). Besides IL-8, *H. pylori* induces production of the chemokines RANTES, GRO α , MIP-1 α , ENA-78, MCP-1 (23). These molecules mediate recruitment of macrophages, neutrophils, mast cells, and T and B lymphocytes to the infected gastric tissue. Furthermore, *H. pylori* proteins upregulate Mac-1 (CD11b/CD18) integrin (90), used by neutrophils and macrophages to adhere to endothelium and extravasate into an inflamed tissue.

1.4.2 Specific immune response

During *H. pylori* infection as well as during other infectious diseases, antigen-specific T cell responses are essential for the clearance, or at least the control, of the pathogen. *H. pylori* antigens are endocytosed by antigen-presenting cells (APCs), processed and presented preferentially to CD4⁺ T (Th) helper cells. Distinct patterns of cytokines are characteristic of two major functionally polarized T helper (Th) cells, the so-called Th1 or Th2 subsets (151) (54). Th1 mediate cellular immunity and are generally associated with proinflammatory responses via production of IFN- γ , IL-2, IL-12 and TNF- α . In contrast, Th2 cells induce strong humoral response and are associated with production of IL-4, IL-5, IL-10, and IL-13 (199). Recognition of *H. pylori* during natural infection is biased for selection of Th1 responses, and a dominant Th1 phenotype is associated with the presence of clinical disease (148). Thus, *H. pylori* infection correlates with expression of a Th1 phenotype in situ characterized by a high incidence of TNF- α , IL-12, IFN- γ secreting leucocytes, but not with the presence of IL-4⁺ cells (1) (24). Despite the Th1 cell immune response is predominant during *H. pylori* infection, humoral immunity is also observed. Thus gastric Th1 clones also stimulate B cell proliferation and provide help for immunoglobulin production (50).

1.5 Bacterial pathogenesis

H. pylori populations are extremely diverse (80), owing to point mutations, substitutions, insertions and/or deletions in their genomes (20). This diversity includes the presence of certain virulence determinants in some *H. pylori* strains. *H. pylori* has a unique array of bacterial virulence factors. One of them is the vacuolating cytotoxin (VacA) that induces vacuole formation in eukaryotic cells and stimulates epithelial cell apoptosis (126) (43). The most important distinguishing factor of *H. pylori* strains is the *cag* pathogenicity island that contains 31 genes (32) (4). Several *cagPAI* genes encode proteins that form a type IV secretion system, which injects various proteins including CagA into epithelial cells (32). This stimulates diverse signaling cascades and induces production of series of cytokines including IL-8 (79) (106) (205). Our group was focused last years on the characterization of the BabA adhesin (175) (78; 177). It is a protein that binds the Lewis b blood group antigens on the gastric epithelium (27). The HP0638 outer inflammatory protein *hp0638* was described as a novel putative virulence factor (226). But the function of Hp0638 remains still unknown.

1.5.1 The *cag* pathogenicity island

In Europe, more than 60% of *H. pylori* strains contain the *cag* pathogenicity island (*cagPAI*), a 40-kb genomic fragment consisting of 31 genes (32) (4; 201). Several *cagPAI* genes possess homology to components of a type IV bacterial secretion system, which, in other prokaryotic species, functions as a conduit for export of multimetric proteins and nucleoproteins across both the inner and outer bacterial membrane (32). One of the *cag* island genes is *cagA*, which encodes the CagA protein. Only a part of the strains harbours the *cagA* gene, especially toxic strains (*vacAsI* positive strains) (37) (204). Therefore the gene was designated as cytotoxin associated gene A (*cagA*) (37). CagA is commonly used as a marker for the entire *cag* locus, although it is becoming apparent that the presence of *cagA* does not always signify the presence of an intact *cag* island (32) (106) (182). Several *cag* island genes are required for translocation of bacterial proteins into host cells, and induction of pro-inflammatory cytokine release (79) (106) (161) (205). When *H. pylori* adheres to host cells, CagA is translocated into and phosphorylated within the epithelial cell, where it induces cytoskeletal changes, including cell elongation, cell spreading, and production of filopodia and lamellipodia (161) (9; 196) (185) (14). Phosphorylated CagA binds to host cell protein SHP2 tyrosine phosphatase that induces growth factor – like cellular responses and cytokine production by the gastric epithelial cells (197) (96). Immunofluorescence studies have revealed that phosphorylated CagA localizes in a cylindrical form directly beneath an attached *H. pylori* bacterium, and within the host cell cytoplasm (185). The ability of *H. pylori* to induce epithelial responses related to pathogenesis, such as IL-8 production, is not uniform across strain populations. Clinical observation revealed that *cagA*-positive strains are more potent in stimulating IL-8 production *in vitro* than *cagA*-negative strains (187). Similar finding *in vitro* showed that carriage of *cagA*⁺ strains augments mucosal IL-8 expression *in vivo* and such increases are directly related to the more severe inflammatory response induced by these strains (169) (223). Numerous *cag* island genes (*cagE*, *cagG*, *cagH*, *cagI*, *cagL* and *cagM*), but not *cagA*, have been demonstrated to be required for NF-κB and mitogen-activated protein kinases-mediated IL-8 production (79) (112) (143). For example, inactivation of *cagE* not only attenuates IL-8 expression but also decreases NF-κB and mitogen-activated protein kinase activation *in vitro* (112) (186). *cagA*⁺ strains appear to be disproportionately represented among persons who develop serious sequelae of *H. pylori* infection, and genes within the *cag* island are necessary for induction of epithelial cell responses relevant to pathogenesis (105). Several studies have shown the correlation between expression of CagA and *H. pylori* virulence and suggest that *cagA*⁺ *H. pylori* strains are associated with an increased risk for the development of severe gastritis, atrophy, ulcer disease and distal gastric cancer (39) (169) (22) (189; 211). Inoculation experiments

with animal models supported these findings. In this context, the loss of *cagE* significantly attenuates the severity of the *H. pylori*-induced gastritis in Mongolian gerbils (106) (163).

1.5.2 The *hp0638* gene

Recently, a novel putative virulence factor has been identified, encoded by the *hp0638* open reading frame (226). HP0638 is a member of the *H. pylori* outer membrane protein (OMP) family. The *hp0638* gene is located on the *H. pylori* chromosome approximately 100 kb from the *cagPAI*. Yamaoka et al. showed that *H. pylori* contain either a functional or non-functional *hp0638* gene with functional status being regulated by a slipped strand repair mechanism based on the number of CT dinucleotide repeats in the 5' region of the gene (switch "on" is the functional status and switch "off" is the non-functional status) (226). The majority of strains with *hp0638* functional status "on" also possessed the *cagPAI* (222) (229). *In vitro* studies by one group showed that the presence of a functional gene was associated with increased IL-8 production from gastric epithelial cell lines. After inactivation of the *hp0638* gene *H. pylori* related induction of IL-8 was reduced and the inactivation of both the *cagPAI* and *hp0638* gene was required for reducing IL-8 production to baseline (226). Furthermore, this group has found that the "*hp0638-on*" status was significantly associated with high mucosal IL-8 level ($p < 0,001$) and severe neutrophil infiltration *in vivo*. However, the *hp0638* status was closely linked to presence of the important virulence factors CagA, VacA and BabA. In mouse infection experiments, the *H. pylori hp0638* switch status influenced both *H. pylori* density and colonisation ability, which were significantly lower in strains with *hp0638* "off" gene status, compared with "on" (221). The *H. pylori hp0638* knockout mutants did not induce inflammation despite the fact that they were able to colonize all of the mice (221). The C-X-C chemokine KC is thought to play an important role in neutrophil trafficking in the mouse (164). *H. pylori* infection was associated with increased KC mRNA and protein levels in the gastric mucosa of infected mice, and these levels were related to *hp0638* status. Furthermore studies in Mongolian gerbils showed that *hp0638* seems to play an essential role in adhesion or colonisation in the gastric mucosa of these animals (2). *hp0638* mutants failed to establish colonisation in all inoculated Mongolian gerbils (2).

The human IL-8 gene is located on the q12-21 of chromosome 4 and its promoter contains binding sites for NF- κ B, AP-1, and, in addition, a novel recently revealed element, the ISRE (interferon-stimulated responsive element). IRF-1 (interferon regulatory factor) is a transcription factor that binds to the ISRE-like element and induces IL-8 transcription. The IRF-1 promoter contains both NF- κ B and STAT1-

binding elements (180) (171). Yamaoka et al showed that STAT1 phosphorylation was dependent on Hp0638 status, and was independent of the *cagPAI* status (225). This *in vitro* evidence was correlated with similar findings *in vivo*, showing that Hp0638, but not *cagPAI*, was related to STAT1 phosphorylation in the antral mucosa (225). Based on those results it is supposed, that Hp0638 regulates STAT1- IRF-1- ISRE (-like element) pathway and *cagPAI* regulates I κ B – NF- κ B – IRF-1 – ISRE (-like element) pathway (225).

The studies by Yamaoka et al suggest an important role of Hp0638 for the development of *H. pylori* related pathologies. However the subsequent investigations by several other groups did not reveal any correlation between IL-8 production *in vitro* and *hp0638* status. Thus, Ando et. al showed in their experiments with AGS cells that the *hp0638* mutant strains were as potent as their corresponding wild-type strains with respect to the capacity to induce tyrosine phosphorylation of the CagA protein and induce IL-8 secretion (6). The same findings were reported by other groups, which did not find any effect of the *hp0638* status on CagA translocation and cytokine induction (2) (160). In addition, studies in 28 Dutch patients with duodenal ulcer and 21 with gastric ulcer did not find any correlation between expression status of *hp0638* and disease outcome (53). The role of Hp0638 protein in gastric colonization *in vivo* was also evaluated in the guinea pig model for *H. pylori* infection (52) *hp0638* mutants were able to colonize all inoculated guinea pigs.

1.5.3 The *vacA* gene

Leunk et al. first reported in 1988 that cell-free supernatants from *H. pylori* broth cultures induced striking vacuolar degeneration when added to cultured eukaryotic cells (126). This effect was subsequently shown to be caused by a secreted protein toxin, designated VacA (41). The *vacA* gene encodes a protoxin approximately 140 kDa in mass, which belongs to the family of secreted gram-negative autotransporter proteins. Both, an amino-terminal signal sequence and a carboxy-terminal fragment are proteolytically cleaved during the process of VacA secretion, and an ~ 88kDa mature toxin is exported (41) (156). Mature toxin molecules are released into the extracellular space, and also may be retained on the surface of the bacterium (200). The single polypeptide forming the mature VacA tends to undergo cleavage at the site of an exposed, protease-sensitive loop into the N-terminal 34 kDa (p37) and C-terminal 54 kDa (p58) fragments (200) (156) (129). These two fragments remain non-covalently associated, and may correspond to distinct structural and functional subunits. When purified from broth culture supernatant, VacA is recovered as a large oligometric complex with mass > 900 kDa (41). Upon imaging by electron microscopy, individual

VacA oligomers appear as flowers or snowflake-shaped complexes about 30 nm in diameter (42) (40). All *H. pylori* strains contain sequences that hybridise with *vacA* probes and nearly all strains secrete a VacA product (44) (11) (73). Nevertheless, there is considerable interstrain variation in vacuolating toxin activity. This variability may be partly due to differences in the level of *vacA* transcription (73), and partly due to the sequence variation among *vacA* alleles. In fact, several distinct families are currently recognised, based on analysis of sequences found at 5' end of the gene (11) Sequences at the 5' end of *vacA* cluster into two families, designated as s1 and s2. In the mid region sequences cluster into two families, designated m1 and m2 (11) (12), (208). With HeLa cells, type s1/m1 toxins are associated with the highest levels of vacuolating activity, s1/m2 toxins with an intermediate toxicity, and s2/m2 toxins lack detectable activity (11). Early studies noted that a higher proportion of *H. pylori* strains cultured from patients with peptic ulcer exhibited cytotoxic activity than did strains from patients without this disease (71). Subsequently, other studies have been reported that patients with peptic ulcer disease are usually infected with strains containing type s1 *vacA* alleles, whereas strains containing type s2 *vacA* alleles are found predominantly in patients without ulcers (78) (11) (209). Recently, one VacA receptor was identified on epithelial cells (219) (76). Despite the intensive investigation of the toxin it is still not clear what the main function of the toxin is. The toxic influence of VacA consists of vacuolating effects and induction of apoptosis (77) (43). Furthermore, it has been shown that VacA inhibits T-cell activation that may contribute to chronic persistence of the bacteria (25).

1.5.4 The *babA2* gene

Biochemical studies have identified a 78 kDa protein from *H. pylori* strains that allows binding to the blood-group antigen Lewis^b present on the surface of gastric epithelial cells (27) (104) (26). The protein has been designated blood group antigen-binding adhesin (BabA) (104). The functionally active gene encoding BabA has been cloned and termed *babA2* (104). The *babA2* gene has almost complete sequence homology to *babA1*, with exception of a 10-bp insert, found only in *babA2*, which creates a translational initiation codon in the signal peptide sequence (104). *H. pylori* strains harbouring the *babA2* gene expresses the adhesin and thereby bind to Lewis antigens on gastric epithelial cells (78) (104) (26). The correlation between *babA2* gene presence and adhesin expression was greater than 90%, indicating that, in most *babA2*-positive strains the adhesin is expressed (104). Some strains may lack the protein because of differential gene regulation, as suggested previously (4). These strains may be able to adapt their outer membrane protein expression to conditions in the environment by

switching gene expression on and off. Furthermore, the expression of the protein may depend further on bacterial growth and may differ at various time points of culture or *in vivo* conditions (78). The presence of the *babA2* genotype can therefore be regarded as a good indicator for the ability of strains to express the Lewis^b adhesin. Falk et al. investigated the role of the BabA/Le^b interaction in transgenic mice, expressing Le^b on the surface of gastric epithelial cells. In this model infection with BabA-positive *H. pylori* strains lead to the development of more severe gastritis and an increased loss of parietal cells, compared to infection with BabA-negative strains (67), (87). *babA2* genotype distribution was determined in different gastric diseases and was a good marker of duodenal ulcer (100% vs. 51.4% in gastritis) and adenocarcinoma (77.8%) (78) (26). Gerhard et al showed that *babA2* gene presence and simultaneous Lewis^b epitope expression in the gastric mucosa plays an important role in the pathogenesis of gastric cancer as well (78). *babA2* status is significantly associated with *cagA*-positive stains and with the *vacAsI* genotype (78) (104). Triple-positive strains (*cagA+*, *vacAsI+*, *babA2+*) showed a significant correlation with the presence of severe lymphocytic and granulocytic infiltration, glandular AT and IM (175). These observations suggest that the adhesion of *H. pylori* to Lewis antigens is important in the pathogenesis of severe gastritis (175). The development of severe gastritis, glandular AT, and finally IM may be initiated and facilitated by a close contact of *babA2*-positive bacteria to the gastric epithelium (175). After attachment via BabA, exposure to VacA toxins and/or *cagPAI* mediated effects may further enhance epithelial damage, achlorhydria, glandular AT, and IM (175). Metaplastic cells may represent a possible target for attachment of bacteria via BabA and Lewis^b interaction, because these cells have been shown to exhibit an increased expression of Lewis antigens (202). Thus simultaneous expression of *babA2* with other virulence factors may lead to severe histological alterations and thereby predispose to gastric carcinogenesis. Furthermore, Rad et al determined the importance of BabA for the development of gastric inflammation and described possible mechanisms involved in this process (177). Adherent bacteria are supposed to have growth advantages based on proximity to the epithelium (i.e., better availability of nutrients). Furthermore, elimination of bacteria by peristaltic movement and washout with the luminal fluid may affect adherent strains to a lesser extent. Therefore, bacteria with better adherence characteristics are supposed to colonize with higher densities. In addition, adherent bacteria may be able to transfer their products more effectively to the host cells. Thus, a higher bacterial density and/or a more efficient delivery of bacterial products to the host may induce a stronger inflammatory response. Indeed, BabA positive strains have been shown to colonize with higher densities, leading to an increased IL-8 response in the mucosa (177). BabA thus facilitates bacterial colonisation and thereby increases a non-specific immune response,

resulting in enhanced granulocytic infiltration and subsequently enhanced mucosal damage.

1.5.5 Urease

In contrast to most other bacteria, *Helicobacter pylori* has the capacity to survive the acidic conditions of the stomach. This is due to production of urease enzyme, a nickel-containing hexadimer composed of two different subunits (60 and 27kDa) (33) (58) (122). There are seven genes in the *H. pylori* urease cluster: ureA and ureB encode the structural subunits of urease, while the ureE, ureF, ureG and ureH encode accessory proteins necessary for assembly and Ni₂⁺ insertion which is required to form active urease (147). The majority of this enzyme is cytoplasmic, although a fraction is present on the bacterial surface after prolonged *in vitro* growth (94). Urease activity increases 10- to 20-fold as the pH falls from 6.0 to 5.0, and thereafter remains constant down to a pH of 2.5 (179) (184). Acid activation of cytoplasmic urease is mediated by expression of the third gene in the urease gene cluster, ureI, which encodes a H⁺ gated urea channel. UreI increases the permeability of the bacterial membrane to urea by at least 300-fold as the pH of the surrounding medium becomes acidic, and the presence of this acid-activated urea channel within the *H. pylori* inner membrane is necessary for efficient utilization of urea present in gastric juice (213). These data explain the absolute requirement for both urease and ureI for survival of *H. pylori* at a medium pH of less than 4.0 as well as for successful colonization of animal models (193) (59). Urease enzymatic activity is conserved among all known *Helicobacter* species and the primary structure of urease shows little divergence among *H. pylori* stains (66).

1.6 The role of Host factors for disease development

Despite *H. pylori* infection is the cause of gastric cancer, duodenal and gastric ulcers, the pathology develops only in a small group of patients (168). The majority (90%) of patients carries the infection asymptotically (168). As it was already described bacterial virulence factors play an important role for the development of the diseases during *H. pylori* infection. However, despite the well defined role of virulence factors, it is unclear why a considerable part of patients infected with the *cagA*⁺, *vacAsI*⁺ or *babA2*⁺ *H. pylori* strains does not develop severe pathologies lifelong.

In addition to bacterial factors, yet mostly unknown host factors seem to influence the inflammatory response and the development of more severe pathology.

1.6.1 The role of IL-10, TNF- α and IFN- γ during pathogenesis of chronic *H. pylori* infection

The *H. pylori* induced inflammation is implicated in the development of mucosal damage and is characterized by a strong granulocytic and lymphocytic infiltration (64) (188). The T helper cell response toward *H. pylori* is generally considered to be of the Th1 phenotype (64) (188), leading to a cell mediated immune response. There is increasing evidence, that the *H. pylori* induced Th1 response contributes to cancer development. Downregulation of the Th1 response was shown to protect against the development of atrophy, intestinal metaplasia and invasive gastric carcinoma (74) (75).

However, the factors influencing the extent of the *H. pylori* induced Th1 response are currently unknown. Cytokines characterizing Th1 mediated immune response are interferon-gamma (IFN- γ) and tumor-necrosis-factor-alpha (TNF- α), both being upregulated during chronic *H. pylori* infection (23) (177) (223). One of the most important cytokines which downregulates cell-mediated immune responses, is interleukin-10 (IL-10) which is also upregulated in the *H. pylori* infected stomach (110).

1.6.2 IL-10, TNF-A, IFN-G Polymorphisms

Genes encoding IFN- γ , TNF- α and IL-10 are polymorphic. Several polymorphisms are considered to influence gene transcription and the subsequent inflammatory processes in response to infectious disease (18) (101).

The gene encoding IL-10 has been mapped to chromosome 1 (115). The IL-10 promoter region contains several single nucleotide polymorphisms (SNP) (18) (206), (65). In Caucasians, SNPs at positions -1082 (G/A), 819(C/T) and -592 (C/A) from the transcription site have been reported to produce mainly 3 haplotypes: GCC, ACC, ATA. Although there may be stimulus- or cell type dependent variations, haplotype GCC seems to be associated with high IL-10 production capacity and ATA with low production capacity from peripheral blood mononuclear cells (PBMC) (206) (47) (49).

Several polymorphisms have been reported in the *TNF-A* promoter, most of which are functionally silent (3). The majority of studies focused on the G/A polymorphism at the position -307 (3) leading to conflicting results: some studies found an increased TNF- α production from LPS stimulated PBMC of allele A carriers (28) (128), whereas other studies reported no significant differences (51) (154).

The *IFN-G* gene is located at chromosome 12q. Allele 2 of a penta –allelic CA repeat polymorphism in the first intron has been associated with higher IFN- γ production from PBMC in vitro (173). Recently, a T/A SNP at position +874, located at the 5' end of the

CA microsatellite region has been described (174). Allele T of this SNP shows an absolute correlation with the presence of the high-producing microsatellite allele 2 (173).

1.7 Epidemiology and Transmission

Helicobacter pylori infects more than half of the world's population. The prevalence of the infection varies among countries and among different groups within the same country (69). The highest rates of infection are associated with low socioeconomic status, crowding, poor sanitation, and unclean water supplies (30) (134). *H. pylori* infection is typically acquired in childhood (69) (181). Children between the ages of 2 years and 8 years in developing nations acquire the infection at a rate about 10% a year (134). This significant difference in the rate of childhood acquisition is responsible for the differences in epidemiology between developed and developing countries (134), (136).

Socioeconomic differences are the most important predictor of the prevalence of infection in any group. Higher standard of living, higher level of education and better sanitation correlate with lower prevalence of infection (30) (136).

The differences in prevalence remained when differences in the following were controlled: income, educational level, current socioeconomic status, housing type, and use of tobacco and alcohol. Race was shown not to be the determining factor. When differences in socioeconomic status during childhood were included in the model, the differences in prevalence among the various groups disappeared, confirming that childhood is the critical period for acquisition of this typically lifelong infection (134).

Although genetic factors were not an important explanation for the differences among racial or ethnic groups, genetic factors are important in determining the bacterium-host interaction and outcome of infection (86). Genetic influences are best demonstrated in studies comparing outcomes in twins raised together and separated at or near birth (70). This allows for comparison of the outcome (eg, infection or not) in monozygotic twins, who share the same genes, with dizygotic twins, who are genetically different. The importance of environmental factors is seen in the differences in prevalence between those reared apart compared with those reared together. Such studies have shown that monozygotic twins reared apart have concordance rates for *H. pylori* infection that are much higher than dizygotic twins. The correlation coefficient (a measure of the influence of heritability on the prevalence of infection) was high (0.66) (86). The major conclusions are that genetics are significant in acquisition of the infection, but

environmental factors are also important. When the effect of socioeconomic class was investigated by studying twins raised apart in families of different economic circumstances, childhood socioeconomic status was found to have a strong influence in acquisition of *H. pylori* infection (86).

Transmissibility of the infection is emphasized by several studies showing that any activity that brings together infected and uninfected individuals in situations where sanitation may be compromised is associated with a high prevalence of *H. pylori* infection (69) - (181). For example, living in crowded circumstances in childhood has repeatedly been shown to be an epidemiologic link to risk that family adult members will acquire the infection. If the index parent was not infected, other members of the household were also unlikely to have *H. pylori* infection. Finally, although *H. pylori* can be found in dental plaque, no data support kissing as a means of transmission. Also, no data support transmission by pets (89) (56).

Studies in relatively economically homogenous populations in China and Russia confirm that density of living is an important factor associated with the risk of infection. Unclean water supplies in Peru and fresh vegetables grown using human wastes for fertilizer in Chile have both been associated with acquisition of *H. pylori* infection (30) (135). However sources of *H. pylori* infection in developing countries are not significant in transmission of the infection in developed countries.

Most studies suggest a fecal-oral mode of transmission. *H. pylori* can reach the mouth via reflux of gastric contents, and can be found in dental plaque. However, dental workers are not at increased risk to develop the infection (133). In addition, the lack of transmission between childless couples suggests that oral-oral transmission is unlikely to be important in transmission between adults. This would be categorised as gastric-oral and would be akin to the increased risk seen in gastroenterologists and endoscopy nurses who work with gastric secretions (215).

1.8 Incidence of gastric cancer in different countries

There are large intercountry variations in incidence of gastric cancer and in *H. pylori* seroprevalence (146) (93). A strong link between *H. pylori* infection and gastric cancer development has been observed. But a mystery during *H. pylori* infection is why some populations, such as Japan, China and Korea, have high incidence of gastric carcinoma, whereas other highly infected Asian populations with similar incidence of *H. pylori* infection, such as populations in India, Bangladesh, Thailand and Indonesia do not

(146) (88). This observation has been claimed as “Asian paradoxon” (146) (93) (141). Furthermore the incidence of stomach cancer is higher in Japan and China than in Western European countries, Australia and USA (93). Although the incidence of gastric cancer has decreased in Western countries during the last decades, its incidence is remaining constant among the leading locations of cancer in many countries, including in Eastern Europe (29). Possible reasons for the discrepancy are differences in genetic susceptibility among populations, environmental factors, strain differences of *H. pylori* and socio-economical conditions. Thus it has been shown that acid secretion and gastric atrophy may be altered according to ethnic background (146). Gastric acid secretion in Japanese is much lower than that in western populations (116). Specifically, gastric atrophy more readily occurs in subjects with lower acid secretion than in those with high acid secretion (121). Therefore, atrophic gastritis is more commonly observed in the Japanese than in Western populations. As it was described above, cytokine gene polymorphisms may contribute to the cancer development (176). Environmental factors also may be important factors for increased cancer risk. Consumption of salt and certain nutrients, such as β -carotene, have been reported to differ among regions where prevalence of gastric cancer is variable (203). Socio-economical factor plays also an important role. In highly developed western countries such as the USA or Germany, the decrease of the gastric cancer is connected to the decreased prevalence of atrophic gastritis due to diminishing *H. pylori* infection. However, in the countries with currently lower socio-economic status such as East Europe, China, Mexico or India, the prevalence of *H. pylori* is high (61). The genetic diversity of *H. pylori* strains has been reported to be associated with pathogenicity of gastroduodenal disorders. Bacterial virulence factors, such as the *cagPAI*, vacuolating cytotoxin (VacA), and the blood group antigen binding adhesion (BabA) are associated with enhanced inflammation and cancer development(32) (175) (78) (22) (46) (11). In Korea and Japan *cagA* and *vacAsI* represent nearly 100% of the *H. pylori* isolates.

Furthermore, diversity of CagA in *H. pylori* strains might be involved in the determination of the type and severity of disease. East-Asian and Western forms of CagA possess distinctly structured tyrosine phosphorylation/SHP2-binding sites — EPIYA-D and EPIYA-C, respectively (95). It has been observed that EPIYA-D shows stronger SHP2 binding and greater morphogenetic activity than EPIYA-C (95). Notably, the grades of inflammation, activity of gastritis, and atrophy are significantly

higher in patients infected with the East-Asian CagA-positive strain than in patients infected with the Western CagA-positive strain (13). Furthermore, the prevalence of the East-Asian *cagA*-positive strains is associated with the high mortality rate of gastric cancer in Asia (13). Therefore, populations infected with East-Asian *cagA*-positive *H. pylori* are at greater risk for gastric cancer than those infected with Western *cagA*-positive strains. Among Western CagA species, the number of EPIYA-C sites directly correlates with levels of tyrosine phosphorylation, SHP2-binding activity and morphological transformation (95). Furthermore, molecular epidemiological studies have shown that the number of EPIYA-C sites is associated with the severity of atrophic gastritis and gastric carcinoma in patients infected with Western CagA-positive strains of *H. pylori* (220).

1.9 The aim of the study

The aim of the study was to investigate the role of certain bacterial and human genetic factors in the pathogenesis of *H. pylori* infection. Due to existence of a gastric biopsy bank in our laboratory it is possible to perform human genetic and histological investigation and simultaneously to study bacterial features in large group of patients. The *in vivo* data should be further supplemented by *in vitro* experiments.

In the first part of our study, we are going to determined if polymorphisms in important cytokine genes (*IL-10*, *TNF-A*, *IFN-G*) influence gastric cytokine expression in response to *H. pylori* infection. Next, the influence of cytokine gene polymorphisms on gastric inflammation, as well as precancerous lesions, such as atrophy and intestinal metaplasia will be investigated. It is also important to find out if there correlation of the genetic variation of the cytokines and bacterial factors.

In the second part we aimed to explore the function of a novel putative *H. pylori* virulence factor Hp0638. The influence of *hp0638* on the severity of gastric inflammation, especially IL-8 secretion will be investigated. The *hp0638* gene encodes an outer membrane protein that could be a good candidate for an adherence factor of *H. pylori*. Therefore it is interesting to study if *hp0638* gene status affects the adherence properties of *H. pylori in vitro*. Finally, the role of *hp0638* for bacterial colonisation in vivo will be analysed.

2 Materials and Methods

2.1 Patients and biopsies.

5 antral and 5 corpus biopsies were collected from each of 742 patients. 207 patients (106 male and 101 female) with chronic gastritis were *H. pylori* infected. Persons taking non-steroidal anti-inflammatory drugs or receiving anti-secretory therapy as well as patients with ulcer disease or gastric carcinoma were excluded. 88.9% of the infected patients had German nationality; the others were from different countries in Southern Europe. The mean age was 62.5 years (32 to 92). Two antral and corpus sections were stained with H&E. Histopathological evaluation was performed according to the Sydney classification system in regard to the presence of IM, AG and the degree of granulocytic infiltration (G1 mild, G2 moderate, G3 severe) and lymphocytic infiltration (L1-L3). The updated Sydney system evaluates histological parameters, topographical distribution, and the etiopathogenesis of the gastritis. Gastritis is differentiated into autoimmune, *H. pylori*-associated, and chemically induced reactive gastritis, as well as other infrequent forms. The group of *H. pylori*-associated gastritis is characterized in regard to the activity and chronicity of inflammation and differentiates several degrees of granulocytic and lymphocytic infiltration (none, mild, moderate, and severe), respectively. Moreover, the presence of gastric atrophy (AT) and IM is assessed.

The three remaining antral biopsy specimens were stored in liquid nitrogen and homogenized before DNA or RNA isolation. After tissue lysis with proteinase K, DNA isolation was performed with a QIAamp tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA was extracted by the phenol/chloroform method.

2.1.1 Immunohistochemistry

2.1.1.1 The principle of immunohistochemistry

Immunohistochemistry is a process to determine the structure of the cells and tissues by using specific antibodies. In the current study a labelled streptavidin-biotin technique [LSAB] was used. Immunohistochemistry requires an addition of the primary antibody

that binds to a specific epitope of the tissue. A secondary biotinylated antibody then binds to primary antibody. Subsequently, streptavidin was added that is conjugated with enzyme alkaline phosphatase. In consequence of high affinity to biotin, streptavidin molecules tightly bind to the secondary antibody. The reaction was followed by addition of chromogen and substrates of alkaline phosphatase (Naphtholphosphate ester). Naphtholphosphate ester is split into phenol components and phosphates by alkaline phosphatase.

2.1.1.2 Reagents

<i>Primary antibody</i>	IgG ₁ - monoclonal, Mouse -anti-Le ^b (Chemikon)	1: 1000
	IgG ₁ - monoclonal Rabbit-anti- <i>H. pylori</i> (DAKO)	1: 200
<i>Secondary antibody</i>	biotinylated Goat-anti-Mouse-Antibody (DAKO)	1: 1000
	biotinylated Goat-anti-Rabbit-Antibody (DAKO)	1: 1000
<i>Komplexlösung</i>	LSAB- Solution (DAKO)	
<i>Chromogene substrate solution</i>	Solutions with Diazonium salts and Naphtholphosphate ester (DAKO)	
<i>Tris-Buffer</i>	Tris (Merck)	1,21 g
	NaCl (Merck)	0,58 g
	H ₂ O dest.	ad 100 ml; pH 7,5 (HCl)
	Mager milk powder	1 g
<i>Other reagents</i>	Xylol (Sigma), different concentrations of Ethanol (Merck), FCS (Gibco BRL)	

Table 1. Reagents for immunohistochemistry

2.1.1.3 Procedure

As material for immunohistochemical staining we used paraffin-embedded, formalin fixed probes from routine biopsies. From paraffin blocks 2-3 µm sections were cut, put on Super Frost Plus Slides (Carl Roth GmbH & Co.) and dried for 2h at 50°C. Then the sections were deparaffinized by 2 min incubation with Xylol, then by 2 min incubation with different concentrations of Ethanol (96 %, 80 % and 50 %). Then sections were put

for 2 min in distilled water and eventually washed in Tris-Buffer. Deparaffinized sections were then stained with specific antibodies.

2.1.1.4 Determination of *H. pylori* colonization densities

For determination of colonization densities, immunohistochemical staining against *H. pylori* was performed. Deparaffinized tissue sections underwent a pre-treatment with steam-pressure boiling for 7 min and were then incubated with a 1/200 dilution of primary rabbit anti-*H. pylori* Ab (DAKO, Hamburg, Germany) for 1 h. The avidin-biotin method was used for the further staining procedure, using goat anti-rabbit secondary Abs (DAKO) for 25 min. Bacterial density was determined semiquantitatively on an ordinal scale ranging from 0 to 3 by one pathologist (I. Becker). Sections without an adequate proportion of epithelial layer and glandular part were excluded from the evaluation.

2.2 Polymerase-Chain-Reaction (PCR)

2.2.1 The Principles of PCR

The PCR process is based on the discovery that the DNA polymerase isolated from thermophiles, the organisms that live in hot springs, are not only active at very high temperatures, but can be subjected to repeated exposures to 100°C without losing activity. The heat-stable DNA polymerase *TaqI* purified from the bacterium *Thermus aquaticus* can be mixed with a DNA template, a synthetic DNA oligonucleotide primer that anneals to the template, and the four deoxynucleoside triphosphates to make a reaction mixture. When the reaction is heated to 100°C for a brief period, the DNA will denature. When allowed to cool, the oligonucleotide primer will anneal to the complementary sequence in the DNA to form a primed template that can be elongated by the *Taq* DNA polymerase. After a short synthesis period, a new copy of DNA results from elongation of the annealed primer. Because of the thermal stability of the *Taq* polymerase, this process can be repeated for many cycles with only a slight loss of enzyme activity during each cycle. Each cycle will result in priming of the DNA template and the subsequent synthesis of a new single strand DNA copy. In the presence of a primer that anneals to a specific place in the DNA template, each cycle will result in the synthesis of a single-stranded DNA copy of one strand of the DNA template, resulting in linear accumulation of copies of one of the two DNA strands.

Primers	Sequence	Annealing temperature
TNF- α 307 sense	5' -GAGGCAATAGGTTTTGAGGGCCAT-3'	59°
TNF- α 307 anti-sense	5' -GGGACACACAAGCATCAAG- 3'	
IL-10 1082 sense	5' - TCGCTGCAACCCAACTGG C-3'	59°
IL-10 1082 anti-sense	5' - GGTCCCTTACCTTGCTCTTACC-3'	
IL-10 819 sense	5' - GACTCCAGCCACAGAAGCTTAC-3'	55°
IL-10 819 anti-sense	5' - AGGTCTCTGGGCCTTAGT-3'	
IL-10 592 sense	5' - ATCCAAGACAACACTACTAA3'	55°
IL-10 592 anti-sense	5' - TAAATATCCTCAAAGTTCC-3'	
Hp0638 sense	5' - CAAGCGCTTAACAGATAGGCT-3'	52°
Hp0638 anti-sense	5' -AAGGCGTTTTCTCGTGAAGC -3'	

Table 2. Primers for PCR

This reaction becomes significantly different in the presence of two different oligonucleotide primers that anneal to opposing strands of the DNA template and direct DNA synthesis towards each other. With two opposing primers, the first synthesis cycle results in two progeny DNA molecules with an annealed primer that has been extended by the polymerase past annealing site for the second primer. Two different types of reaction product occur in all subsequent cycles: primers can anneal to the original two template molecules and to the elongated products synthesized in the preceding synthesis cycle. While copies of the original DNA template accumulate at a linear rate, copies of the DNA sequences located between the annealing sites for the two primers accumulate at an exponential rate. The region between the primers becomes amplified as a DNA fragment with ends defined by the annealing sites for the primers. When the PCR reaction is allowed to proceed for 20-30 cycles, sufficient yield is obtained to allow the use of gel electrophoresis for observation of the resultant DNA fragments. The utility of this DNA amplification process is striking. Once a gene fragment has been isolated and sufficient nucleic acid sequence determined to allow the design and chemical synthesis of the oligonucleotide primers, portions of the gene can be rapidly amplified to such high levels that they can be easily examined by gel electrophoresis. Sufficient yields are obtained to circumvent the need for cloning and vector-mediated amplification of DNA

fragments in a biological host. Since each amplification cycle can often be completed in a few minutes, the total time required to complete a DNA fragment analysis can as brief as a few hours, in contrast to the several days required by conventional recombinant DNA methods.

2.2.2 Materials

PCR reactions were performed by using *Taq* PCR Master Mix Kit (Qiagen, Hilden). The Kit contains the following materials: Master Mix, including Reaction-buffer, *Taq* Polymerase und dNTP (10 nM each), and also sterile H₂O_{dest.} The following numbers of materials were pipetted each time in a 200 µl Reaction-tube:

DNA	2,0 µl
Master Mix	12,0 µl
Sense Primer (20pmol/ µl)	0,5 µl
Antisense Primer (20pmol/ µl)	0,5 µl
H₂O dest.	10,0 µl
Total:	25,0 µl

Table 3: Standard protocol for PCR

2.2.3 Procedure of PCR

The PCR reaction was performed in the Thermo-Cycler 9600 (MWG Biotech, Ebersberg). The contents of a reaction-tube were mixed carefully and centrifuged briefly. The following temperature profile was used:

1 Cycle	Denaturation:	94° C	5 min
	Annealing:	X° C	1 min
	Extension:	72° C	1 min
30 - 35 Cycles	Denaturation:	94° C	45 s
	Annealing:	X° C	55 s
	Extension:	72° C	1 min
Final Extension	Extension:	72° C	10 min

Table 4: Procedure of PCR

X° C = the annealing temperature of the corresponding Primers

2.3 Gel-Electrophoresis

2.3.1 The Principles of Electrophoresis

Separation of DNA fragments according to size is generally accomplished by gel electrophoresis. The phosphate molecules that are present throughout the backbone of DNA have a strong negative charge and when DNA is placed in an electric current, the DNA fragments will migrate towards the positive terminus. In the absence of anything to impede the migration of the fragments, fragments will all migrate at about the same rate, because the charge-to-size ratio for DNA is constant.

Electrophoresis is performed in a matrix, such as agarose or polyacrilamide that resist the migration of the DNA fragments by forcing the fragments to work their way through small pores in the matrix. The matrix can be imagined as a fine mesh or grid trough which the DNA molecules must filter in order to move with electric current. Because it is easier for smaller fragments to weave through the grid than it is for larger fragments, the DNA fragment will separate according to size, with small fragments migrating much faster than large fragments. When a DNA sample is loaded at one end of a gel in a small slot called a sample well, fragments of different sizes will resolve into bands as electrophoresis proceeds.

A gel must contain sufficient ions to allow electric current to flow, and must be buffered to prevent changes in pH that occur when ions move during electrophoresis. Gel buffer systems often contain the compounds Tris, EDTA, and boric or acetic acid at pH of about 8. DNA bands are most often visualized by staining of the gel with ethidium

bromide, which binds to the DNA fragments. Exposure to ultraviolet light then causes the DNA fragments to fluoresce, allowing routine detection of as a little as 2 nanograms (2×10^{-9} gram) of DNA in a band. Because ethidium bromide and UV light can both act as mutagens to introduce changes in DNA, some elements of caution is necessary in handling the ethidium solutions.

Agarose or polyacrilamide gel matrices are useful for characterizing DNA fragments of different size ranges. With special electrophoretic techniques, such as pulsed field electrophoresis, fragments of several hundred thousand basepairs can be resolved by agarose gel electrophoresis.

Mobility of DNA fragments during electrophoresis is inversely dependent on fragment size, but the relationship between size and mobility is non-linear. A 4,000 base pair fragment does not migrate twice as fast as an 8,000 base fragment and one-half as fast as a 2,000 base pair fragment.

2.3.2 Materials und Procedure

2.3.2.1 Electrophoresis buffer

The buffer we used is TAE-buffer (Tris acetate EDTA buffer), that can be stored as 50 times Stock solution.

1x Tris-Acetate-EDTA (TAE) Buffer	
<i>Tris-HCL (pH 7.8)</i>	<i>40 mM</i>
<i>Sodium acetate</i>	<i>20 mM</i>
<i>EDTA (pH 8,0)</i>	<i>1mM</i>
<i>H₂O dest.</i>	<i>ad 1000 ml</i>

Table 5: Reagents for gel electrophoresis

2.3.2.2 Preparation of agarose gel

2-3 g of agarose (Gibco BRL) was dissolved in 100 ml of 1x TAE electrophoresis buffer. Agarose was boiled in the microwave oven till the complete dissolution. Agarose was cooled to 50°C and ethidium bromide solution was added to a final concentration of 0,5 µg/ml (Merck). The liquid gel was then poured into a flat bed tray containing comb so that wells were generated when the gel had solidified. Once the gel had set the gel tray was placed into a gel electrophoresis tank with the wells facing the

cathode. The comb was carefully removed and the tank was filled with 1xTAE buffer, enough to cover the gel. DNA samples were resuspended in a tenth volume of 10x loading buffer and were loaded into the wells of the gel using a yellow pipette tip. The samples were electrophoresed at between 50 and 90 volts and visualised under ultra-violet light. Gel was photographed using a Video-Documentation system (MWG Biotech).

2.3.2.3 The loading buffer

Because DNA products have almost the same density as electrophoresis buffer, it was difficult to pipette directly to the gel, therefore 3 µl of loading buffer were mixed with PCR products to increase the density and enable the DNA sample to load to the gel.

The 5-fold loading buffer consists of:

- *Ficoll 400 20% w/v*
- *EDTA 50 mM*
- *Bromphenolblue 0,001 % w/v*
- *Xylencyanol 0,001 % w/v*

2.4 Isolation of DNA – fragments from agarose gels

DNA fragments were separated in the TAE-buffered gel and the corresponding DNA band was identified between other unspecific products. A clean scalpel was taken to excise the desired DNA fragment under UV-light. Only gel containing the fragment was excised to minimize the gel volume. The weight of the gel slice was determined and was transferred to a clean tube. Eventually DNA fragment was extracted from the agarose gel with help of Nucleo Spin Kits (Machery-Nagel GmbH & Co. KG, Düren). The procedure was followed concerning the protocol of the kit.

2.5 Cloning of DNA-Fragments

2.5.1 The Principles of Cloning

Cloning is a process of DNA introduction in one vector (Plasmid) that is capable to replicate and produce many copies of desired DNA fragments. The vector DNA could be inserted into bacteria with help of chemical or physical methods or phags.

2.5.2 Materials

<i>LB Agar Plates + Ampicillin</i>	
LB Agar (USB)	30g/L
Aqua dest.	ad 500 ml
Ampicillin	50mg/L

Table 6: Materials for preparation of LB agar plates

After Autoclaving LB agar medium was cooled to $< 60^{\circ}$ C and 50 μ g/ml of ampicillin were added, the liquid medium was then poured into Petri plates, and was leaved for one day to solidify. Plates were stored at 4° .

<i>LB Medium</i>	
LB Broth (USB, Cleveland, OH, USA)	20 g
Aqua dest.	ad 1000 ml

Table 7: Materials for preparation of LB medium Autoclaving and storage at 4° in dark is necessary.

2.5.3 The procedure of cloning

2.5.3.1 Ligation of DNA Fragments into Plasmid Vectors

For ligation of restriction enzyme fragments, the molar ratio of vector to insert was 30fmol of vector and 90fmol of insert. Ligation was performed in 1x ligation buffer containing 1mM ATP. These components were incubated at 22° C overnight using 1unit of T4 DNA ligase per reaction.

2.5.3.2 Cloning of PCR products

All the reagents used were from TA cloning Kit supplied by Invitrogen. A ligation reaction was carried out with 1µl of PCR product, 1µl 10x ligation buffer, 2µl resuspended vector, 5µl sterile water, 1µl T4 DNA ligase. This ligation mixture was then transformed into TOP 10 competent bacteria and plated onto LB agar containing ampicillin and X-gal selection at 37° overnight.

2.5.3.3 Transformation of bacteria

Competent bacterial cells were provided by Invitrogen and the protocol described in the manuals was as follows. A vial of One Shot competent cells thawed on ice and 2ml of 0.5M b-mercaptoethanol was added to each vial and mixed gently. Then between 1-10ml of ligation reaction was gently pipetted on the competent cells and the cells were left to incubate with the DNA on ice for 30 minutes. The cells were then heat shocked at 42°C for 30 seconds and placed onto ice for 2 minutes. Then 250 ml of pre-warmed SOC media (20g/litre bacto-tryptone, 5g/litre bacto-yeast extract, 0.5g/litre NaCL, 20mM glucose) was added and the vial was placed in an orbital shaker at 37°C for 30 minutes. Then between 20-200ml of each transformation was plated onto LB agar containing antibiotic selection. The plates were inverted and incubated overnight at 37°C for colonies to grow.

2.5.3.4 Isolation of plasmids

The single colony was picked from a freshly streaked selective plate and incubated in 2-5ml LB medium containing the appropriate selective antibiotic. Bacteria were incubated for ~8h at 37°C with vigorous shaking (~300 rpm). For isolation of large plasmid amounts 100ml selective LB medium was added to this starter culture. Bacteria grew at 37°C for 12-16 h with vigorous shaking. Subsequently, plasmid purification was performed with Qiagen Midi Kit, according to the manufacturer's instructions.

2.6 Restriction fragment length polymorphism and RFLP analysis

2.6.1 Principle of RFLP analysis

RFLP analysis is based on the observation that there are slight differences in the nucleotide sequences of the same gene isolated from two different individuals. These nucleotide sequence differences can be used to distinguish the gene isolated from one individual from the same gene isolated from a different individual. Isolation and determination of the nucleotide sequences of the genes from both individuals would be a slow and expensive method of identification. Fortunately, the variation in nucleotide sequence will occasionally either inactivate or create the cleavage site for a restriction enzyme. When the DNA from two individuals is digested with the same restriction enzyme, the same gene can be present on DNA fragments of different sizes. The lengths of the restriction fragments carrying a specific gene are polymorphic, or of variable sizes, when an entire population of individuals is examined.

Medical research is identifying an increasing number of polymorphisms that are associated with specific genetic disorders. These polymorphisms may affect the gene involved in the disorder or may be simply associated with DNA that is very close to the defective gene.

2.6.2 Procedure of RFLP analysis

IL-10 SNPs were genotyped by RFLP analysis. For genotyping of the -1082 polymorphism PCR products were digested with 10 units of *MnII* (New England Biolabs) at 37°C for 3h. This gave products of 98 bp + 46 bp (-1082G) and 144 bp (-1082A). An additional *MnII* restriction site at position -1059 was eliminated from the sequence by introducing a point mutation into the amplified PCR product through a base exchange in the antisense primer (C to G, underlined in the primer sequence). To analyse the *IL-10-819* polymorphism, PCR products were digested with 1 unit of *RsaI* (Roche) at 37°C for 2h. Digestion yielded five bands (-819A: 240, 11, 85, 42, 8 bp) or four bands (-819C: 351, 85, 42, 8 bp). Typing of the *IL-10-592* SNP was performed by digestion of the PCR products with *MaeIII* (New England Biolabs) at 55°, which gave products of 217, 144, and 125bp (-592C) or of 361 and 125 bp (-592T).

Typing of the *TNF-A-307* SNP was also performed by RFLP analysis. Primer sequences are shown in table 1. PCR products were digested with 10 units of *NcoI* (New England Biolabs) at 37°C for 3h giving fragments of 126 + 21 bp (-307G). The -307A allele was

not cut. The lengths of digested products were analyzed 2-3% agarose gels. All primer sequences used for RFLP analysis are shown in chapter 2.2.1.

2.7 Real-time quantitative PCR (TaqMan PCR)

2.7.1 Principles of Taq Man PCR

2.7.1.1 Fluorogenic Probes.

Real-time systems for PCR were improved by using fluorogenic probes. Holland et al. (98) were the first to demonstrate that cleavage of a target probe during PCR by the 5' nuclease activity of Taq DNA polymerase could be used to detect amplification of the target-specific product. In addition to the components of a typical amplification, reactions included a probe labelled with ^{32}P on its 5' end and blocked at its 3' end so it could not act as a primer. During amplification, annealing of the probe to its target sequence generates a substrate that is cleaved by the 5' nuclease activity of Taq DNA polymerase when the enzyme extends from an upstream primer into the region of the probe. This dependence on polymerisation ensures that cleavage of the probe occurs only if the target sequence is being amplified.

The development of fluorogenic probes by Lee et al. (125) made it possible to eliminate post-PCR processing for the analysis of probe degradation. The probe is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached. While the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye by Förster resonance energy transfer (FRET) through space. Probe design and synthesis has been amplified by the finding that adequate quenching is observed for probes with the reporter at the 5' end and the quencher at the 3' end.

If the target sequence is present, the probe anneals downstream from one of the primer sites and will be cleaved by the 5' nuclease activity of Taq DNA polymerase once this primer is extended. This cleavage of the probe separates the reporter dye from the target strand, allowing primer extension to continue to the end of the template strand. Thus inclusion of the probe does inhibit the overall PCR process. Additional reporter dye molecules are cleaved from their respective probes with each cycle, affecting an increase in fluorescence intensity proportional to the amount of amplicon produced.

The advantage of fluorogenic probe over DNA binding dyes is that specific hybridisation is required to generate fluorescent signal. Thus, with fluorogenic probes, non-specific amplification due to mis-priming or primer-dimer artefact does not generate signals. Another advantage of fluorogenic probes is that they can be labelled

with different, distinguishable reporter dyes. By using probes labelled with different reporters, amplification of two distinct sequences can be detected in a single PCR reaction. The disadvantage of fluorogenic probes is that different probes must be synthesized to detect different sequences.

Primers and Probes	Sequence
GAPDH sense	ACGGATTTGGTCGTATTGGGC
GAPDH anti-sense	TTGACGGTGCCATGGAATTTG
GAPDH probe	CCTGGTCACCAGGGCTGCTTTTAA
IFN- γ sense	CTTGGCTTTTTCAGCTCTGCATC
IFN- γ anti-sense	CTTCAAAATGCCTAAGAAAAGAGTTCC
IFN- γ probe	TTGGGTTCTCTTGGCTGTTACTGCCA
TNF- α sense	GCCCAGGCAGTCAGATCATCTTC
TNF- α antisense	TGAGGTACAGGCCCTCTGATGG
TNF- α probe	CGAACCCCGAGTGACAAGCCTGTAGC
IL-8 sense	GCCAACACAGAAATTATTGTAAAGCTT
IL-8 antisense	AATTCTCAGCCCTCTTCAAAAACCTT
IL-8 probe	AGAGCTCTGTCTGGACCCCAAGGAAAAC

Table 8. Primer and Probes for TaqMan PCR.

2.7.1.2 Double –Stranded DNA Binding Dyes.

Small molecules that bind to double-stranded DNA can be divided into two classes: intercalator and minor groove binder (157). We used the SYBR Green 1 dye. PE Biosystems has developed conditions that permit the use of the SYBR Green I dye in PCR without inhibition and with increased sensitivity compared to ethidium bromide. The mechanism of SYBR Green I dye's interaction with DNA is not known.

Both the advantage and disadvantage of using a DNA binding dye for real-time detection of PCR are that the dye allows detection of any double stranded DNA

generated during PCR. On the plus side, this means versatility because the same dye can be used to detect any amplified product. Thus, any PCR amplification can be monitored simply by including the generic DNA binding dye with the other PCR reagents. On the negative side, both specific and non-specific products generate signal. Thus, any mispriming events that lead to spurious bands observed on electrophoretic gels will generate false positive signals when a generic DNA binding dye is used for real-time detection.

Another aspect of using DNA binding dyes is that multiple dyes bind to a single amplified molecule. This increases the detecting amplification products. A consequence of multiple dye binding is that the amount of signal is dependent on the mass of double-strand DNA produced in the reaction. Thus, if the amplification efficiencies are the same, amplification of a longer product will generate more signals than a shorter one. This is in contrast to the use of a fluorogenic probe, in which a single fluorophore is released from quenching for each amplified molecule synthesized, regardless of its length.

2.7.1.3 Instrumentation.

The ABI Prism 7700 Sequence Detection Systems was used. It is a more flexible system designed to take full advantage of the benefits of fluorogenic probe detection. The 7700 system has a built-in thermal cycler and a laser directed via fiber optic cables to each of the 96 sample wells. The fluorescence emissions travels back through the cables to a CCD camera detector. Because each well is irradiated sequentially, the dimensions of the CCD array can be used for spectral resolution of the fluorescent light. Because the 7700 instrument detects an entire fluorescence spectrum, the system is capable of distinguishing and quantification multiple fluorophores in each sample well. The software analyses the data by first calculating the contribution of each component dye to the experimental spectrum. Each reporter signal is then divided by fluorescence of an internal reference dye (ROX) in order to normalize for non-PCR related fluorescence occurring well-to-well or over time. The use of this internal reference dye increases the precision of the data obtained with the 7700 systems. The fluorescence dye is obtained for SYBR Green I dye and ROX dye detection of PCR on the 7700 system. The other advantage of distinguishing fluorophores is that probes labelled with different reporter dyes can be used so that more than one PCR target can be detected in a single tube.

2.7.2 Real-time PCR Quantification.

The ability to monitor the real-time progress of the PCR completely revolutionizes the way one approaches PCR-based quantification of DNA and RNA. Reactions are

characterized by the point in time during cycling when amplification of a PCR product is first detected. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

An amplification plot is the plot of fluorescence signal versus cycle number. In the initial cycles of PCR, there is a little change in fluorescence signal. This defines the *baseline* for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline. The parameter C_T (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The entire process of calculating C_{TS} , preparing a standard curve, and determining starting copy number for unknowns is performed by the software of the 5700 and 7700 systems.

2.7.3 Effect of limiting reagents

The early cycles of PCR are characterized by an exponential increase in target amplification. As reaction components become limiting, the rate of target amplification decreases until a plateau is reached and there is little or no net increase in PCR product. During the exponential phase, none of the reaction components is limiting; as a result, C_T values are very reproducible for reactions with the same starting copy numbers. This leads to greatly improved precision in the quantification of DNA and RNA. The amount of PCR product observed at the end of the reaction is very sensitive to slight variation in reaction components. For example, side reactions, like formation of primer dimer, can consume reagents to different extents from tube to tube. It is possible for a sample with a higher starting copy number to end up with less accumulated product than sample with lower starting copy number.

2.7.4 Allelic-discriminating (AD) TaqMan-PCR

The mutation of single or polynucleotide polymorphisms could be determined by using AD-TaqMan PCR. We used this method to check the Single Nucleotide Polymorphisms (SNP) of different alleles. The principles of method based on using of 2 Probes during PCR and each probe is specific for one of two SNP alleles. The probes are marked with different Reporter dyes, the probe specific for one allele with FAM and probe specific for another allele with VIC. The procedure of PCR is similar to quantitative TaqMan analysis, with the difference that instead of using cDNA genomic DNA was taken. The case of homozygosity for allele 1 leads to effective binding and cleavage of the allele 1-specific probe. As a result of this the level of fluorescence specific for allele 1 is high. Simultaneously, the level of allele 2-specific fluorescence is lower. In case of

homozygosity for allele-2 the opposite reaction is observed. In case of heterozygosity the fluorescence level is equally high.

The *IFN-G+874* SNP was genotyped by allelic discriminating TaqMan PCR, using the following primers and probes:

<i>forward primer</i>	5´-ATTCAGACATTCACAATTGATTTTATTCTTAC-3´
<i>reverse primer</i>	5´-ACTGTGCCTTCCTGTAGGGTATTATT-3´
<i>probe-1</i>	5´-FAM-AATCAAATCTC <u>C</u> ACACACAC-TAMRA-3´
<i>probe 2</i>	5´-VIC-ATCAAATC <u>A</u> CACACACAC-TAMRA-3´

PCR and endpoint analysis was performed in a volume of 25 µl on a ABI PRISM 7700 Sequence Detection System (Perkin-Elmer, Weiterstadt, Germany).

2.8 Isolation of nucleic acids

2.8.1 Isolation of deoxyribonucleic acid DNA

At the beginning biopsies were minced using a mortar and then 180µl of ALT-buffer, 20 µl Proteinase K were added to biopsies for lyses. The reaction was shaken for 3h at 55°C. The addition of 200µl buffer AL to the tissue and incubation at 70°C for 10 minutes allowed selective binding of DNA to the DNeasy membrane. Next, a washing step with 210 µl Ethanol (96-100%) enabled to remove contaminants with a simple washing spin. The samples with ethanol were mixed thoroughly by vortexing and centrifuged by 6000g for 1 minute, thus the DNA was bound to the membrane of the collection tubes and the contaminants flow-through. After two new wash steps with 500µl AW-buffer DNA was eluted two times by using 200 µl of 70° - pre-warmed buffer AE and centrifuged at 6000g.

2.8.2 Isolation of ribonucleic acid (RNA)

RNA was isolated by using the RNeasy isolation kit. This method combines the selective binding properties of a silica-gel-based membrane with the speed of microspin technology.

A specialised high-salt buffer system allows up to 100µg of RNA longer than 200 bases to bind to the RNeasy silica-gel membrane. Biological samples are first lysed and homogenised in the presence of a highly denaturing guanidine isothiocyanate (GITC)-containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy mini column where the total binds to the membrane and contaminants are efficiently washed away. High quality RNA is then eluted in 30µl, or more of water.

2.9 Photometrical assay of nucleic acids concentration

The optical density (OD) of isolated DNA and RNA was possible to measure by using of the Photometer (Beckman Instruments, Spectrometer DU 640). The following formula was used to count a concentration:

$$\text{Dilution factor} * 40 * \text{OD}_{260\text{nm}} = \text{concentration } (\mu\text{g/ml})$$

The factor 40 should be applied for the quantitative determination of DNA and RNA because OD-1 corresponds the concentration of 40 µg/ml.

2.10 Reverse Transcription-PCR (RT-PCR)

2.10.1 Principles of RT-PCR

The heat-stable DNA polymerases used in the PCR amplification generally works efficiently only with a DNA template and cannot be used to directly amplify RNA molecules. However, the enzyme reverse transcriptase (RT), which elongates a DNA primer annealed to an RNA template to make a complementary cDNA copy of the RNA molecule, has been combined with PCR to allow amplification of RNA.

The RT/PCR first anneals an oligonucleotide primer to an RNA sample, then uses reverse transcriptase to elongate the primer to an RNA sample, then uses reverse transcriptase to elongate the primer and make a cDNA copy of the RNA molecules. The primer may be general, such as oligo dT annealed to the poly-A tail of a eukaryotic mRNA population, or a specific primer that will anneal preferentially to a particular RNA, such as rRNA that forms the structural backbone of the ribosome. Following annealing of the primer, reverse transcriptase and deoxynucleoside triphosphates are

added to extend the primer and make a single-stranded cDNA copy of the desired RNA molecules.

2.10.2 Procedure of RT-PCR

RNA	10 μ l
H ₂ O dest.	30 μ l
MgCl ₂	22 μ l
dNTP-Mix	20 μ l
RT-Puffer	10 μ l
Random Primer	5 μ l
RNase Inhibitor	1 μ l
Reverse Transkriptase	2 μ l
Total:	100 μ l

Table 9: The following protocol with reagents was used for 100 μ l

The reaction was carefully mixed and centrifuged, and the following temperatures were programmed in Thermocycler:

- 25° C (10 min)
- 48° C (30 min)
- 95° C (5 min)

2.10.3 Reagents

To perform RT PCR the TaqMan Reverse Transcription Reagents Kit (Perkin Elmer) was used.

<i>10x TaqMan RT Buffer</i>	
TRIS	100 mM
KCl	500 mM
pH 8,3	
<i>Reverse Transkriptase</i>	
MultiScribe Reverse Transkriptase	100 units/ μ l MMLV
<i>RNase Inhibitor</i>	
RNase Inhibitor	20 mM
<i>Deoxynucleotid triphosphate (dNTP) Mixture</i>	
dATP	2,5 mM
dTTP	2,5 mM
dCTP	2,5 mM
dGTP	2,5 mM
<i>Random Primer</i>	
Random Hexamer	50 mM [d(N) ₆]
<i>MgCl₂ solution</i>	
MgCl ₂	25mM

Table 10: Reagents for the reverse transcription with Multiscribe Reverse Transcriptase (TaqMan Reverse Transcription Reagents, Perkin Elmer, Weiterstadt)

2.11 Culture of *Helicobacter pylori*

The *H. pylori* culturing was performed in BHI – Medium (Brain heart infusion) and on WC-Agar plates. In the tables 13 and 14 the compounds of the medium are shown. To prepare BHI medium, BHI and yeast extract were solved in 1000 ml of distilled water. The solution was autoclaved, and then 10% of FCS and antibiotic were added. BHI

medium was stored at 4°C. To prepare WC-Agar plates WC-Agar and distilled water were mixed and the solution was autoclaved. The WC-Agar mixture was the sterilized using an autoclave. Antibiotics and 10% of FCS were added once the agar had cooled to 50°C in a water bath and the solution was poured into Petri dishes to solidify. Plates were stored at 4°C prior to use.

BHI (Sigma)	36g/l
Yeast extract (Sigma)	0,25%
Aqua dest	ad 1000 ml
FCS ^a	10%
DENT-Supplement (Oxoid) ^a - Vancomycin 5mg - Trimethroprim Lactat 2,5 mg - Cefsulodin 2,5 mg - Amphotericin B 2,5 mg	2 tubes/1000ml

Table 11. Reagents for preparation of BHI medium (Brain Heart Infusion)

WC-(Wilkins Chalgren)-Agar (Oxoid)	43g/l
Yeast extract (Sigma)	0,25%
Aqua dest	ad 1l
FCS ^a	10%
DENT-Supplement (Oxoid) ^a	2 tubes/1000ml

Table 12. Reagents for preparation of WC-Agar-Platten (Wilkins Chalgren)

In the present study we worked with two *H. pylori* strains G27 and B128. The both strains had *hp0638*- gene -knockout strains, that were also used in the study. *H. pylori* is an S2 human pathogen that obligate to work in special lab-areas with S2 status. 200µl of glycerol stock (see below) were taken in blood – Agar plates and spread over the whole surface of the plate using a ‘hockey stick’. The culture was put in microaerophilic incubator at 37°C. An incubator set at 10% CO₂, 5% O₂ and 85% N₂ has been shown to

be satisfactory for *H. pylori* culture. Under these incubation conditions *H. pylori* was cultured for 2-3 days. After harvest of strains the vitality was checked by using light microscopy. In light microscopy the morphology (coccoid or helical) and motility of *H. pylori* were checked.

2.11.1 Culture of *H. pylori* in liquid medium

To obtain a big amount of *H. pylori* we used a liquid culture. *H. pylori* bacterial cells were resuspended in the medium till solution had an OD 528 of 0.1-0.2. The liquid medium with bacteria was poured to cell culture flasks and incubated in a microaerophilic incubator at 37°C by shaking at 130 rpm. Approximately after 24 hours the OD 528 of the bacterial suspension reached 0.7-1 and the bacterial cells were harvested.

2.11.2 Long-time storage of *H. pylori*

For long-time storage bacterial cells were harvested by scraping growth from two plates per strain into 2-3 ml of BHI-glycerol (about 10^9 viable spiral, non coccoid bacteria per ml). BHI-glycerol solution consists of 1ml BHI, 20% glycerol and 10% of FCS. 0,5ml of the bacterial suspension were subjected into small plastic cryovials and frozen immediately at -70° to -80°C or in liquid nitrogen at -196°C.

2.12 Co-culture of *H. pylori* and gastric epithelial cells

<i>Culture medium for KATO III Cells</i>	
Fetal Bovine Serum (Gibco BRL)	20%
RPMI 1640 Medium (Gibco BRL, Karlsruhe)	79%
Penicillin (100U/ml) Streptomycin (100µg/ml)	1%
<i>Culture medium for AGS Cells</i>	
Fetal Bovine Serum (Gibco BRL)	10%
DMEM (Gibco BRL, Karlsruhe)	89%
Penicillin (100U/ml) Streptomycin (100µg/ml)	1%
<i>0,15M PBS (phosphate buffered saline)</i>	
KCl (Merck)	0,2 g
NaCl (Merck)	8,0 g
KH ₂ PO ₄	0,2 g
Na ₂ HPO ₄	1,44 g
H ₂ O dest.	ad 1000 ml, pH 7,4

Table 13: Materials for cell-culturing

2.12.1 Procedure of co-culture of *H. pylori* with gastric epithelial cells

KATO-III cells (3×10^5) and AGS cells (3×10^5) were plated into six-well plates and cultured for 24 hours in 2 ml of RPMI 1640 supplemented with 20% FCS, 1% antibiotic for KATO-III and DMEM medium supplemented with 10% FCS, 1% antibiotic for AGS respectively in a humidified incubator containing 5% CO₂ (all Sigma-Aldrich, Munich, Germany). After 24 hours cells were washed once with PBS and 2 ml of antibiotic-free medium was added to each dish. For stimulation with *H. pylori* different concentrations of bacteria were co-cultured (bacteria/cell: 1:100, 1:50, 1:10, 1:1) with the cells in a 5% CO₂ incubator. Incubation time depended on the type of experiment. For determination of IL-8 protein level by ELISA, *H. pylori* was co-cultured with

gastric cell lines for 24 hours. In contrast, for investigation of IL-8 mRNA levels by TaqMan PCR the incubation time was only 2 hours. In case of adherence assays, 20 and 40 minutes of incubation time were used.

2.13 Adherence assay

For this experiment we used 3 different cancer cell lines KATOIII, AGS, and HeLa. The cancer cells were seeded in a six-well plate on coverslides and grown to a pre-confluent monolayer. After harvesting the bacteria from plates, cells were infected with different concentrations of bacteria: from MOI-10 to MOI-20. B128-wild type, *babA* and *hp0638* knockouts were added to the cells and incubated for 20 and 40 min, with and without shaking. Non-adherent bacteria were washed away with PBS (3x), and cells were fixed with 4% para-formaldehyde at least for 15 min. At this reaction adherence assay was finished in case using of *H. pylori* transformed with GFP (green fluorescent protein) tagged plasmid. Since its isolation from the luminescent jellyfish *Aequorea Victoria*, GFP has become a powerful tool for researches studying mammalian and microbiological systems. This is, because GFP absorbs the (short-wavelength) blue light and concomitantly emits (longer-wavelength) green light (149). Because the cDNA sequence of GFP is known, it can in principle be expressed in any organism for which a gene delivery system is available. In our study GFP was expressed in wild type and *hp0638* knockout strains. The fluorescence properties of GFP enabled us to detect the strains using microscope equipped for fluorescence with filters for the commonly used fluorophore fluorescein isothiocyanate (FITS). For strains without GFP the reaction was continued by blocking for 1 hour with 10% of normal goat serum. After washing with PBS (3x) the cover slides were incubated with primary Anti-*H. pylori* Ab (diluted 1:20) (Dako). Alexa 488 goat anti-rabbit IgG (diluted 1:200) was applied for the second antibody. Adherent *H. pylori* and cells were fixed at room temperature using 4% paraformaldehyde. Visualisation was performed by fluorescence microscopy. Bacteria/cell ratios were then determined by enumeration of adherent bacteria. In addition we stained membrane and nucleus of the cells by using Nile-red and TO-PRO-3 stains. After fixation with paraformaldehyde cell were washed two times and incubated in dark with saponin buffer for 25 minutes at room temperature. TO-PRO-3 solution was added and the reaction was incubated again for 40 minutes. After washing with PBS (3x), Nile-red solution was put on the cells. The long-wavelength TO-PRO-3 dye provide blue staining of nuclear. Nile-red dye was used for staining of cell membrane. Membrane lipids then fluoresce orange to red, depending on their relative hydrophobicity.

2.14 Enzyme-linked Immuno - Sorbent Assay (ELISA)

2.14.1 Principles

Enzyme-linked Immunosorbent Assays (ELISAs) combines the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily-assayed enzyme that possesses a high turnover number. ELISAs can provide a useful measurement of antigen or antibody concentrations.

A widely used immunoassay is the two-antibody "sandwich" ELISA. This assay is used to determine the antigen concentration in unknown samples. This ELISA is fast and accurate, and if a purified antigen standard is available, the assay can determine the absolute amounts of antigen in unknown samples. The sandwich ELISA requires two antibodies that bind to epitopes that do not overlap on the antigen. This can be accomplished with either two monoclonal antibodies that recognize discrete sites or one batch of affinity-purified polyclonal antibodies.

To utilize this assay, one antibody (the "capture" antibody) is purified and bound to a solid phase. Antigen is then added and allowed to complex with the bound antibody. Unbound products are then removed with a wash, and a labeled second antibody (the "detection" antibody) is allowed to bind to the antigen, thus completing the "sandwich". The assay is then quantitated by measuring the amount of labelled second antibody bound to the matrix with help of a colorimetric substrate. A major advantage of this technique is that the antigen does not need to be purified prior to use and that these assays are very specific. However, one disadvantage is that not all antibodies can be used. Monoclonal antibody combinations must be qualified at "matched pairs", meaning that they can recognize separate epitopes on the antigen.

2.14.2 Solution formulation

<i>Solutions for IL-8 ELISA (Biosource, Nivelles, Belgien)</i>		
Coating Buffer A	NaCl (Merck)	8,0 g
	Na ₂ HPO ₄ * H ₂ O (Merck)	1,42 g
	KH ₂ PO ₄ (Merck)	0,2 g
	KCl (Merck)	0,2 g
	H ₂ O dest.	ad 1000 ml, pH 7,4
Blocking Solution	NaCl	8,0 g
	Na ₂ HPO ₄ * H ₂ O	1,42 g
	KH ₂ PO ₄	0,2 g
	KCl	0,2 g
	BSA (Sigma)	5,0 g
	H ₂ O dest.	ad 1000 ml, pH 7,4
Assay Buffer	NaCl	8,0 g
	Na ₂ HPO ₄ * H ₂ O	1,42 g
	KH ₂ PO ₄	0,2 g
	KCl	0,2 g
	BSA	5,0 g
	Tween 20 (Biosource)	1 ml
	H ₂ O dest.	ad 1000 ml, pH 7,4
Wash buffer	NaCl	9,0 g
	Tween 20 (Biosource)	1 ml
	H ₂ O dest.	ad 1000 ml, pH 7,4
Stop Solution	H ₂ SO ₄ (Biosource)	1,8 N
Coating-Antibody (Biosource)	Maus IgG1	1:1000 Dilution

		in Coating-Buffer-A
Detections-Antibody (Biosource)	Maus IgG1	1:1250 Dilution in Assay-Buffer
Streptavidin-Horseradish- Peroxidase Conjugate (Biosource)		1:5000 Dilution in in Assay-Buffer
Tetramethylbenzidin (Biosource)		
Standards Dilution (Biosource)	1:2 Dilutions start with 800 pg/ml	

Table 14. Reagents for IL-8 ELISA (Biosource, Nivelles, Belgien).

2.14.3 Procedure of ELISA

96-well plates were coated with 100µl/well coating antibody at 1 µg/ml for 12-18 hours at 2-8°C. The coating antibody was aspirated from the wells and tap on absorbent paper to remove the excess liquid. The plates were washed one time then blocked for 2 hours at room temperature by adding 300µl Blocking Solution to each well. If the plates were not used immediately, they were stored sealed for up to 5 days at 4°C in Blocking solution. After 4 washes, diluted standards were added at 100µl/well followed immediately by addition of 50µl of biotinylated antibody and incubated for 2 hours at room temperature with continual shaking (700 rpm). After 4 washes, 100µl/well of Streptavidin-HRP 1/2500- was added and incubated for 30 minutes at room temperature with continual shaking (700rpm). After 4 washes, 100µl/well of the Chromogen TMB (tetramethylbenzidine) was added and incubated in dark for 30 minutes at room temperature with continual shaking (700 rpm) and then 100µl/well of Stop Solution were added. At the end, the optical density of the plates was measured at 450 nm (reference filter 450 nm.)

2.15 Statistical analysis.

Statistical analysis was performed using the Mann Whitney Rank Sum test, a t-test, Chi Square test , depending on the data set of concern. The test applied is indicated in the figure and table legends. P values of less than 0.05 were considered to be significant.

3 Results

3.1 Patients and biopsy bank

The large biopsy bank was established in our laboratory in previous years (177). Gastric antrum and corpus biopsy specimens were collected from 742 patients undergoing routine gastroscopy. RNA and DNA were isolated from these biopsies. The histological and immunohistochemical staining also obtained from these samples. Antral and corpus sections were used for RNA and DNA isolation, histological and immunohistochemical staining. This gave us a chance to perform genetic analysis of *H. pylori* and the human host. *H. pylori* infection was detected from the patient biopsy by histopathology and *vacA* PCR. The patients were between the ages of 32-92. Among the infected patients, 88.9% were German, 7.6% Turkish, and the others from different parts of Europe, such as Greece, Bosnia, Serbia, Croatia and Italy. African, Asian and American patients were not included in the study.

3.2 Pathogenesis during *H. pylori* infection: The role of cytokine gene polymorphisms

3.2.1 Patient population, polymorphism frequencies and *H. pylori* strain characteristics

Strain characteristics of *H. pylori* were investigated in 207 infected patients by PCR. The *vacAs1* genotype was found in 78.3% (162/207), *cagA* in 70.5% (146/207) and *babA2* in 35.3% (73/207). Almost all *cagA*⁺ strains were simultaneously *vacAs1*⁺ (143/146) and almost all *babA2*⁺ strains were *cagA*⁺ (67/73). Genotyping of cytokine gene polymorphisms was performed after DNA isolation from the biopsies by RFLP analysis and allelic discriminating TaqMan PCR. Allele frequencies of *IL-10*, *TNF-A* and *IFN-G* polymorphisms and of different *IL-10* haplotypes are shown in figure 1. For all control populations (G0/G1; L1; Non-IM; Non-AG) alleles at the different loci were in Hardy-Weinberg equilibrium, with non-significant χ^2 values. Since the development of severe histological changes is influenced by age and gender, these parameters were investigated in different patient groups. There were no significant differences in age and gender between carriers of different cytokine gene polymorphisms or patients infected with different strain types.

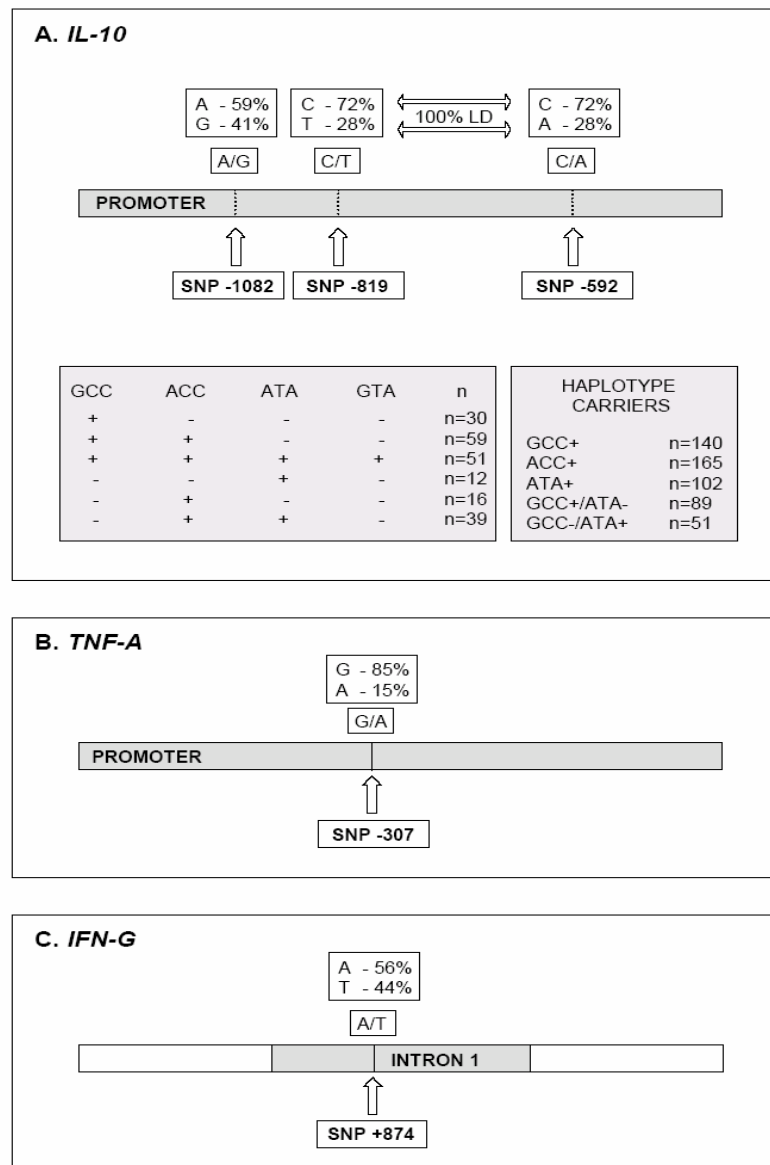


Figure 1. Allele/haplotype frequencies of *IL-10*, *TNF-A* and *IFN-G* polymorphisms. Single nucleotide polymorphisms (SNPs) at position -819 and -592 in the *IL-10* promoter were in complete linkage disequilibrium (LD). Four *IL-10* haplotypes were observed and the occurrence of each haplotype and of haplotype combinations is listed in the gray boxes.

3.2.2 *IL-10* polymorphisms influence mucosal *IL-10* expression during chronic *H. pylori* infection

To investigate the influence of *IL-10* polymorphisms on cytokine expression, *IL-10* mRNA levels were determined from the biopsies by quantitative real-time TaqMan RT-PCR. Correlating mucosal *IL-10* mRNA amounts to host *IL-10* polymorphisms, -1082AA was associated with low *IL-10* expression, -1082GA with intermediate and -1082GG with high mucosal *IL-10* mRNA levels (figure 2A). "Similarly, the *IL-10*-

592C and -819C alleles, which are in complete linkage disequilibrium, were associated with higher IL-10 mRNA levels. As shown in figure 2A, -592CC carriers had almost two times higher mRNA levels than -592AA carriers. We then explored the influence of *IL-10* haplotypes and haplotype combinations on IL-10 expression. Figure 2B shows, that the GCC haplotype (-1082G, -819C, -592C) was associated with significantly higher IL-10 mRNA levels than the ATA haplotype. ACC haplotype carriers had intermediate IL-10 mRNA levels. Furthermore, the highest IL-10 mRNA levels were observed in patients with the haplotype combination GCC^+/ATA^- , whereas GCC^-/ATA^+ carriers were associated with the lowest IL-10 mRNA levels.

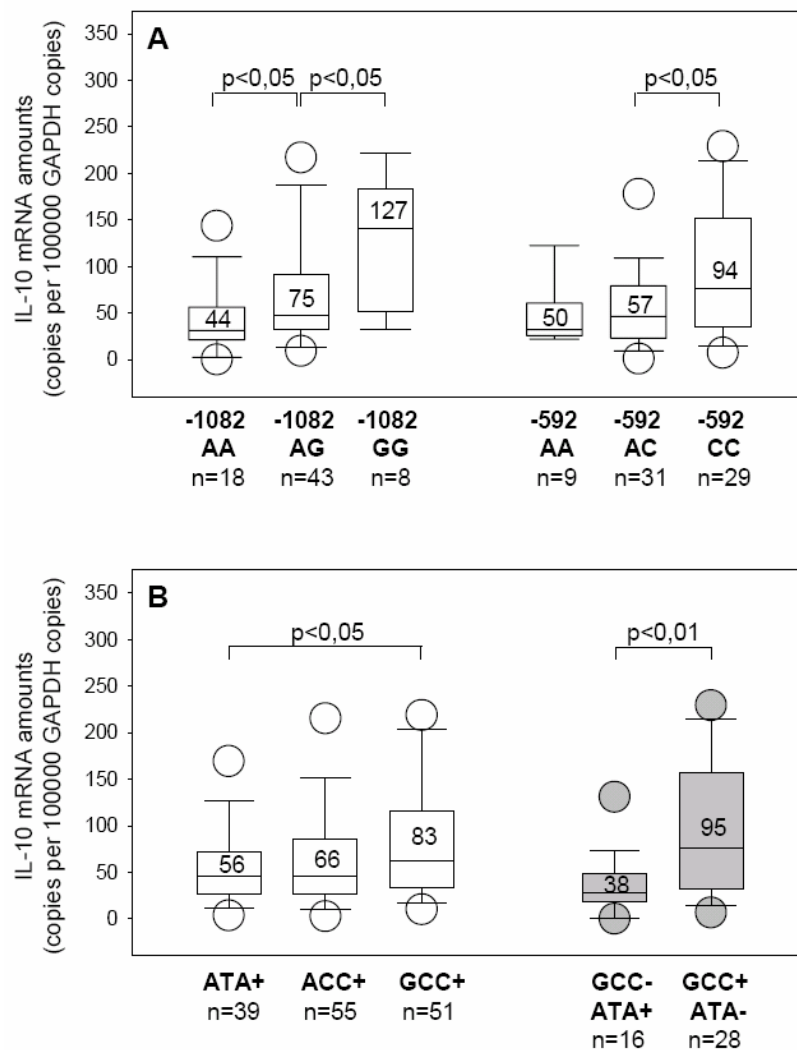


Figure 2. Interleukin 10 (*IL-10*) mRNA amounts in *Helicobacter pylori* infected patients harboring different *IL-10* single nucleotide polymorphisms (SNP) alleles (A) and different *IL-10* SNP haplotypes/haplotype combinations (B). As *IL-10*-592C allele is in complete linkage disequilibrium with *IL-10*-819C, data are not shown for the latter. Bars within the plots represent median values and numbers above indicate mean *IL-10* mRNA amounts for each group. *P* values were calculated using Mann-Whitney *U* test. GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

3.2.3 High *IL-10* secreting haplotypes are associated with infection by virulent *cagA*⁺, *vacAs1*⁺ and *babA2*⁺ strains

Next, the infecting strain type was correlated to the different *IL-10* haplotypes. Interestingly, the prevalence of *cagA*⁺ strains was higher among GCC carriers than among ATA carriers [104/140 (74.3%) vs. 64/102 (62.8%); $p=0.05$, χ^2 test]. Patients with the ACC haplotype had an intermediate frequency of *cagA*⁺ strains [115/165 (69.7%)]. Similarly, the frequency of *vacAs1*⁺ and *babA2*⁺ strains was higher among GCC carriers than among ATA carriers [*vacAs1*⁺ strains: 115/140 (82.1%) vs. 72/102 (70.6%); $p<0.05$, χ^2 test; *babA2*⁺ strains: 59/140 (42.1%) vs. 31/102 (32.4%); $p=0.06$, χ^2 test].

More pronounced were the differences between carriers of combined pro- and anti-inflammatory haplotypes. Figure 3 shows that the prevalence of *cagA*⁺, *vacAs1*⁺, and *babA2*⁺ strains was lower among GCC⁻/ATA⁺ patients [*cagA*⁺ strains: 29/51 (56.9%); *vacAs1*⁺ strains: 35/51 (68.6%); *babA2*⁺ strains: 9/51 (17.6%)] than among GCC⁺/ATA⁻ patients [*cagA*⁺ strains: 69/89 (77.5%); *vacAs1*⁺ strains: 78/89 (87.6%); *babA2*⁺ strains: 37/89 (41.6%)]. The most obvious difference was observed for *babA2*⁺ strains: the prevalence of *babA2*⁺ strains was 2.4 times higher among patients with the high *IL-10* secreting haplotype combination compared to the low *IL-10* secreting haplotype combination. Interestingly, polymorphisms in other cytokine genes investigated in this study were not associated with certain bacterial strain types.

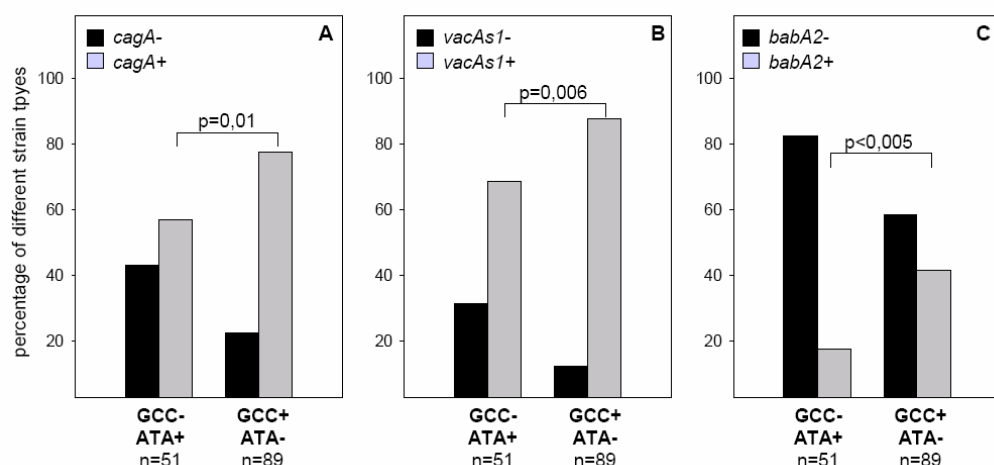


Figure 3. Prevalence of infection with *cagA*⁺ and *cagA*⁻ strains (A) as well as *vacAs1*⁺ and *vacAs1*⁻ strains (B) and *babA2*⁺ strains (C) in carriers of different *IL-10* haplotype combinations (GCC⁻/ATA⁺, low *IL-10* secreting haplotype combination; GCC⁺/ATA⁻, high *IL-10* secreting haplotype combination). *P* values were calculated by a χ^2 test.

3.2.4 The role of *IL-10* polymorphisms for the gastric inflammatory response and the development of IM/AG

To investigate the role of *IL-10* polymorphisms on the development of gastric inflammation and IM/AG, the frequency of *IL-10* haplotypes was compared in different patient groups. Interestingly, although *IL-10* polymorphisms influenced IL-10 expression, the frequency of *IL-10* haplotypes was similar in patients with high or low degrees of gastritis and in patients with or without IM/AG. Table 15 shows frequencies of IL-10 haplotype combinations in patients with different degrees of granulocytic (G) and lymphocytic infiltration (L) as well as presence or absence of intestinal metaplasia (IM) and atrophic gastritis (AG). G0, absent; G1, mild; G2, moderate; G3, severe granulocytic infiltration; L1, mild; L2, moderate; L3, severe lymphocytic infiltration. GCC⁻/ATA⁺, low IL-10 secreting haplotype combination; GCC⁺/ATA⁻, high IL-10 secreting haplotype combination. Odds ratios with exact 95% confidence intervals were calculated using StatXact-4.0.1 software. Table 15 shows that even the frequency of high or low IL-10 secreting haplotype combinations did not differ significantly among groups. Although the frequency of the GCC⁺/ATA⁻ haplotype combination tended to be higher among patients with IM/AG (OR 1.2 to 4.2), the differences compared to the GCC⁻/ATA⁺ group were mostly not significant.

Antrum												
	G2/G3	G0/G1	OR	L2/L3	L1	OR	IM	Non-IM	OR	AG	Non-AG	OR
	n=152	n=55	(95% CI)	n=162	n=45	(95% CI)	n=64	n=143	(95% CI)	n=49	n=158	(95% CI)
GCC-ATA+	35	16	1.0	44	7	1.0	11	40	1.0	7	44	1.0
	23%	29%	reference	27%	16%	reference	17%	30%	reference	14%	28%	reference
GCC+ATA-	65	24	1.2	69	20	0.6	34	55	2.3	24	65	2.3
	43%	44%	(0.5-1.8)	43%	44%	(0.2-1.5)	53%	39%	(1.0-5.5)	49%	41%	(0.9-6.9)
Corpus												
	G2/G3	G0/G1	OR	L2/L3	L1	OR	IM	Non-IM	OR	AG	Non-AG	OR
	n=84	n=123	(95% CI)	n=62	n=145	(95% CI)	n=12	n=195	(95% CI)	n=12	n=195	(95% CI)
GCC-ATA+	15	36	1.0	13	38	1.0	3	48	1.0	1	50	1.0
	18%	29%	reference	21%	26%	reference	25%	25%	reference	8%	26%	reference
GCC+ATA-	38	51	1.8	32	57	1.6	6	83	1.2	7	82	4.2
	45%	42%	(0.8-4.0)	52%	39%	(0.7-3.9)	50%	43%	(0.2-7.5)	58%	42%	(0.5-196.1)

Table 15. Frequencies of *IL-10* haplotype combinations in patients with different degrees of granulocytic (*G*) and lymphocytic infiltration (*L*) as well as presence or absence of intestinal metaplasia (*IM*) and atrophic gastritis (*AG*). *G0*, absent; *G1*, mild; *G2*, moderate; *G3*, severe granulocytic infiltration; *L1*, mild; *L2*, moderate; *L3*, severe lymphocytic infiltration. *GCC-ATA+*, low *IL-10* secreting haplotype combination; *GCC+ATA-*, high *IL-10* secreting haplotype combination. Odds ratios with exact 95% confidence intervals were calculated using StatXact-4.0.1 software.

3.2.5 The role of *TNF-A-307* and *IFN-G+874* polymorphisms for mucosal cytokine expression and the gastric inflammatory response

Next, the influence of the *TNF-A-307* and the *IFN-G+874* polymorphisms on mucosal cytokine expression was assessed. Figure 4 shows that there were no significant differences in IFN- γ and TNF- α levels between different allele carriers. This suggests, that those polymorphisms do not influence cytokine expression in the course of chronic *H. pylori* infection. Accordingly, there were no significant associations of these polymorphisms with gastric inflammation and the presence of IM and AG (tables 16 and 17). Although the frequency of the *TNF-A-307A* allele tended to be higher among patients with IM/AG, the differences to the *TNF-A-307TT* group did not reach statistically significant levels.

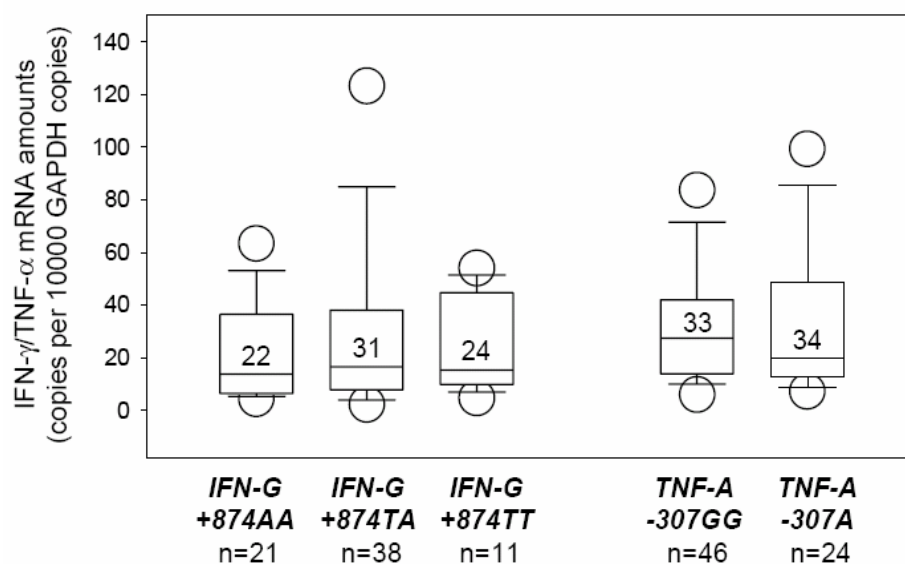


Figure 4. *TNF- α* and *IFN- γ* mRNA amounts in *H. pylori* infected patients harboring different *TNF-A-307* and *IFN-G-874* alleles. Bars within the plots represent median values, numbers above indicate mean cytokine mRNA amounts for each group. *P* values were calculated by a Mann-Whitney *U* test, but were not significant between groups.

Antrum												
	G2/G3	G0/G1	OR	L2/L3	L1	OR	IM	Non-IM	OR	AG	Non-AG	OR
	n=152	n=55	(95% CI)	n=162	n=45	(95% CI)	n=64	n=143	(95% CI)	n=49	n=158	(95% CI)
GG	113	34	1.0	115	32	1.0	40	107	1.0	34	113	1.0
	74%	62%	reference	71%	71%	reference	62%	75%	reference	69%	72%	reference
A carrier	39	21	0.6	47	13	1.0	24	36	1.8	15	45	1.1
	26%	38%	(0.3-1.1)	29%	29%	(0.5-2.3)	38%	25%	(0.9-3.5)	31%	28%	(0.5-2.3)
Corpus												
	G2/G3	G0/G1	OR	L2/L3	L1	OR	IM	Non-IM	OR	AG	Non-AG	OR
	n=84	n=123	(95% CI)	n=62	n=145	(95% CI)	n=12	n=195	(95% CI)	n=12	n=195	(95% CI)
GG	59	88	1.0	43	104	1.0	6	141	1.0	6	141	1.0
	70%	72%	reference	69%	72%	reference	50%	72%	reference	50%	72%	reference
A carrier	25	35	1.1	19	41	1.1	6	54	2.6	6	54	2.6
	30%	28%	(0.6-2.0)	31%	28%	(0.6-2.2)	50%	28%	(0.7-10.1)	50%	28%	(0.7-10.1)

Table 16. Frequencies of TNF-A-307 alleles in patients with different degrees of granulocytic (G) and lymphocytic infiltration (L) as well as presence or absence of intestinal metaplasia (IM) and atrophic gastritis (AG). G0, absent; G1, mild; G2, moderate; G3, severe granulocytic infiltration; L1, mild; L2, moderate; L3 severe lymphocytic infiltration. Odds ratios with exact 95% confidence intervals were calculated using StatXact-4.0.1 software.

Antrum												
	G2/G3	G0/G1	OR	L2/L3	L1	OR	IM	Non-IM	OR	AG	Non-AG	OR
	n=152	n=55	(95% CI)	n=162	n=45	(95% CI)	n=64	n=143	(95% CI)	n=49	n=158	(95% CI)
AA	47	18	1.0	54	11	1.0	20	45	1.0	17	48	1.0
	31%	33%	reference	33%	24%	reference	31%	31%	reference	35%	30%	reference
T carrier	105	37	1.1	108	34	0.7	44	98	1.0	32	110	0.8
	69%	67%	(0.5-2.2)	67%	76%	(0.3-1.4)	69%	69%	0.5-2.0	65%	70%	0.4-1.7

Corpus												
	G2/G3	G0/G1	OR	L2/L3	L1	OR	IM	Non-IM	OR	AG	Non-AG	OR
	n=84	n=123	(95% CI)	n=62	n=145	(95% CI)	n=12	n=195	(95% CI)	n=12	n=195	(95% CI)
AA	28	37	1.0	25	40	1.0	4	61	1.0	4	61	1.0
	33%	30%	reference	40%	28%	reference	33%	31%	reference	33%	31%	reference
T carrier	56	86	0.9	37	105	0.6	8	134	0.9	8	134	0.9
	67%	70%	(0.5-1.6)	60%	72%	(0.3-1.1)	67%	69%	(0.2-4.3)	67%	69%	(0.2-4.3)

Table 17. Frequencies of *IFN-G+874* alleles in patients with different degrees of granulocytic (G) and lymphocytic infiltration (L) as well as presence or absence of intestinal metaplasia (IM) and atrophic gastritis (AG). G0, absent; G1, mild; G2, moderate; G3, severe granulocytic infiltration; L1, mild; L2, moderate; L3 severe lymphocytic infiltration. Odds ratios with exact 95% confidence intervals were calculated using StatXact-4.0.1 software.

3.3 The role of *hp0638* in pathogenesis during *H. pylori* infection

3.3.1 Sequence Analysis in the Signal-Peptide Coding Region of the *hp0638* Gene

The functional status of *hp0638* is regulated by slipped strand repair mechanism based on the number of CT dinucleotide repeats in the signal sequence coding region of the *hp0638* gene. Samples from 59 patients who were *H. pylori* positive were examined. The signal sequences of the *hp0638* gene including the CT repeats were amplified by PCR. Resulting amplified DNA fragments were purified and sequenced by GATC Company (Konstanz, Germany).

CT dinucleotide repeats present in the signal sequence coding region of the *hp0638* gene ranged from 4 to 11. Strains with a functional *hp0638* gene contained six or nine CT dinucleotide repeats or 5 CT+TT. These sequences keep the peptide in frame and the gene status is “on”. Strains with a non-functional *hp0638* gene contain all remaining variations of CT dinucleotide repeats in the signal peptide coding region of the *hp0638* gene. In such cases the peptide is out-of-frame and the gene status will be “off”, suggesting that a slipped strand repair mechanism modulates this gene (Table 18).

	NUMBER OF PATIENTS	SEQUENCE OF THE SIGNAL-PEPTIDE CODING REGION	NUMBER OF CT REPEATS	GENE STATUS
1	9	ATGAAAAAAGCCCTCTTACTCTCTCTCTTTCTCT.....CGTTTGGCTC M K K A L L L S L F L S F W L	5 + 2	On
2	1	ATGAAAAAAGCTCTTACTAACTCTCTCTCT.....CGTTCTGGCT M K K A L L L T L S L V L A	5	Off
3	1	ATGAAAAAAGCTCTTTTACTCTCTCTCTCTCT.....CGTTTTGGCTC M K K A L L L S L S L S F W L	8 (2+6)	On
4	19	ATGAAAAAAGCTCTTACTAACTCTCTCTCTCT.....CGTTCTGGCTC M K K A L L L T L S L S F W L	6	On
5	6	ATGAAAAAAGCTCTTACTAACTCTCTCTCTCTCT.....CGTTCTGGC M K K A L L L T L S L S R S G	7	Off
6	1	ATGAAAAAAGCCCTCTTACTCTCTCTTTCTCT.....CGTTTGGCT M K K A L L L S L S L V L A	4 + 2	Off
7	1	ATGAAAAAAGCTCTTACTAACTCTTTCTCTCT.....CGTTCTGGCTC M K K A L L L T L S L S F W L	2 + 3	On
8	5	ATGAAAAAAGCTCTTACTAACTCTCTCTCTCTCTCT.....CGTTCTGGCTC M ^a K K A L L L T L S L S L S F W L	9	On
9	11	ATGAAAAAAGCTCTTACTAACTCTCTCTCTCTCTCT.....CGTTCTGGCT M K K A L L L T L S L S L V L A	8	Off
10	3	ATGAAAAAAGCTCTTACTCACTCTCTCTCTCTCTCT... CGTTTCTGG M K K A L L L T L S L S L S S G S ATGAAAAAAGCTCTTACTAACTCTCTCTCTCTCTCT... CGTTTTGGC M K K A L L L T L S L S L S R F G	10	Off
11	1	ATGAAAAAAGCTCTTACTAACTCTCTCTCTCTCTCTCTCGTTCTGGCT M K K A L L L T L S L S L S L V L A	11	Off
12	1	ATGAAAAAAGCTCTTACTCTCTCTCTCT.....CGTTTGGC M K K A L L L S L S R F G	6 (2+4)	Off

Table 18. Signal-Sequence coding region of the *hp0638* gene. Variations in *hp0638* CT repeats, sequenced in 59 *H. pylori* infected patients.

3.3.2 Association of *hp0638* with other virulence factors

DNA was isolated from gastric biopsies and the presence of *H. pylori* virulence factors was determined by PCR in regard to the presence of *vacAs1/2* alleles, *cagA*, and *babA2*. We initially sequenced *hp0638* gene from 59 patients, however, genotyping of the genes failed in six patients. Therefore, in the current experiment we present the data from 53 patients. Figure 5 A shows that the *vacAs1* genotype was found in 75%, *cagA* in 59%, *babA2* in 34%, and *hp0638-on* in 57% of all *H. pylori* antral biopsies. The *hp0638* gene showed a strong association with *cagA* gene presence (Figure 5 B), because nearly all *hp0638-on* strains (26 of 30 patients; 87%) were simultaneously *cagA* positive. Furthermore, *hp0638-on* strains were also associated with presence of the *vacAs1*, and *babA2* genes. All of *hp0638-on* strains were *vacAs1* positive (30 of 30 patients; 100%), and 50% of *hp0638-on* strains harboured the *babA2* gene (15 of 30 patients).

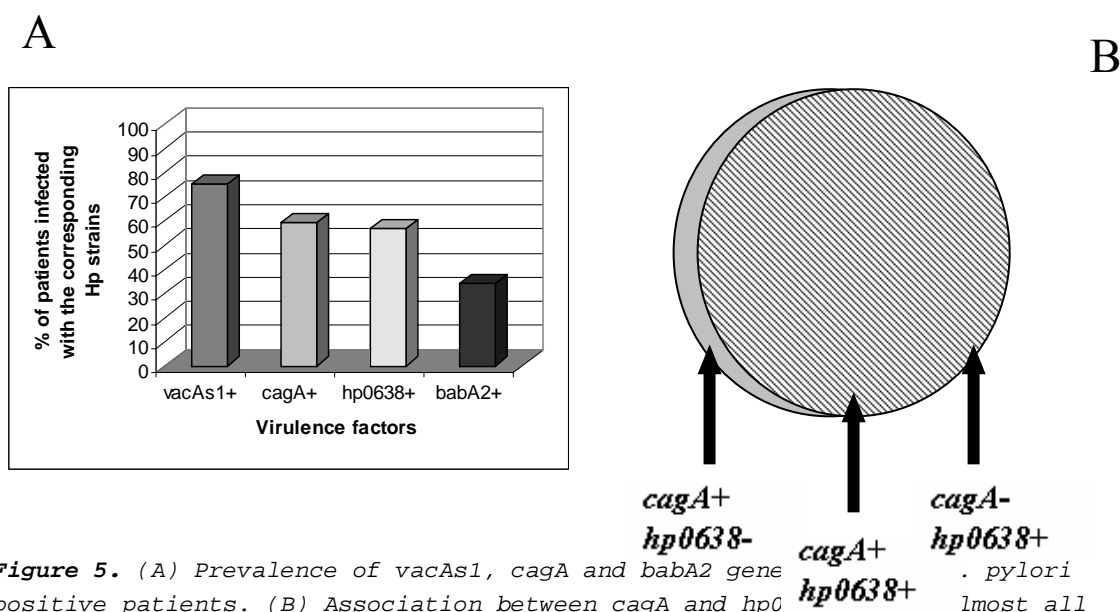


Figure 5. (A) Prevalence of *vacAs1*, *cagA* and *babA2* gene positive patients. (B) Association between *cagA* and *hp0638*. *H. pylori* most all (87%) *hp0638-on* *H. pylori* strains were simultaneously *cagA* positive.

3.3.3 Construction of *hp0638* Isogenic Mutant Strains of *H. pylori*

To evaluate the function of *hp0638* we generated *H. pylori* knockout mutants for the *hp0638* gene. Therefore, we performed gene targeting by homologous recombination, which allows the introduction of specific mutations into any gene locus. In our case the *hp0638* gene was inactivated by interrupting its coding sequence with a positive selectable marker – chloramphenicol. Homologous recombination occurring between host chromosomal DNA sequence and similar sequences present in the introduced DNA

caused the chromosomal integration of the transformed DNA molecule. For construction of isogenic mutant strains, we used the B128 *H. pylori* strain. Three independent *hp0638* mutant strains (A, B, C) were constructed. The *hp0638* gene was amplified by PCR. Primer sequences were chosen to create *KpnI* restriction sites at both ends of the PCR product. The amplified fragment was then digested by *KpnI* and inserted into the *KpnI* restriction site of UP1 plasmid, a modified pBluescript-II SK(+) vector. Subsequently, a chloramphenicol resistance cassette was cleaved from pAV36 vector by *PvuII* enzyme. Thereby blunt ends were created. The cassette was inserted into a *NheI* restriction site of the inserted *hp0638* DNA. The plasmid (1 to 2 μ g) was then used for inactivation of the *H. pylori* *hp0638* gene by natural transformation. For selection of transformants suspension were spread onto Wilkins-Chalgren agar plates containing 5 μ g of chloramphenicol. Single colonies were selected on fresh antibiotic containing plates, and transformants were grown. Correct insertion of the targeting construct into the chromosomal *hp0638* locus was proved by PCR (Figure 6).

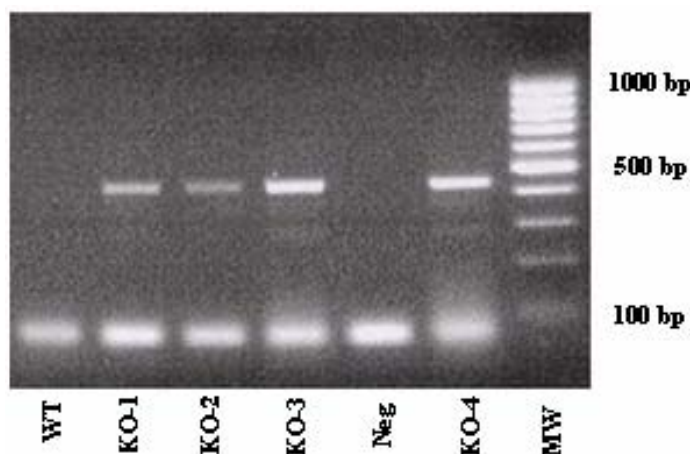


Figure 6. PCR to check the *hp0638* knockout construct. The forward primer was chosen to lay in the *hp0638* gene, whereas the reverse primer is in the antibiotic resistance cassette. All four mutants, used in the experiments showed the presence of the resistance cassette. Wild type and negative control are also shown. MW, Molecular weight standard.

3.3.4 The *hp0638*-knockout mutant of *H. pylori* showed reduced adherence to gastric cancer cell lines

Adherence is an important virulence determinant for *Helicobacter pylori* that may contribute to differences in pathogenicity (159).

The *hp0638* gene encodes protein that belongs to the outer membrane protein gene family and could be a good candidate as an adherence factor of *H. pylori*. Therefore, we investigated the role of *hp0638* for bacterial adherence.

Adherence of the wild type B128 strain and the corresponding *hp0638* mutant to gastric epithelial cells was assessed by quantitative adherence assay, using fluorescence and confocal microscopy (Figure 7 A and 7 B). Bacteria were resuspended in PBS and standardized numbers were coincubated with KATO III epithelial cells (bacteria –to cell ratio 10:1 and 20:1) at 37° for 40 minutes. Nonadherent bacteria were removed by two further washes with PBS, before KATO III cells with adherent bacteria were fixed with 4% paraformaldehyde. The reaction was visualized by fluorescence microscopy (Figure 7 A). The adherence phenotype was assessed by the calculation of the number of bacteria adhering to each cell.

Both the mutant and the wild type strain were able to adhere to KATO III cells (Figure 8). However we observed a significant difference in binding to the cells between the mutant and the wild type strains. After 40 min of coincubation, KATO III cells incubated with the mutant had less adherent bacteria than the cells infected with the wild type strain. Quantification of bacteria per cell showed that the *hp0638* mutant had significantly reduced effect in binding to KATO III cells (about 30% reduction for the *hp0638* mutant, $p < 0.001$ in Chi-Square compare to the wild type) (Figure 8). Similar patterns were observed with AGS cells and using different strains (data not shown).

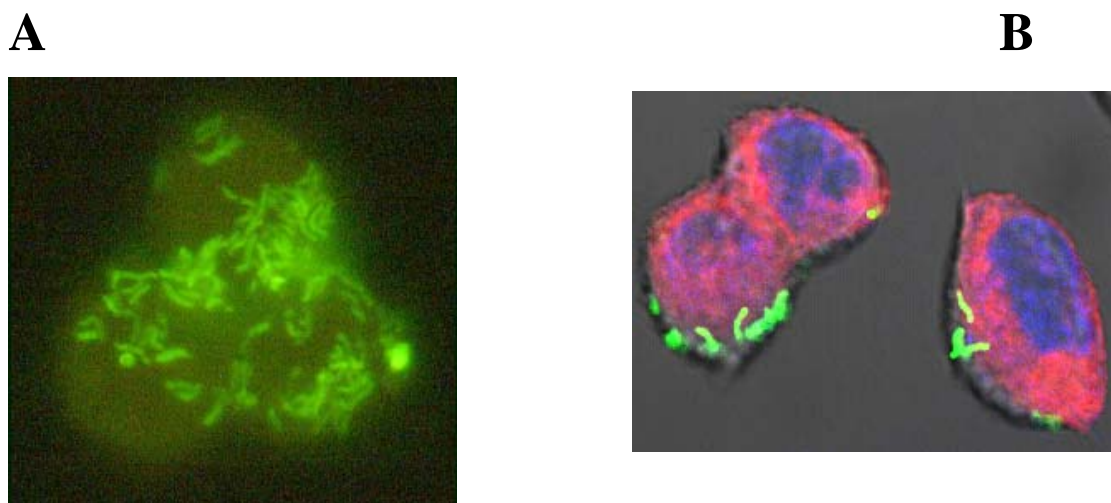


Figure 7. Adherence assay for binding of *H. pylori* to gastric epithelial cell lineages. (A) Fluorescence microscopy of *H. pylori* cells expressing GFP. Bacteria bind tightly to gastric epithelial cells (magnification: 400x). (B) Confocal microscopic image of GFP expressing bacteria adhering to KATO III gastric cancer cells (600x). Nuclear and membrane staining of the cells was performed (nucleus, TO-PRO-3 staining; membrane, Nile red staining;).

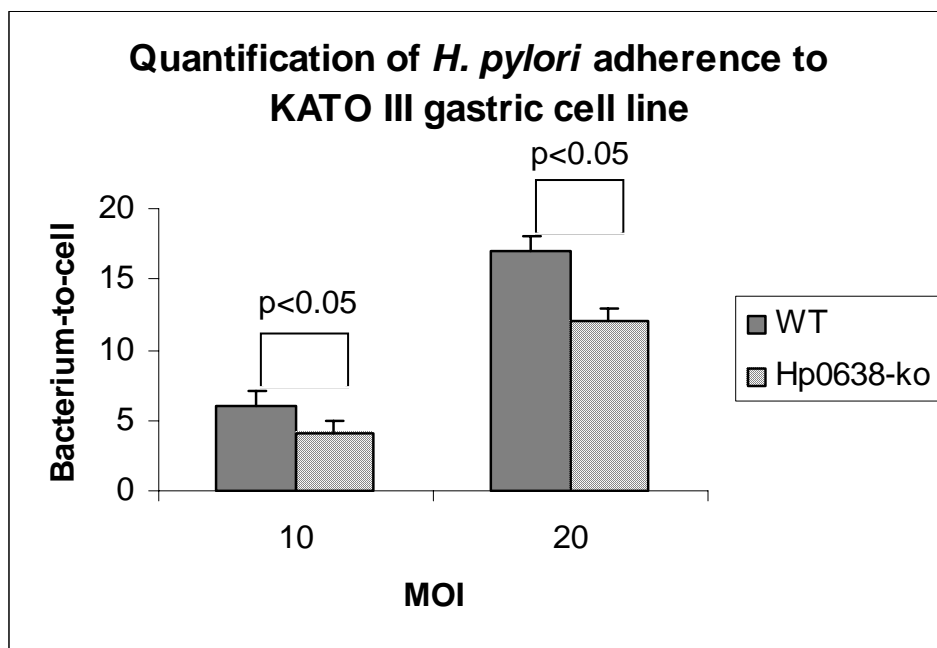


Figure 8. Adherence properties of *H. pylori* strains to gastric epithelial cells. KATO III cells were cocultured with a B128 wild type strain and its *hp0638* knockout mutant (bacterium-to-cell ratio 10:1 and 20:1). After different washing steps adherent bacteria were enumerated. Statistical analysis was performed using a Chi-Square test. One of three independent experiments, yielding similar results, is shown.

3.3.5 Influence of *hp0638* on bacterial colonization

We have shown that the *hp0638* gene mediates adherence *in vitro*. It is known that adherence protects *H. pylori* from clearance of the bacterium from the stomach by peristaltic movements or washout with the luminal fluid (177). Therefore bacterial colonization densities would be expected to be higher in patients harbouring *H. pylori* strains with good adherence properties. Therefore we explored the association of *hp0638* with *in vivo* colonization density of *H. pylori*. 37 antral sections were evaluated in regard to bacterial colonization density. After immunohistochemical *H. pylori* staining, bacterial density was determined semiquantitatively on an adequate proportion of epithelial layer and glandular parts were excluded from the evaluation. Degrees of colonisation were defined as follows 0, sporadic occurrence of single bacteria; 1, frequent occurrence of single bacteria or sporadic occurrence of bacterial clusters, 2, numerous bacterial clusters on the epithelial surface and in the glands; 3, dense continuous bacterial layer in many arrears of biopsies.

The association of colonisation densities with the presence of functional *hp0638* gene is shown in figure 9. Colonization densities were significantly lower in patients infected with the *hp0638*-off strains than in patients infected with the *hp0638*-on strains

($p < 0,05$; Mann-Whithney Test). Patients infected with the *hp0638*-on strains had mostly grade 3 colonisation densities, whereas patients infected with the *hp0638*-off strains showed the low degrees of bacterial colonization 0-2. Based on the association of the *hp0638* with increased *H. pylori* density, we supposed that the *hp0638* ameliorates bacterial colonisation in the gastric mucosa.

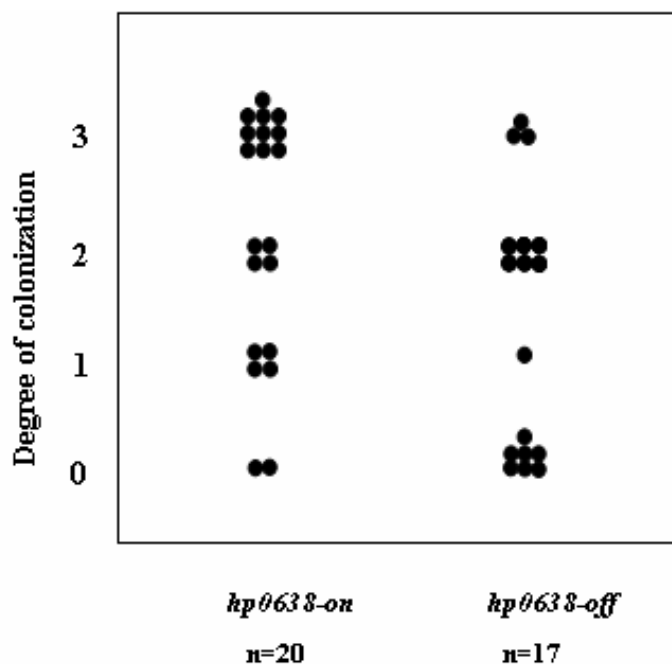


Figure 9. Bacterial colonization densities in patients infected with *Hp0638*-positive and -negative *H. pylori* strain types. Semiquantitative determination of colonization densities in the gastric mucosa after immunohistochemical *H. pylori* staining. Each circle represents the density of one patient infected with the indicated strain type.

3.3.6 Effect of *hp0638* on IL-8 secretion from epithelial cells *in vitro*

Gastric mucosal IL-8 levels correlate significantly with the extent of inflammatory cellular infiltration in gastric tissues of *H. pylori* positive persons (32). The induction of IL-8 *in vitro* has been attributed to a functional *cagPAI* (32). Yamaoka et al reported that *hp0638* reading frame status affects the ability of *H. pylori* strains to induce IL-8 secretion. To further evaluate those findings we constructed the *hp0638*-knockout mutant strains and examined their ability to induce IL-8 from gastric epithelial cells in comparison to the wild type strains. Each *H. pylori* strain was incubated with KATO-III

cells for 24hrs and the supernatants were assayed by ELISA for IL-8 release. Various concentrations of bacteria were used (10, 50, 100 bacteria per cell). As it is shown in figure 10 the *H. pylori* the B128 WT strain induced high levels of IL-8 secretion from KATO-III cells. The effect was dose dependent. The increase of bacterial concentrations was followed by a correspondent increase of IL-8 secretion. Importantly, there was no difference in IL-8 induction between the B128 WT strain and three other independent *hp0638* mutants (Figure 10). As a control the *cagE* knockout mutant was used. The *cagE* gene is one of the *cagPAI* island genes, which is a functional marker of the *cagPAI*. After deletion of *cagE*, pathogenicity island loses its functional activity (39). The *cagE* mutant strain induced significantly lower IL-8 production from gastric cancer cell lines, compared to the wild type strain (data not shown).

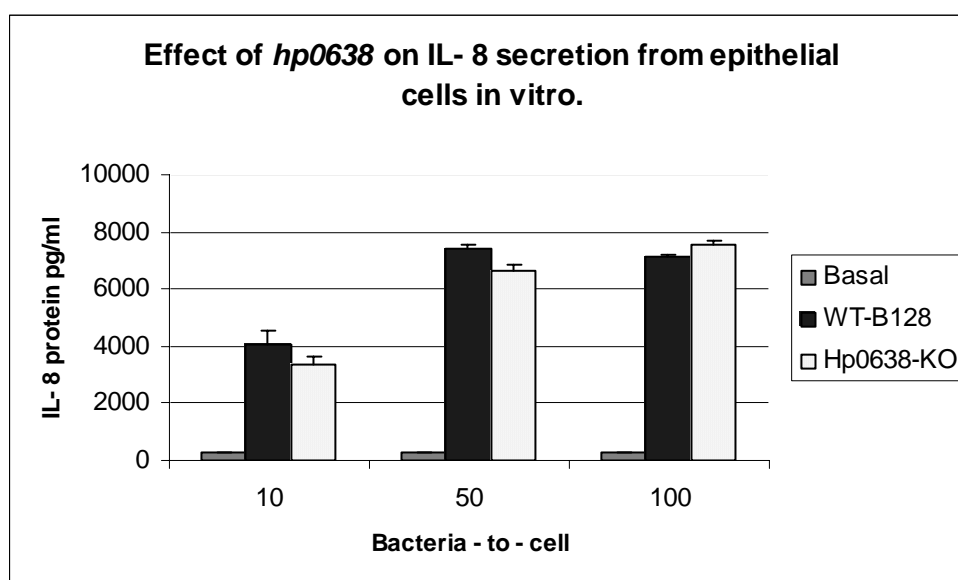


Figure 10. Influence of *hp0638* on IL-8 secretion from gastric cancer cell lines. IL-8 protein production from KATO III cells after incubation with wild type and mutant *H. pylori* strains. The graphic encloses data from 5 independent experiments $n=5$.

3.3.7 The role of *hp0638* for IL-8 expression *in vivo*

The *hp0638* gene has been reported to increase IL-8 mRNA expression in the gastric mucosa of *H. pylori* positive patients and influence the clinical outcome of infection (221). In the current study we also investigated effect of *hp0638* on the IL-8 expression *in vivo*. Patients infected with different strain types were divided into four groups,

depending on their *cagA* and *hp0638* status (Figure 11). IL-8 mRNA levels were measured by quantitative TaqMan PCR in the gastric mucosa of the patients. Then the relationship between IL-8 mRNA levels and the *hp0638* presence were determined (Figure 11). The amount of IL-8 mRNA was significantly higher in *H. pylori* positive compared to non-infected patients (data not shown). IL-8 amounts were found to be differ significantly in patients infected with the different strain types. As shown in Figure 11, the lowest IL-8 mRNA levels were detected in patients that were infected with *cagA*⁻ strains, independently of the *hp0638* status, furthermore no significant differences between *cagA*⁻/*hp0638*-off and *cagA*⁻/*hp0638*-on strains were found. *cagA*⁺ strains induced higher levels of IL-8 mRNA in comparison to patients that were infected with *cagA*⁻ strains ($p < 0.05$ Mann-Whitney Test). This effect is independent of the *hp0638* gene status, as the IL-8 mRNA levels were similar in patients infected with *hp0638*-on/*cagA*⁺ or with *hp0638*-off/*cagA*⁺ strains ($p = 0.413$, Mann-Whitney Test;). Therefore, *hp0638* has no significant effect on IL-8 expression *in vivo*. In contrast, infection with *cagA*-positive *H. pylori* strains was significantly associated with the high IL-8 mRNA expression *in vivo*.

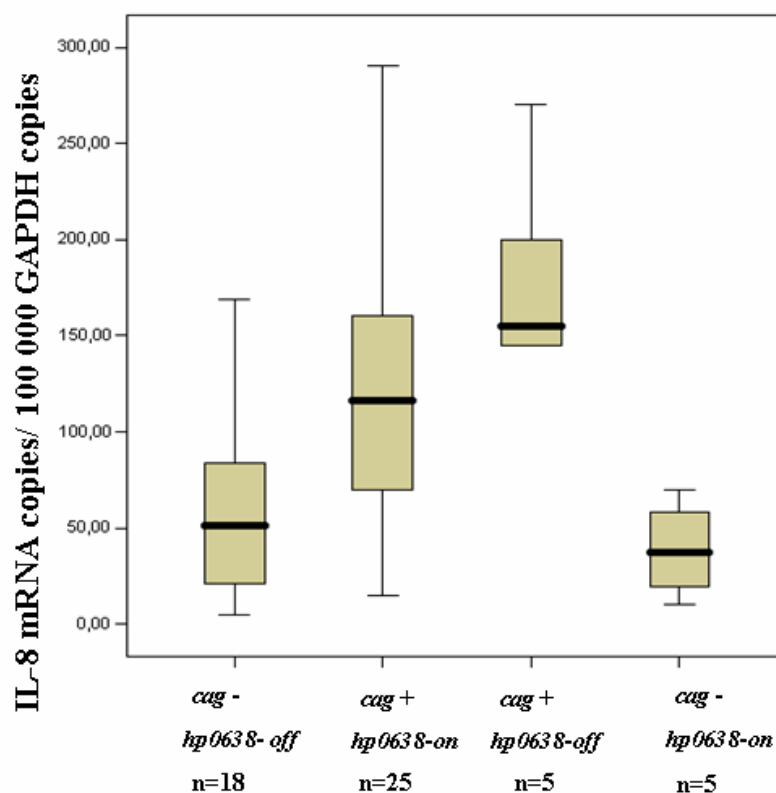


Figure 11. IL-8 mRNA amounts in gastric biopsies infected with different bacterial strain types.

4 Discussion

4.1 General discussion

Infection with *H. pylori* leads to persistent colonisation and chronic inflammation of the gastric mucosa, thereby increasing the risk for the development of peptic ulceration, distal gastric adenocarcinoma and gastric lymphoma (39) (197). There are high inter-individual differences in the extent of gastric inflammation among *H. pylori* infected patients, and clinical consequences develop in only a small subgroup. Bacterial virulence factors, like the *cag* pathogenicity island (*cagPAI*), the vacuolating cytotoxin (*VacA*) and the blood group antigen-binding adhesin (*BabA*) are associated with enhanced inflammation and cancer development (32) (175) (46) (11). However, despite the well defined role of virulence factors, it is unclear why a considerable part of patients infected with the *cagA*⁺, *vacAs1*⁺ or *babA2*⁺ *H. pylori* strains does not develop severe pathologies lifelong. In addition to bacterial factors, yet mostly unknown host factors seem to influence the inflammatory response and the development of more severe pathology. The T helper cell response toward *H. pylori* is generally considered to be of the Th1 phenotype (64) (188), leading to a cell mediated immune response. There is increasing evidence that the *H. pylori* induced Th1 response contributes to cancer development (63). Downregulation of the Th1 response in mice by concurrent enteric helminth infection or p53 mutation was shown to protect against the development of atrophy, intestinal metaplasia and invasive gastric carcinoma (74) (75). However, the factors influencing the extent of the *H. pylori* induced Th1 response are currently unknown. Important cytokines characterizing Th1 mediated immune responses are interferon-gamma (IFN- γ) and tumor-necrosis-factor-alpha (TNF- α) both being up-regulated during chronic *H. pylori* infection (23) (177) (224). IL-10, which is also highly expressed in the *H. pylori* infected stomach (110) is one of the most important regulatory cytokines, inhibiting cell-mediated immune responses.

In the first part of the current study we investigated a large group of *H. pylori* positive patients with chronic gastritis and have shown how genetic polymorphisms in cytokine genes influence cytokine expression, gastric inflammation and bacterial strain selection in *H. pylori* infection.

In the second part of the study we explored the function of the novel putative virulence factor Hp0638 and its influence on bacterial adherence and the development of gastric pathology.

4.2 Influence of cytokine gene polymorphisms on colonisation with different *H. pylori* strain types

While inflammation is advantageous for a host when microbes can be eliminated, it may be deleterious when infection cannot be eradicated, since it leads to a continuous impairment of tissue structure and function. An interesting question is therefore how genetic variants in candidate genes influence the *H. pylori* induced inflammatory response and the accompanying development of gastric pathology. Genes encoding cytokines and related molecules harbour polymorphic regions, which are considered to alter gene transcription and thereby influence inflammatory processes in response to infectious diseases (18) (101). Studies investigating the role of genetic polymorphisms on cytokine secretion are controversial and the experimental approaches used (mainly *in vitro*) have several disadvantages (18) (3). For an example, experiments with stimulated PBMC from different polymorphism carriers are often not generally applicable, since the cytokine response may differ depending on the stimulus (pathogen) (3). Furthermore, the stimulus dose *in vitro* may not reflect the situation *in vivo*. To overcome these difficulties we determined actual cytokine mRNA levels *in vivo*, in the infected mucosa.

In the current study, we found that *IL-10* SNPs markedly influenced mucosal IL-10 expression in the course of chronic *H. pylori* infection: GCC haplotype carriers (*IL-10* – 1082G; –819C; –592C) were associated with high and ATA carriers with low IL-10 expression. These results show the functional relevance of *IL-10* SNPs *in vivo*.

Interestingly, *IL-10* polymorphisms were associated with infection by different bacterial strain types. *H. pylori* strains have co-evolved to colonize specific human host populations via the selection of certain bacterial properties (38) (117), however it is unknown which evolutionary forces influence strain selection. Ethnic differences in the frequency of host genetic polymorphisms may account for the diversity of *H. pylori* strain distribution in human populations. In the current study, the prevalence of virulent *cagA*⁺/*vacAsI*⁺/*babA2*⁺ *H. pylori* strains was higher among GCC than ATA carriers. There are two possible explanations for this association: either (i) initial infection with *cagA*⁺ strains may be more successful in GCC carriers, suggesting a host-specific colonization, or (ii) host-specific adaptation of the bacterium may occur in the course of chronic infection.

Regarding the first hypothesis (host-specific colonization), there is evidence from animal models that individuals differ in susceptibility to *H. pylori* strains during initial stages of colonization. For example, after challenge of rhesus monkeys with a mixture of 7 different *H. pylori* strains, different animals were colonized by different strains

(57). In addition, recently Ferrero et al. have shown in the mouse model, that *cagPAI* *H. pylori* strains have a selective advantage for colonisation. Since IL-10 lowers gastric inflammation (198), *cagPAI*⁺ strains (which usually induce a strong inflammatory reaction) may be more successful in initial colonization of hosts with the high *IL-10* producing haplotype GCC.

Regarding the second hypothesis (host-specific bacterial adaptation during chronic infection), it has been shown that *H. pylori* exists within its ecological niche as a quasispecies, a bacterial population in a continuous state of genetic flux, which allows optimal adaptation to changing conditions (120) (107). Exaggerated inflammation may be deleterious for the bacterium, since it leads to impairment of tissue structure/function and finally to a loss of its niche (20). Therefore, in patients with low IL-10 production (pro-inflammatory ATA haplotype carriers), it may be beneficial for *H. pylori* to lose its *cagPAI*, which is an unstable locus, (32) (113) to avoid an exaggerated immune response. Alternatively, in case of mixed infections, *cagPAI* strains may have a selective advantage in such hosts. Figure 12 summarizes the two hypotheses.

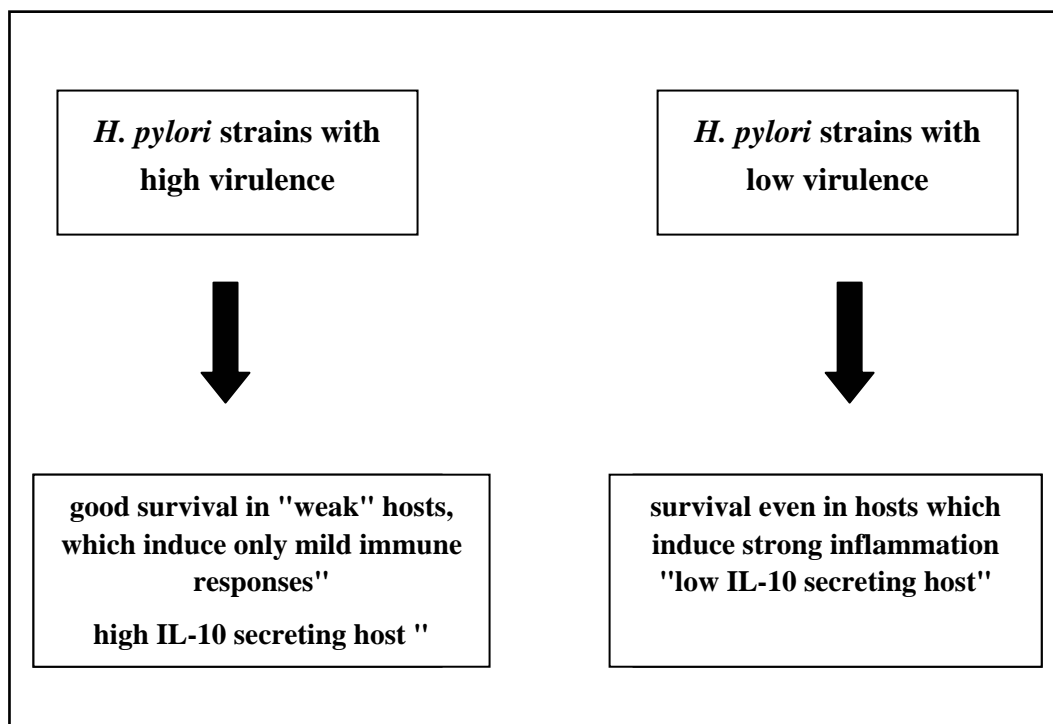


Figure 12. Possible explanation for the association of different *H. pylori* strains with distinct *IL-10* polymorphisms. In the course of infection, host-specific colonization or adaptation of *H. pylori* occurs. Thereby, virulent *cagA*⁺/*vacAs1*⁺ *H. pylori* strains may have a selective advantage in hosts which mount only low immune responses.

Correlating *IL-10* SNPs to gastric histopathology, we could not find significant associations to gastric inflammation or atrophy and intestinal metaplasia. In a mouse model, IL-10 has been shown to protect from development of severe gastritis and mucosal damage through down-regulation of the *H. pylori* induced Th1 response (198), (60). In humans, two recent large studies associated *IL-10* polymorphisms with gastric cancer, leading to conflicting results. Whereas one report found a higher prevalence of gastric cancer in patients with the pro-inflammatory (low IL-10 secreting) haplotype ATA (62), the other study reported an association of carcinoma with the anti-inflammatory (high IL-10 secreting) haplotype GCC (217). The apparent paradoxical observation in the latter study may be explained by our finding that *cagA*⁺ strains were more prevalent among GCC carriers. Further studies are needed to clarify the role of *IL-10* polymorphisms in *H. pylori* infection.

The *TNF- α -307* and *IFN- γ +874* SNPs were not associated with changes in mucosal cytokine expression and the inflammatory response to *H. pylori*. Several polymorphisms have been reported in the *TNF- α* promoter, almost all of them being functionally silent (3). The G/A polymorphism at position -307 is considered to be related to differences in TNF- α secretion by some authors (3). However, the results of the available studies are conflicting: two studies found a 20-40% increased TNF- α production from LPS stimulated PBMC of allele A carriers (28) (128), whereas the majority of reports found no significant differences (51) (100) (172), (154). Our data are in agreement with the latter reports and indicate, that the -307 polymorphism is either generally functionally silent or does not play a functional role for the *H. pylori* induced TNF- α expression. An alternative explanation for associations of cytokine gene polymorphisms with heightened inflammatory responses could be a linkage of these loci with other immunologically important genes (such as association of *TNF- α -307A* with the HLA-DR3 allele). However, in this study we could not find an influence of the *TNF- α -307* polymorphism on the severity of gastric inflammation.

There is only one study examining the relationship between *IFN- γ* polymorphisms and IFN- γ transcription, reporting an association of allele 2 of a polymorphic microsatellite marker (which shows an absolute correlation with the *IFN- γ +874T* allele (174), with heightened IFN- γ secretion (173). However, in the *H. pylori* infected stomach, the +874A/T SNP seems to have no influence on the *H. pylori* induced IFN- γ expression and the subsequent inflammatory response.

To summarize, the current study shows how genetic polymorphisms influence cytokine expression, gastric inflammation and bacterial strain selection in *H. pylori* infection. In future, genotyping of host polymorphisms may allow the identification of patients with increased risk for the development of gastric pathology. The association of polymorphisms with certain bacterial strain types indicates that host-specific

colonization or adaptation occurs, which may explain the heterogeneity of *H. pylori* strains in different population (39). Taken together, these findings contribute to the understanding of the complex host-pathogen interaction and support the model of a multifactorial development of gastric pathology.

4.3 Role of the *H. pylori* outer membrane protein HP0638 for bacterial adherence and cytokine response

In recent years, the entire *H. pylori* genome has been sequenced from two strains (4). Among the newly described *H. pylori* genes, it has been suggested that the *hp0638* gene encoding an outer membrane protein influences the gastric inflammatory response to *H. pylori* (226). The gene product of *hp0638* has been shown to be associated with IL-8 secretion from epithelial cells *in vitro* and *in vivo*, thereby inducing the influx of polymorphonuclear cells into the gastric mucosa (226) (221). Therefore, the gene product of *hp0638* was termed Outer inflammatory protein (OipA) (226).

It has been reported that *H. pylori* strains have either a functional or non-functional *hp0638* gene. Functional status of the *hp0638* gene is regulated by a slipped strand repair mechanism based on the number of CT dinucleotide repeats in the 5' region of the gene (226) In the current study, we also found that European *H. pylori* strains have functional or non-functional *hp0638* gene, depending on the number of CT dinucleotide repeats in the coding region of the gene.

Bacterial adherence to the host epithelia is an important virulence determinant for many mucosal pathogens and for some bacteria it is an essential pre-requisite for pathogenicity (109) (162). For *H. pylori*, adherence to the gastric mucosa is likely to be an important virulence factor *in vivo* since infection is associated with a high degree of species specific tissue tropism (127). In addition *H. pylori* leads to the formation of so called “attaching – effacing lesions”, which have been associated with a more severe gastritis (159) (218) (118). The *hp0638* gene encodes a protein that belongs to the outer membrane protein gene family and that could be a good candidate for a bacterial adherence factor. In their previous reports, Yamaoka et al provided different evidences in regard to influence of the *hp0638* gene on adherence properties of *H. pylori*. Thus, whereas in the first report no difference in the cell adherence between wild type and mutant strains was seen (226), in the later studies they found that the *hp0638* knockout strains showed reduced adherence compared to the wild type strain (221) (225). In addition, in different reports they observed dissimilar data (10% and 30% of reduction).

This creates very unclear evidence and makes it difficult to explain why the same research group in three different publications provide contradictory data. In the current study we showed that *hp0638* ameliorates the adherence properties of *H. pylori* and thereby augments the density of bacterial colonisation. *In vitro* co-culture experiments of KATO III cells with the wild type and the *hp0638* knockout strains showed that both strains were able to adhere to gastric cancer cells. However, the *hp0638* mutants had significantly reduced adherence to KATO III cells. Approximately 30% reduction for *hp0638* KO compared with wild type strain was observed.

Mathematical as well as experimental models have hypothesized that the outcome of bacterial colonization is influenced by the ability of bacteria to adhere to epithelial cells (68) (21). The bacterium colonizes the gastric mucosa by adhering to epithelial cells and the mucus layer lining the gastric epithelium (117) (68) (21). Adherent bacteria are supposed to have growth advantages based on proximity to the epithelium, because of better availability of nutrients from exudates gastric epithelium that has been damaged by the infection (132). In addition, these adherence properties protect the bacteria from the extreme acidity of the gastric lumen and displacement from the stomach by forces such as those generated by peristaltic movements, gastric emptying and washout with luminal fluid (104). Therefore, bacteria with better adherence characteristics are supposed to colonize with higher densities (177). The *hp0638* mediated adherence seems to facilitate bacterial colonisation. Analyzing biopsies from infected patients, we found that both *hp0638*-on and *hp0638*-off strains were able to colonize the gastric mucosa. However, colonization densities were significantly lower in patients infected with the strains with *hp0638*-off in comparison with patients infected with the *hp0638*-on strains ($P < 0.05$). Our findings are supported by recent studies (222) (221) (2) which investigated the role of *hp0638* on bacterial colonisation and inflammation in the *H. pylori* infected mucosa of humans and mice. In these studies strains with *hp0638* “off” colonized both humans and mice, however naturally switched “off” strains infected patients and mice at significantly lower bacterial densities ($p < 0.001$) compared with the parental strains. Thus, the *hp0638* “on” status was related to *H. pylori* density in humans and mice. The influence of *hp0638* on adherence and colonisation was also investigated in the Mongolian gerbil model. After inoculation of Mongolian gerbils with the wild type and the *hp0638* mutant strains, *H. pylori* was recovered in all animals inoculated by the wild strain, however the mutant failed to establish colonization in all 15 Mongolian gerbils (2). Thus, the *Hp0638* outer membrane protein seems to play an essential role in adhesion or colonization in the gastric mucosa of Mongolian gerbils. *H. pylori* is a human-specific pathogen and does not cause intense inflammation in conventional experimental animals. The Mongolian gerbil is an exception. Several studies have indicated that ulcers, intestinal metaplasia, and even adenocarcinoma

develop during long term *H. pylori* infection in the animal (97) (103). Therefore, the gerbil model may be valuable not only in educating *H. pylori* induced neoplasia but also in evaluating the role of virulence factors *in vivo* (2).

IL-8 is produced by numerous cell types, particularly monocytes/macrophages, epithelial cells, neutrophils, fibroblasts, and endothelial cells in response to bacterial infection (153) (15). IL-8 activates neutrophils in several pathways. On the one hand, it increases the adherence of neutrophils to endothelial cells and promotes their recruitment to the site of infection. On the other hand, it induces the release of granular contents from neutrophils (elastase, lactoferrin and MPO) (214) (81). IL-8 is also a chemoattractant for other cell types such as basophils, T lymphocytes, and NK cells, and also enhances permeability of endothelial cells (15) (158) (152). In many invasive infections, the level of IL-8 expression correlates with the severity of the disease (108), (142) particularly with neutrophilic inflammation and mucosal destruction. The PMN infiltrate was shown to be responsible for bacterial dissemination, massive rupture and subsequent inflammatory destruction of the epithelial barrier in human and animal studies (183) (140). Yamaoka et al reported that the functional status influenced IL-8 secretion from gastric epithelial cells (226). However, this finding awaits independent confirmation. In our study we found no influence of the *hp0638* functional status on IL-8 secretion from gastric epithelial cells *in vitro*. Our data were in agreement with several other reports that could not reproduce the results of Yamaoka et al (161) (2) (6). In addition, Yamaoka et al have shown that *in vivo* (in humans as well as in mice) there is a strong relationship between *hp0638* switch status and the development of duodenal ulcer, severe gastric inflammation, high *H. pylori* density and high levels of mucosal IL-8 expression (222) (221). In the current study we also investigated the effect of *hp0638* on IL-8 production *in vivo* (in the gastric mucosa of 53 *H. pylori* positive patients). We sequenced *hp0638* gene from 53 patients and determined the number of CT repeats that enabled us to define the functional status of the gene. The functional *hp0638* gene was found in 30 of our 53 *H. pylori* clinical isolates. The *hp0638* frame status correlated strongly with the *cagA* status. Almost all of the strains with the in-frame *hp0638* gene were *cagA*⁺, whereas most of the strains with the out of frame *hp0638* gene were *cagA*-negative. Our findings are in agreement with other studies, where the functional *hp0638* gene was also associated with the presence of *cagA* (221) (5; 229). In addition, in our study *in vivo* the functional *hp0638* status had no effect on IL-8 production; only *cagA* positive status induced increased levels of IL-8 in the gastric mucosa. Thus in the patient group, the *hp0638* status does not directly affect IL-8 induction, in contrast to the report by Yamaoka et al (221). The reason for the diverging results could be explained by the fact that Yamaoka et al excluded from their study the *cag*⁻/*hp0638*-on strains, they compare only the *cag*⁺/*hp0638*-on with the *cag*⁻/*hp0638*-off strains. Given

that the *hp0638*-on strains are almost always *cag*⁺, the influence of *hp0638* on IL-8 production and the association of *hp0638* with gastro-intestinal pathology are results of an association with *cagPAI*. Therefore there is no independent effect of *hp0638* on clinical outcome and IL-8 production.

Taken together, these findings contribute to the understanding of the interaction between gastric epithelium and *H. pylori*. In the current study we showed the influence of *hp0638* on adherence and bacterial colonization. However, on our opinion the Hp0638 can not be claimed as OipA (outer inflammatory protein), because no influence on IL-8 production *in vivo* and *in vitro*, as well as no effect on gastric inflammation were observed. Further investigations are needed, to determine specific receptor to Hp0638.

Summary

Infection with *H. pylori* leads to persistent colonisation and chronic inflammation of the gastric mucosa, thereby increasing the risk for the development of peptic ulceration, distal gastric adenocarcinoma and gastric lymphoma (39) (197). *H. pylori* causes persistent inflammation in the human stomach, that can progress to precancerous lesions such as atrophy, intestinal metaplasia, dysplasia, and finally convert to invasive carcinoma (105). Although *H. pylori* was recognised as a primary cause of gastric cancer, only a minority of persons harbouring this organism develop gastric malignancy. Enhanced risk for cancer development may be related to differences in the expression of specific bacterial products, to variations in the host inflammatory response to the bacteria, or to specific interactions between the host and microbe. The aim of the study was to investigate in a large patient group the role of bacterial factors, host factors and their interaction for development of gastric cancer.

In the first part of the study we determined the influence of polymorphisms in important cytokine genes (*IL-10*, *TNF-A*, *IFN-G*) on the development of gastric pathology in response to *H. pylori* infection. The T helper cell response toward *H. pylori* is generally considered to be of the Th1 phenotype leading to a cell mediated immune response (64) (188). There is increasing evidence that the *H. pylori* induced Th1 response contributes to cancer development (63). Important cytokines characterizing Th1 mediated immune responses are interferon-gamma (IFN- γ), Tumor-necrosis-factor-alpha (TNF- α) both being up-regulated during chronic *H. pylori* infection (23) (177) (224). IL-10, which is also highly expressed in the *H. pylori* infected stomach (110) is one of the most important regulatory cytokines, inhibiting cell-mediated immune responses. Allelic variants in cytokine genes have been shown to influence the susceptibility to and severity of a variety of infectious and autoimmune diseases (18) (101). We investigated large *H. pylori* infected patient group with chronic gastritis and determined the infecting bacterial strain type, host cytokine gene polymorphisms, the corresponding mucosal cytokine expression, and the histopathological features of gastritis. Three single nucleotide polymorphisms (SNPs) were genotyped in the *IL-10* promoter. Two SNPs at position -819 and -592 were in complete linkage disequilibrium (LD). We found that *IL-10* SNPs markedly influenced mucosal IL-10 expression in the course of chronic *H.*

pylori infection: GCC haplotype carriers (*IL-10* -1082G; -819C; -592C) were associated with high and ATA carriers with low IL-10 expression. Furthermore, we found that the prevalence of *cagA*⁺, *vacAsI*⁺, and *babA2*⁺ strains was significantly higher among patients with the high IL-10 secreting GCC haplotype than among low secreting haplotype ATA carries. These findings suggest that either initial infection with *cagA*⁺ strains may be more successful in GCC carriers, suggesting a host-specific colonization. Alternatively, host-specific adaptation of the bacterium may occur in the course of chronic infection. Correlating *IL-10* SNPs to gastric histopathology, we could not find significant associations to gastric inflammation or atrophy and intestinal metaplasia. The *TNF-A-307* and *IFN-G+874* SNPs were not associated with changes in mucosal cytokine expression and the inflammatory response to *H. pylori*.

In the second part of the study we explored the function of a novel putative virulence factor *hp0638* and its influence on bacterial adherence and the development of gastric pathology. Recently, it has been suggested that the *hp0638* gene influences the gastric inflammatory response to *H. pylori* (226). Functional status of *hp0638* gene has been reported to be regulated by a slipped strand repair mechanism based on the number of CT dinucleotide repeats in the coding region (226). The gene product of *hp0638* has been shown to be associated with IL-8 secretion from epithelial cells *in vitro* and *in vivo* (226) (221). In the current study, we also found that European *H. pylori* strains have the functional or non-functional *hp0638* gene, depending on the number of CT dinucleotide repeats in the coding region of the gene. However, the *hp0638* status did not directly affect IL-8 induction neither *in vivo* nor *in vitro*, in contrast to the previous study (221).

Given that *hp0638*⁺ strains are almost always *cag*⁺, the influence of *hp0638* on IL-8 production and the association of *hp0638* with gastro-intestinal pathology are results of an association with the *cagPAI*. Therefore there is no independent effect of *hp0638* on clinical outcome and IL-8 production. We also showed that *hp0638* ameliorates the adherence properties of *H. pylori* and thereby augments the density of bacterial colonisation. Adherence assays *in vitro* showed that both the wild type and its *hp0638* mutant strains were able to adhere to gastric epithelial cells. However, we observed a significant difference in binding to the cells between the mutant and wild type strains. The *hp0638* mutant had about 30% reduction in adherence to epithelial cells in comparison to the wild type. The *hp0638* mediated adherence seems to facilitate bacterial colonisation. Analyzing biopsies from infected patients, we found that both the

hp0638-on and the *hp0638*-off strains were able to colonize gastric mucosa. However, colonization densities were significantly lower in patients infected with strains lacking *hp0638* than in patients infected with *hp0638*-positive strains ($P < 0.05$).

To summarize, in the first part of the study it has been shown how genetic polymorphisms influence cytokine expression, gastric inflammation and bacterial strain selection in *H. pylori* infection. In the second part of the study we explored the function of the novel putative virulence factor *hp0638* and its influence on bacterial adherence and the development of gastric pathology. The current data contribute to the understanding of the complex host-pathogen interaction and support the model of a multifactorial development of gastric pathology.

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