

*Vesa-Matti Pohjanen*

TOLL-LIKE RECEPTOR 4  
AND INTERLEUKIN 6 GENE  
POLYMORPHISMS IN  
*HELICOBACTER PYLORI*  
RELATED DISEASES

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UNIVERSITY OF OULU,  
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*VESA-MATTI POHJANEN*

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PYLORI* RELATED DISEASES**

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## **Pohjanen, Vesa-Matti, Toll-like receptor 4 and interleukin 6 gene polymorphisms in *Helicobacter pylori* related diseases.**

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### ***Abstract***

*Helicobacter pylori* is a Gram-negative bacterium, which infects the stomach of more than 50% of the population worldwide. In addition to being the most important risk factor for gastric cancer and peptic ulcers, *H. pylori* infection is a risk factor for several extra-digestive diseases including dyslipidemia. The consequences of having an *H. pylori* infection are significantly influenced by the inflammatory response of the host. The pattern recognition receptor Toll-like receptor 4 (TLR4) and the cytokine interleukin 6 (IL6) are important mediators of inflammation in *H. pylori* related diseases.

We have analyzed a series of control subjects and patients with dyspepsia, peptic ulcers or gastric cancer for frequent genetic polymorphisms of the TLR4 and IL6 genes. The prevalence of *H. pylori* infection, the histologic features of gastritis and cancer and serum endocrine markers and lipid concentrations were also analyzed. Furthermore, the expression of TLR4 was analyzed in specific cell types of gastric mucosa by immunohistochemistry.

The TLR4 wild type genotypes of polymorphisms +896 and +1196 were associated with an increased risk of peptic ulcers. The same genotypes also associated with higher serum gastrin levels, but not with atrophy or other features of gastritis. The TLR4 expression was seen in the gastrin and somatostatin secreting cells of gastric mucosa. These results suggest a regulatory link between TLR4 and gastrin secretion. Such a link indicates the presence of a novel effector mechanism for innate immunity in modifying the host endocrine function. The IL6 -174 polymorphism associated significantly with a risk of the diffuse type of gastric carcinoma but not with the intestinal type or its precursor conditions. Finally, we demonstrated that *H. pylori* infections modify HDL serum levels significantly only in IL6 -174 CC genotype patients, which suggests that the detrimental effects of *H. pylori* infections on HDL levels are transmitted through IL6. These results clarify the mechanisms of *H. pylori* related diseases and open new possibilities for research on peptic ulcer disease, gastric cancer and dyslipidemia.

**Keywords:** dyslipidemia, gastric cancer, *Helicobacter pylori*, interleukin 6, non-ulcer dyspepsia, peptic ulcer, Toll-like receptor 4



## **Pohjanen, Vesa-Matti, Tollin kaltainen reseptori 4:n ja interleukiini 6:n geneettisten polymorfioiden merkitys Helicobacter pylorin aiheuttamissa sairauksissa.**

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Medical Research Center Oulu; Oulun yliopistollinen sairaala

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### ***Tiivistelmä***

*Helicobacter pylori* on yleinen ihmisen mahalaukussa esiintyvä Gram-negatiivinen bakteeri. Helikobakteeri on tärkein mahasyövän ja maha- ja pohjukaissuolihaavan riskitekijä ja se on myös muun muassa rasva-aineenvaihdunnan häiriöiden riskitekijä. Ihmisen tulehdusvaste vaikuttaa merkittävästi helikobakteeri-infektion seurauksiin. Tollin kaltainen reseptori 4 (TLR4), joka on hahmontunnistusreseptori ja tulehduksenvälittäjäaine interleukiini 6 (IL6) ovat tärkeitä ihmisen tulehdusvasteeseen osallistuvia proteiineja.

Olemme tutkineet dyspepsiaa, maha- ja pohjukaissuolihaavaa ja mahasyöpää sairastavilta potilailta sekä kontrollihenkilöiltä TLR4:n ja IL6:n geenien yleisiä emäsjärjestyksen polymorfioita. Tutkimme myös helikobakteeri-infektion yleisyyttä ja histologisia piirteitä, mahasyövän histologisia piirteitä ja seerumin merkkiaineita ja lipidipitoisuuksia. Lisäksi tutkimme TLR4:n ilmenemistä mahan limakalvolla immunohistokemiallisesti.

TLR4:n polymorfismien +896 ja +1196 villin tyypin genotyypit liittyivät kohonneeseen maha- ja pohjukaissuolihaavan riskiin. Samat genotyypit liittyivät myös korkeampiin gastriinitasoihin. TLR4:ä esiintyi mahalaukun limakalvolla gastriinia tai somatostatiinia ilmentävissä soluissa. Täten TLR4:n ja maha- pohjukaissuolihaavariskin yhteys näyttää välittyvän gastriinin erityksen kautta, mikä viittaa uuteen säätely-yhteyteen luontaisen immunitietin ja mahalaukun umpieritysjärjestelmän välillä. IL6 -174 -polymorfismi yhdistyi diffuusin tyypin mahakarsinooman riskiin mutta ei intestinaalisen tyypin karsinooman riskiin. Helikobakteeri-infektio yhdistyi pienentyneisiin HDL-kolesterolipitoisuuksiin vain potilailla, joilla oli IL6 -174 CC genotyyppi, mikä viittaa helikobakteerin kolesterolitasoille haitallisen vaikutuksen välittyvän IL6:n kautta. Nämä tulokset antavat lisätietoa helikobakteerin aiheuttamien sairauksien mekanismeista ja avaavat uusia tutkimuspolkuja myös mahahaavan, mahasyövän ja rasva-aineenvaihdunnan häiriöiden kliniseen tutkimukseen.

**Asiasanat:** dyslipidemia, dyspepsia, *Helicobacter pylori*, interleukiini 6, mahasyöpä, Tollin kaltainen reseptori 4, ulkustauti



*To Riikka*



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Oulu, March 2016

Vesa-Matti Pohjanen

## Abbreviations

APC	adenomatous polyposis coli
cagA	cytotoxin associated gene A
CD	cluster of differentiation
CI	confidence interval
COX-1	cyclooxygenase 1
DNA	deoxyribonucleic acid
<i>e.g.</i>	exempli gratia
G17	gastrin 17
gp130	glycoprotein 130
HDL	high density lipoprotein
HR	hazard ratio
HP	Helicobacter pylori
<i>i.e.</i>	id est
IL	interleukin
IQR	interquartile range
JAK	Janus kinase
LDL	low density lipoprotein
LPS	lipopolysaccharide
MyD88	myeloid differentiation factor 88
NSAID	non-steroidal anti-inflammatory drug
OR	odds ratio
PG	pepsinogen
PCR	polymerase chain reaction
SD	standard deviation
STAT	signal transducer and activator of transcription
TIR	Toll/interleukin 1 receptor
TLR	Toll-like receptor
TNF	tumor necrosis factor
TP53	tumor protein p53
vacA	vacuolating cytotoxin
Wnt	wingless-type mouse mammary tumor virus integration site family



## Original publications

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:

- I Pohjanen VM, Koivurova OP, Huhta H, Helminen O, Mäkinen JM, Karhukorpi JM, Joensuu T, Koistinen PO, Valtonen JM, Niemelä SE, Karttunen RA & Karttunen TJ (2015) Toll-like receptor 4 wild type homozygosity of polymorphisms +896 and +1196 is associated with high gastrin serum levels and peptic ulcer risk. *PLoS One* 10(7): e0131553.
- II Pohjanen VM, Koivurova OP, Mäkinen JM, Karhukorpi JM, Joensuu T, Koistinen PO, Valtonen JM, Niemelä SE, Karttunen RA & Karttunen TJ (2013) Interleukin 6 gene polymorphism -174 is associated with the diffuse type gastric carcinoma. *Genes Chromosomes Cancer* 52(10): 976–982.
- III Pohjanen VM, Koivurova OP, Niemelä SE, Karttunen RA & Karttunen TJ (2016) The role of *Helicobacter pylori* and interleukin 6 -174 gene polymorphism in dyslipidemia: a case-control study. *BMJ Open* 6(1): e009987.



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# 1 Introduction

Although the incidence of *Helicobacter pylori* infections have steadily decreased in Finland over the last century, (Rautelin & Kosunen 2004) the *H. pylori* related gastroduodenal diseases cause a major disease burden globally (Bosman *et al.* 2010). *H. pylori* is a major risk factor for gastric adenocarcinoma, which is the second leading cause of cancer death worldwide, and of peptic ulcers, which ail 10 % of global population in their lifetime (Bosman *et al.* 2010, Kusters *et al.* 2006). *H. pylori* infection has also been documented as a cause of atherogenic changes in the host lipid metabolism and to be a possible risk factor for coronary heart disease (Sharma & Aggarwal 2015).

Before 1982, when Barry Marshall and Robin Warren made the Nobel Prize worthy discovery of *H. pylori* as the main etiologic factor for peptic ulcers, (Marshall & Warren 1984) the disease was thought to be caused by having a stressful lifestyle and eating spicy foods. Nowadays, these factors are thought to have at most a minor role in the pathogenesis of peptic ulcers, especially when compared to *H. pylori* and the other major risk factor, the use of non-steroidal anti-inflammatory drugs (NSAID). But as approximately 80% of patients with *H. pylori* infection do not develop peptic ulcers or gastric cancer and only a part of the risk of cancer could be explained by known traditional risk factors as age, sex, tobacco, alcohol and some aspects of diet, etiological research has turned to genetic studies of both *H. pylori* and the human host (Bosman *et al.* 2010, Kusters *et al.* 2006). As a result, some *H. pylori* genetic variants have been documented as being more pathogenic than others (Murata-Kamiya 2011, Roesler *et al.* 2014). Furthermore, research on human genetics has provided many disease associating polymorphic gene loci of mostly immune and inflammation related proteins (Kusters *et al.* 2006).

The decreased incidence of *H. pylori* is due to active elimination of *H. pylori* and improved hygiene and housing conditions and has resulted in lower peptic ulcer and gastric cancer rates in Finland and in many other developed countries (Kusters *et al.* 2006, Rautelin & Kosunen 2004, Roesler *et al.* 2014). NSAIDs have become a proportionally more prevalent etiology for gastric ulcers. In gastric cancers the number of the more frequent intestinal histological subtype has decreased proportionally more than the diffuse histological subtype, which has further emphasized the differences in the pathogenesis of the different subtypes (Sostres *et al.* 2014, Bosman *et al.* 2010).

Toll-like receptor 4 (TLR4) is a pattern recognition receptor that recognizes bacterial lipopolysaccharide (LPS). TLR4 genetic polymorphisms have been

documented as minor risk factors for gastric cancer. The activation of TLR4 can lead to the production of several different cytokines, and the pathways which contribute to the pathogenesis of gastric cancer and the role of TLR4 in the pathogenesis of peptic ulcers are not known (Kutikhin 2011; Castano-Rodriguez *et al.* 2013). Interleukin 6 (IL6) is a cytokine, which has been shown to play a role in *H. pylori* infections, peptic ulcers and gastric cancer (Furukawa *et al.* 1998, Tsai & Hsu 2010, Ashizawa *et al.* 2005). IL6 has been also documented as associating with serum lipid concentrations (Fernandez-Real *et al.* 2000).

The aims of our study were to analyze whether the polymorphic loci of TLR4 and IL6 are associated with the risk of *H. pylori* related diseases and to assess what the possible pathophysiological pathways of these associations could be.

## 2 Review of the literature

### 2.1 *Helicobacter pylori*

*Helicobacter pylori* is a spiral-shaped Gram-negative bacterium found on the luminal surface of the gastric epithelium. The first isolation of *H. pylori* was in 1982 by Barry Marshall and Robin Warren (Marshall & Warren 1984), who were awarded the Nobel Prize in Physiology or Medicine in 2005. Since the pathogen isolation, *H. pylori* infection has been associated with the development of various digestive and extra-digestive diseases, such as chronic gastritis, peptic ulcer disease, gastric adenocarcinoma, dyslipidemia and cardiovascular diseases (Kusters *et al.* 2006, Roesler *et al.* 2014).

#### 2.1.1 Epidemiology

The prevalence of *H. pylori* shows large geographical and socio-economical variations. In northern European and North American populations about one-third of the adult population is *H. pylori* positive, whereas in southern and eastern Europe, South America and Asia *H. pylori* prevalence is often higher than 50% (Eusebi *et al.* 2014). In Finland, the serologic prevalence of *H. pylori* varies from 4% to 80% from the 1980–1990 cohort to the 1910–1920 cohort respectively (Rautelin & Kosunen 2004).

The infection is probably acquired by interpersonal transmission, although the specific mechanisms of transmission are not known and environmental transmission through contaminated water also remains a possible route. Parental transmission has been frequently reported. Several socioeconomic factors, such as low family income and living in rural areas and crowded homes have been associated as risk factors for *H. pylori* infections (Eusebi *et al.* 2014). The highest risk period for acquiring an *H. pylori* infection is in childhood and early adolescence and the infection usually prevails lifelong in the absence of antibiotic treatment. The active elimination of *H. pylori* and improved hygiene and housing conditions have resulted in a lower infection rate in children and in the cohort effect of declining prevalences seen in Finland and many other developed countries (Kusters *et al.* 2006, Rautelin & Kosunen 2004, Roesler *et al.* 2014). *H. pylori* recurrence after successful eradication is an infrequent event with less than a 5% rate in long term follow ups (Eusebi *et al.* 2014).

### **2.1.2 Pathophysiology and virulence factors**

Human gastric mucosa is the only known natural habitat for *H. pylori*. The initial colonization of the gastric mucosa is dependent on the helical shape and the urease activity of *H. pylori*. *H. pylori* is guided by chemotactic mechanisms towards the surface of gastric epithelial cells and it uses its helical shape and flagella to penetrate the gastric mucus, where the pH gradient is higher. *H. pylori* has a highly active urease production. The urease catalyzes the degradation of urea to carbon dioxide and ammonia, which raises the local pH and allows *H. pylori* to survive in the acidic environment of the stomach (Kusters *et al.* 2006, Roesler *et al.* 2014). However, the increase of the local pH when the host develops atrophic changes in the gastric corpus mucosa, decreases the number bacteria, leading eventually to the disappearance of the bacteria (Karttunen *et al.* 1991). *H. pylori* also uses the produced ammonia in its amino acid biosynthesis (Kusters *et al.* 2006, Roesler *et al.* 2014).

*H. pylori* adheres to the luminal epithelia, which protects it from exfoliation and the low pH of the gastric juice. The adhesion is enabled by bacterial cell surface proteins called adhesins. The adhesins demonstrate genetic variability between *H. pylori* strains. The adhesins affect the host inflammatory response and disease outcome as virulence factors. (Roesler *et al.* 2014).

The secreted virulence factors also demonstrate genetic variability. The most studied *H. pylori* virulence factor is cytotoxin associated gene A (CagA), which is the effector protein and marker of cytotoxin associated gene pathogenicity island. The pathogenicity island is a 40 kb region of chromosomal DNA encoding approximately 31 genes, which form a type IV secretion system. This secretion system includes a pilus that delivers the CagA protein into the gastric epithelial cells, which leads to the dephosphorylation of the host cell proteins and leads to cellular morphological changes. The cagA positive *H. pylori* strains have been documented as causing more severe gastric mucosal inflammation than cagA negative strains and are known to increase the risk of peptic ulcers and gastric carcinomas. (Murata-Kamiya 2011, Roesler *et al.* 2014).

Vacuolating cytotoxin (VacA) is a secreted cytotoxin with multiple cellular effects in different host cell types. VacA forms cell membrane channels, induces the formation of intracellular vacuoles and can cause apoptosis and lead to necrosis in vitro. There is variation in the activity of VacA between *H. pylori* strains

determined by the differences in the signal (s1 or s2) and middle regions (m1 or m2). In humans, s1 and m1 genotypes have been associated with higher levels of neutrophilic and lymphocytic infiltrates, epithelial damage, gastric atrophy, and intestinal metaplasia compared to those with s2 or m2 strains, and patients infected with vacA s1 or m1 strains may be at increased risk of developing peptic ulcers and gastric cancer. (Ferreira *et al.* 2014, Yamaoka & Graham 2014).

### 2.1.3 Diagnostics

The diagnosis of an *H. pylori* infection can be made by histology, serology, stool antigen test, urea breath test, rapid urease test, bacterial culture and polymerase chain reaction (PCR). The sensitivities and specificities of the methods vary based on the used methodology and the choice of golden standard. The accuracy of all the methods mentioned is high when conducted in optimal conditions. Typical sensitivities and specificities are compiled in Table 1 (Garza-Gonzalez *et al.* 2014, Megraud & Lehours 2007, Redeen *et al.* 2011, Wang *et al.* 2015).

**Table 1. Sensitivities and specificities of the *H. pylori* diagnostic methods.**

Method	Sensitivity	Specificity
Histology	80–95%	90–100%
Serology	80–100%	70–95%
Stool antigen test	80–95%	85–95%
Urea breath test	80–100%	80–98%
Rapid urease test	80–95%	90–100%
Bacterial culture	80–95%	95–100%
Polymerase chain reaction	95–100%	95–100%

## 2.2 Peptic ulcer disease

Both gastric and duodenal ulcers are strongly associated with *H. pylori* and approximately 95% of duodenal ulcers and 85% of gastric ulcers develop in the presence of *H. pylori*. It has been estimated that 10–15% of *H. pylori* positive patients develop a peptic ulcer in a long time follow up period. As the prevalence of *H. pylori* has declined in the most western world and in Finland, the increasing use of nonsteroidal anti-inflammatory drugs (NSAIDs) is becoming a more significant risk factor. The risks from *H. pylori* infection and NSAID use are cumulative. The diagnosis of peptic ulcers is based on direct visual identification

of a mucosal break with a diameter of 5 mm or larger, covered with fibrin in the lining of stomach or duodenum during gastroduodenal endoscopy (Kusters *et al.* 2006, Malmi *et al.* 2014, Malfertheiner *et al.* 2009)

### **2.2.1 Epidemiology**

The incidence of peptic ulcers has steadily decreased in western countries for decades following the decreasing prevalence of *H. pylori* infections (McColl & El-Omar 2002). In Finland, the annual incidence of all peptic ulcers has still decreased between 2000–2008 from 121/100 000 to 79/100 000 (Malmi *et al.* 2014).

### **2.2.2 Pathophysiology**

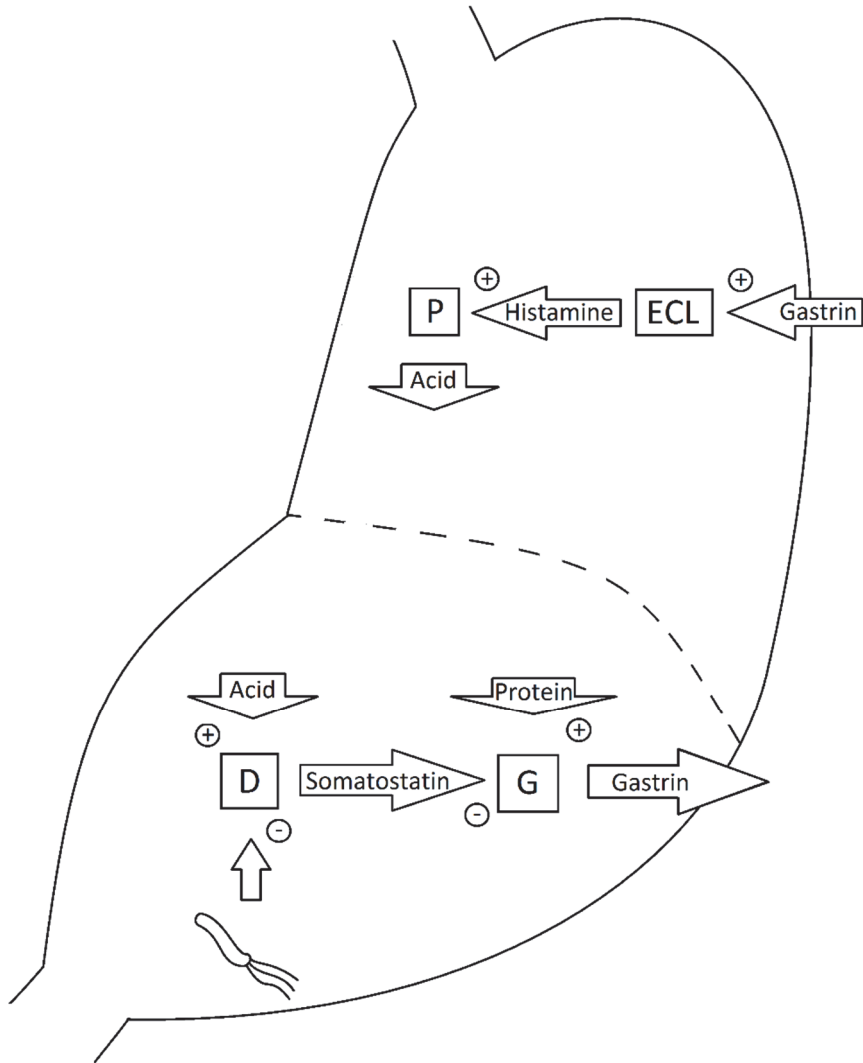
The infection of the stomach with *H. pylori* virtually always leads to the colonization of both gastric antrum and corpus and to chronic active gastritis. When the colonization becomes persistent, the distribution of gastritis is associated with the level of acid production. Antrum dominant gastritis is associated with high gastric acid production, while corpus dominant gastritis and pangastritis are associated with low acid production (Kusters *et al.* 2006). The key mechanism in the pathogenesis of duodenal, pyloric and pre-pyloric ulcers is *H. pylori* induced excessive gastrin secretion and the following excessive acid secretion. The increased acid load together with inflammation and secreted proteinases then damage the gastric or duodenal mucosa. The increased acid load also triggers gastric metaplasia in the duodenum, which makes the colonization of the duodenum by *H. pylori* possible. The inflamed duodenal mucosa becomes more susceptible to peptic acid attack and ulceration (Chu & Schubert 2013, Ernst & Gold 2000, McColl *et al.* 2000, Malfertheiner *et al.* 2009).

Gastrin, a polypeptide hormone secreted by antral G cells, is the most important stimulator of gastric acid secretion. There are two main forms of gastrin present in the serum, 17 and 34 amino acid peptides. Gastrin-17 (G17) is the prevalent form in serum postprandially. Gastrin release is stimulated by the presence of amino acids in the stomach, as well as due to mechanical antrum distension and vagal stimulation. Gastrin stimulates the release of histamine from enterochromaffin-like cells and the histamine in turn binds to the histamine 2 receptors in parietal cells in the fundus and corpus of the stomach. Histamine 2 receptor activation triggers the hydrochloric acid secretion through cell membrane proton pumps (H<sup>+</sup>/K<sup>+</sup>-adenosinetriphosphatase) and chloride channels (Fig. 1).

Gastrin also triggers the release of pepsinogen I (PGI) from chief cells of fundic mucosa, and pepsinogen II (PGII) from the chief cells of fundic, pyloric and duodenal mucosa, which are then cleaved to form proteinases. Gastrin secretion is inhibited by somatostatin, a hormone secreted by antral D cells. The release of somatostatin is triggered by a low pH of the gastric content. (Di Mario & Goni 2014, Kusters *et al.* 2006, McColl *et al.* 2000).

The mechanism of hypergastrinemia in antrum dominant gastritis seems to be related to the impairment of the acid-mediated inhibitory control and lower concentrations of somatostatin, but the precise mechanisms by which *H. pylori* decreases somatostatin levels are not fully understood. It has been proposed that the high concentrations of ammonia produced by the *H. pylori* urease could raise the local antral surface pH and thus prevent the D cells from secreting somatostatin, but experimental evidence has not fully supported this theory. It has been also proposed that cytokines might affect G and D cell function. (Chu & Schubert 2013, McColl *et al.* 2000). Animal models have suggested that somatostatin and gastrin secretion also might be regulated by the innate immune system when it recognizes bacteria or bacterial products (Fukui *et al.* 2006, Kidd *et al.* 2009, Zavros & Merchant 2005). The pathogenesis of other gastric ulcers than of pyloric and pre-pyloric locations seem to more related to the pathogenesis of atrophic gastritis and gastric carcinoma (Kusters *et al.* 2006).

NSAID related gastric ulcers are mainly caused by the inhibition of cyclooxygenase 1 (COX-1). COX-1 is responsible for the synthesis of prostaglandins, which protect the stomach lining from secreted acid, maintain blood flow in gastric mucosa, and produce bicarbonate. The inhibition of prostaglandins also leads to the activation of proinflammatory pathways, which might be an additional factor in the pathogenesis. NSAIDs have also been documented as having direct cytotoxic effects on gastric mucosal cells. By this mechanism NSAIDs create a gastric environment that is also more susceptible to damage induced by other endogenous or exogenous factors, such as *H. pylori*. NSAIDs, *H. pylori* are considered to be independent risk factors for gastric ulcers and the risks are considered to be cumulative. (Malmi *et al.* 2014, Sinha *et al.* 2013, Sostres *et al.* 2014).



**Fig. 1. Schematic drawing of the basic regulatory mechanisms of gastric acid secretion and the effect of *H. pylori* in peptic ulcer disease. G cells (G) secrete gastrin in response to stimulation by intragastric proteins. Gastrin stimulates the release of histamine from enterochromaffin-like cells (ECL). Histamine triggers the hydrochloric acid release from parietal cells (P). As a feed-back mechanism, D cells (D) secrete somatostatin in response to low pH and somatostatin suppresses gastrin secretion. *H. pylori* is thought to cause hypergastrinemia and increased acid secretion by suppressing somatostatin production. Increased acidity promotes mucosal barrier breakdown.**

## 2.3 Gastric carcinoma

Gastric carcinomas are malignant epithelial neoplasms. *H. pylori* infection is the most important etiological factor in the development of distal gastric carcinoma and it has been estimated that *H. pylori* colonization increases the risk of non-cardia carcinomas 10-fold (Kusters *et al.* 2006). *H. pylori* is not considered to be a risk factor for proximal cardia carcinomas. Other risk factors for gastric carcinomas include smoking, a high intake of salt and smoked food and a low intake of fresh fruit and vegetables (Bosman *et al.* 2010).

The risk of gastric cancer is also increased in several cancer disposition syndromes such as with the familial adenomatous polyposis syndrome (germline mutation of APC, adenomatous polyposis coli gene), Lynch syndrome (germline mutations of mismatch gene repair genes) and Li-Fraumeni syndrome (germline mutation of TP53, tumor protein p53 gene) (Bosman *et al.* 2010). Several single nucleotide polymorphisms in genes associated with the immune response to *H. pylori* have also been associated with an increased risk of gastric cancer. These include genes of, *e.g.*, IL1 $\beta$ , IL6, IL10, tumor necrosis factor (TNF)  $\alpha$  and TLR4 (Bosman *et al.* 2010, Castano-Rodriguez *et al.* 2013, Correa & Piazuelo 2011, Gatti *et al.* 2007).

### 2.3.1 Classification

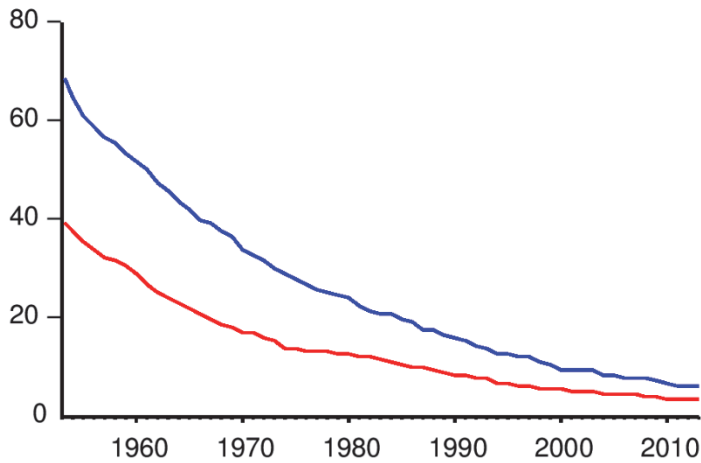
One of the most commonly used gastric carcinoma classifications is the Lauren classification, in which gastric adenocarcinomas are histologically divided into intestinal, diffuse, mixed and indeterminate types (Lauren 1965, Bosman *et al.* 2010). The intestinal type is described by the formation of glandular structures and the diffuse type as consisting of poorly cohesive cells with little or no glandular structures. Mixed type carcinomas display a mixture of intestinal and diffuse type structures of approximately equal quantities. The Lauren classification has significance from epidemiological, clinical, and molecular points of view (Lochhead & El-Omar 2008, Ristamaki *et al.* 2010, Vauhkonen *et al.* 2006).

The current World Health Organization classification recognizes five main types of gastric adenocarcinoma on the basis of morphological growth patterns: tubular, papillary, mucinous, poorly cohesive and mixed carcinomas. Rare histological variants are classified separately. The corresponding type in the Lauren classification for tubular, papillary and mucinous types is the intestinal type and for

poorly cohesive carcinoma the corresponding type is the diffuse type. (Bosman *et al.* 2010, Hu *et al.* 2012).

### **2.3.2 Epidemiology**

Gastric cancer is the fourth most common cancer accounting for 7.8% of cancers globally and the second leading cause of cancer mortality. High incidence areas include eastern Asia, eastern Europe and Latin America while low incidence areas include northern Europe, including Finland, in addition to North America (Bosman *et al.* 2010). The yearly (2013) incidence rate for all stomach cancers in Finland is 3.7/100 000 for women and 6.3/100 000 for men and the 5-year survival rate is 26% and 21% respectively (Finnish Cancer Registry 2014; Fig. 2). As a result of the declining prevalence of *H. pylori*, the age standardized incidence and mortality of gastric cancer have steadily declined over the last 15 years. Especially the incidence of the intestinal type has declined significantly. Thus the intestinal type is relatively more prevalent in the high incidence regions, whereas the diffuse type is relatively more prevalent in the low incidence areas. Gastric carcinoma incidence increases progressively with age. The intestinal type carcinoma demonstrates a male predominance while the diffuse type does not show sex predominance. The diffuse type is also relatively more prevalent in younger people (Bosman *et al.* 2010, Correa & Piazuelo 2011).



**Fig. 2. Age standardized incidence of gastric cancer in Finland (/100 000 persons, blue for men, red for women; Finnish Cancer Registry, [www.cancer.fi](http://www.cancer.fi)).**

### **2.3.3 Pathophysiology**

Although *H. pylori* infections increase the risk of both intestinal and diffuse type gastric adenocarcinomas, the pathogeneses differ significantly. The intestinal type of gastric carcinoma is known to develop through chronic *H. pylori* related gastritis, which leads to gastric atrophy and intestinal metaplasia and after that dysplasia. This carcinogenetic route is also known as the Correa sequence (Lochhead & El-Omar 2008, Yuasa 2003, Correa & Piazuelo 2011). Especially the corpus dominant or pangastric gastritis patterns are associated with hypochlorhydria and the development of gastric atrophy (Kusters *et al.* 2006). An atrophic stomach is a risk factor for carcinoma regardless of its cause and it has been documents that *H. pylori* density decreases in an atrophic stomach. Thus the role of *H. pylori* is thought to be less significant in the later stages of gastric carcinogenesis. Gastric atrophy leads to the colonization of the stomach by other bacteria, some of which produce carcinogenic metabolites, which might have a role in the carcinogenesis (Atherton 2006). The pathogenesis of the diffuse type gastric cancer is less well understood. The diffuse type cancers do not have recognizable precursor lesions and at least a subset of diffuse type gastric cancers develop independently of atrophic gastritis or intestinal metaplasia (Yamamoto *et al.* 2011).

The intestinal and diffuse type carcinomas also differ from a genetic standpoint. The loss of the cell adhesive function of E-cadherin has been documented as a fundamental defect in diffuse and mixed type gastric cancers (Chan & Wong 2001), but is rarely documented in intestinal type cancers (Bosman *et al.* 2010). The dysregulation of E-cadherin can be caused by genetic alterations, transcriptional repressors or promoter hypermethylation (Carneiro *et al.* 2012). A hereditary diffuse gastric cancer susceptibility syndrome caused by germline mutations of the E-cadherin gene has also been discovered (Bosman *et al.* 2010). The loss of E-cadherin function results in a loss of cell adherence, cell polarity, contact inhibition and unregulated growth (Graziano *et al.* 2003).

Of the other known carcinogenetic pathways, the Wnt (wingless-type mouse mammary tumor virus integration site family)/ $\beta$ -catenin pathway is activated in approximately 30% of gastric cancers. The activation of the Wnt/ $\beta$ -catenin signaling in gastric cancer is often due to mutations in the APC gene, which are mainly documented in intestinal type cancers, or due to mutations in the  $\beta$ -catenin gene, which are documented in both intestinal and diffuse type carcinomas. These mutations cause the accumulation of cytoplasmic  $\beta$ -catenin and the binding of  $\beta$ -catenin to cell growth promoting transcriptional factors (Bosman *et al.* 2010, Chiurillo 2015, Clements *et al.* 2002). The Janus kinase (JAK)/ signal transducer and activator of transcription (STAT) 3 pathway has also been implicated in gastric carcinogenesis (Jackson *et al.* 2007, Yakata *et al.* 2007).

There have been also attempts to classify gastric cancers based on molecular pathways and genetic changes. A recent classification proposed four subtypes: (1) tumors positive for Epstein-Barr virus, which display *e.g.* frequent amplification of JAK2 gene, (2) microsatellite unstable tumors which show elevated mutation rates, including mutations of TP53 and APC, (3) genomically stable tumors which are enriched for the diffuse histological type carcinomas and mutations of E-cadherin gene and (4) tumors with chromosomal instability which show marked aneuploidy and focal amplification of receptor tyrosine kinases (Cancer Genome Atlas Research Network 2014).

## **2.4 *Helicobacter pylori* related dyslipidemia**

*H. pylori* infection has been associated with high total serum cholesterol levels, high low density lipoprotein (LDL) levels, low high density lipoprotein (HDL) levels and high serum triglyceride levels in several studies (Table 2). The association between *H. pylori* and low HDL has been documented most often. *H.*

*pylori* infection has also been associated with the risk of coronary heart disease, but the evidence is still equivocal (Sharma & Aggarwal 2015). *H. pylori* eradication has been documented as affecting serum lipid levels favorably (de Luis *et al.* 1999, Gen *et al.* 2010, Kanbay *et al.* 2005, Pellicano *et al.* 2009, Scharnagl *et al.* 2004) and to decrease coronary artery lumen reduction after percutaneous coronary intervention in coronary heart disease patients (Kowalski *et al.* 2001).

**Table 2. Significant associations with *Helicobacter pylori* and serum lipids in previous studies. The mean or median difference in lipid variables in *Helicobacter pylori* positive patients compared to *Helicobacter pylori* negative patients is presented.**

Reference	Total cholesterol	LDL	HDL	Triglycerides
Murray <i>et al.</i> 1995	0.21 mmol/l higher	-	0.06 mmol/l lower	-
Niemelä <i>et al.</i> 1996	NS	-	NS	0.33 mmol/l higher
de Luis <i>et al.</i> 1998	-	NS	0.43 mmol/l lower	0.11 mmol/l higher
Laurila <i>et al.</i> 1999	0.47 mmol/l higher	-	NS	0.17 mmol/l higher
Hoffmeister <i>et al.</i> 2001	NS	-	0.08 mmol/l lower	-
Takashima <i>et al.</i> 2002	NS	-	0.05 mmol/l lower	NS
Sung <i>et al.</i> 2005	0.08 mmol/l higher	0.07 mmol/l higher	0.04 mmol/l lower	0.07 mmol/l higher
Longo-Mbenza <i>et al.</i> 2007	NS	-	0.63 mmol/l lower	0.27 mmol/l higher
Gunji <i>et al.</i> 2008	-	Higher in linear regression model	Lower in linear regression model	NS
Jia <i>et al.</i> 2009	NS	NS	0.05 mmol/l lower	NS
Gen <i>et al.</i> 2010	0.62 mmol/l higher	0.52 mmol/l higher	0.21 mmol/l lower	0.24 mmol/l higher
Satoh <i>et al.</i> 2010	-	0.10 mmol/l higher	0.05 mmol/l lower	-
Moretti <i>et al.</i> 2014	-	NS	0.39 mmol/l lower	NS

NS, No significant results; LDL, Low density lipoprotein; HDL High density lipoprotein

### 2.4.1 Pathophysiology

The effects of the low intensity inflammation caused by *H. pylori* have been proposed as mechanisms for dyslipidemia. *H. pylori* infection associated cytokines, *e.g.* IL6, have been documented as altering the activity of the triglycerides lipases, which affect the serum lipid concentrations (Zuliani *et al.* 2007). Other proposed mechanisms in which *H. pylori* could contribute to the development of atherosclerotic diseases are: hyper-homocysteinemia, hypercoagulability, impaired

glucose metabolism, endothelial dysfunction (Vijayvergiya & Vadivelu 2015) and increased blood leukocytosis (Karttunen & Niemela 1990).

## 2.5 Toll-like receptor 4

TLR4 is a part of the Toll-like receptor pattern recognition molecule family, which functions as a part of the innate immunity recognizing pathogen associated molecular patterns. TLR4 binds the bacterial LPS of *H. pylori* and other gram negative bacteria and the subsequent downstream signaling stimulates cytokine production (Kutikhin 2011).

In the binding process, LPS is first bound by circulating a lipopolysaccharide-binding protein which works as an opsonin for cluster of differentiation 14 (CD14). CD14 catalyzes the binding of LPS to myeloid differentiation protein-2. This complex interacts directly with TLR4 and the TLR4 cytoplasmic domain dimerizes, thus initiating signal transduction. There are two pathways of downstream signal transduction: the myeloid differentiation factor 88 (MyD88) dependent and the MyD88 independent routes. The MyD88 dependent route activates nuclear factor- $\kappa$ B and the production of cytokines TNF $\alpha$ , IL1, IL6, IL8 and IL12, of which many have been implicated in gastric carcinogenesis. The independent pathway is mediated by the Toll/IL1 receptor (TIR) domain-containing protein and by TIR-domain-containing adapter inducing interferon  $\beta$ . Initiation of this pathway leads to the activation of interferon regulatory factor 3 and the expression of type I interferons. The interaction between TLR4 and antigen presenting cells results in the attraction of neutrophils and activated macrophages to the inflammation site, T-cell activation, as well as the inhibition of regulatory T-cell activity, and activation and maturation of B cells. (Kutikhin 2011).

TLR4 is highly expressed in lymphocytes and monocytes (Zarembler & Godowski 2002) and TLR4 expression has also been documented in the epithelia of gastric foveolar mucosa (Schmausser *et al.* 2004) and duodenal mucosa (Eiro *et al.* 2012). The *H. pylori* LPS is a weak activator of TLR4 in vitro (Cullen *et al.* 2011) but the TLR4 expression has been documented as being upregulated in gastric mucosa during *H. pylori* infection (Ishihara *et al.* 2004) and TLR4 has been implicated as having role in gastric carcinogenesis (Castano-Rodriguez *et al.* 2013).

### 2.5.1 Genetic polymorphisms

The gene encoding TLR4 in humans is located on chromosome locus 9q32-q33 and contains 4 exons. Two non-synonymous polymorphisms, TLR4 +896 adenine/guanine (A/G) (rs4986790) and TLR4 +1196 cytosine/thymine (C/T) (rs4986791), have been located in the fourth exon and they cause amino acid substitutions: glycine for aspartic acid at position 299 and isoleucine for threonine at position 399 respectively. These substitutions lead to the alteration of the ligand-binding receptor site. Both of these mutations are associated with LPS hyporesponsiveness. The double mutant receptor is even less responsive than the receptor containing a single mutation and might be less well expressed than the wild type receptor (Kutikhin 2011). These two polymorphisms co-segregate, and 6–14 % of Indo-European individuals are double heterozygotes for these alleles, whereas both of these mutations are very rare in Asian populations (Ferwerda *et al.* 2007).

Both of these polymorphisms have been associated with increased susceptibility to bacterial diseases, *e.g.* blood stream infections of Gram-negative bacteria (Kutikhin 2011). Increased susceptibility to *H. pylori* has not been documented. In addition to possible increased susceptibility to Gram-negative bacteria, the TLR4 polymorphisms have been proposed to affect the probability of disease through altered inflammatory states (El-Omar *et al.* 2008, Kutikhin 2011). Previous studies on TLR4 polymorphisms and gastric inflammation have provided conflicting results: Achyut *et al.* (Achyut *et al.* 2007) reported higher neutrophil scores for TLR4 +896 mutants and higher plasma cell scores for +1196 mutants. Rigoli *et al.* (Rigoli *et al.* 2010) associated +896 mutants with both antrum and corpus predominant gastritis and +1196 mutants with corpus predominant gastritis. On the other hand, Bagheri *et al.* (Bagheri *et al.* 2014) reported that the wild type genotypes were more prevalent in active chronic gastritis patients than in chronic gastritis patients. There are also several studies where no associations between these TLR4 polymorphisms and gastric inflammation were seen (Hofner *et al.* 2007, Kato *et al.* 2007, Murphy *et al.* 2009). The TLR4 polymorphisms have been also documented as affecting cytokine levels; The TLR4 +896 and +1196 mutant allele carriers have increased levels of TNF $\alpha$ , IL10, monocyte chemoattractant protein-1 and macrophage inflammatory protein 1 $\alpha$  expression but decreased levels of IL1 $\beta$ , IL6, IL8, and growth-related oncogene factor  $\alpha$  expression (Trejo-de la *et al.* 2008).

There is only one study reporting an association between these TLR4 polymorphisms and peptic ulcers. The TLR4 +896 mutants were associated with

duodenal ulcers in the study, which, however, had technical problems, as in over 40% of the duodenal ulcer patients the TLR4 +896 polymorphism could not be analyzed (Trejo-de la *et al.* 2008).

There have been several studies with a few significant results regarding the associations of TLR4 genotypes and gastric cancer and precancerous lesions (Table 3). Meta-analyses based on these studies have documented that mutant genotypes of TLR4 +896 (Castano-Rodriguez *et al.* 2013, Zhang *et al.* 2013, Zhou *et al.* 2014, Zhu *et al.* 2013, Zou *et al.* 2013) and +1196 polymorphisms (Castano-Rodriguez *et al.* 2013, El-Omar *et al.* 2008, Zhou *et al.* 2014, Zhu *et al.* 2013) are associated with a modest risk increase for gastric carcinoma. The TLR4 +896 mutant allele carriers have been associated with both intestinal and diffuse type carcinomas (Hold *et al.* 2007), and the TLR +1196 mutant allele carriers have been specifically associated with the intestinal type carcinoma, but not the diffuse type carcinoma (Santini *et al.* 2008). The TLR4 +896 mutants have also been associated with an increased risk of hypochlorhydria and gastric atrophy, which are considered to be premalignant conditions (Hold *et al.* 2007, Zou *et al.* 2013).

**Table 3. Original studies on the relationship between Toll-like receptor 4 polymorphisms +896 and +1196 and gastric cancer or precancerous lesions.**

Reference	+896	+1196
Kato <i>et al.</i> 2007	No associations with precancerous lesions	-
Achyut <i>et al.</i> 2007	No associations with precancerous lesions	Mutant allele (T) associated with gastric atrophy (OR=4.2) and intestinal metaplasia (OR=4.7)
Garza-Gonzales <i>et al.</i> 2007	No associations with gastric cancer	No associations with gastric cancer
Hold <i>et al.</i> 2007	Mutant allele (G) associated with atrophy (OR=11.0) and non-cardia gastric carcinoma (OR=2.3)	-
Santini <i>et al.</i> 2008	No associations with gastric cancer	Mutant allele (T) associated with gastric cancer (HR=3.6) and intestinal type cancer (HR=5.4)
Trejo-de la <i>et al.</i> 2008	No associations with gastric cancer	No associations to gastric cancer
Rigoli <i>et al.</i> 2010	Mutant allele (G) associated with corpus dominant gastritis or pangastritis (OR=4.80)	Mutant allele (T) associated with corpus dominant gastritis or pangastritis (OR=3.66)
Schmidt <i>et al.</i> 2011	No associations with gastric cancer	-
de Oliveira <i>et al.</i> 2012	Mutant allele (G) associated with gastric cancer (OR=3.19)	No associations with gastric cancer
Qadri <i>et al.</i> 2013	No associations with gastric cancer	No associations with gastric cancer
Companiononi <i>et al.</i> 2014	No associations with gastric cancer	No associations with gastric cancer
Kutikhin <i>et al.</i> 2014	No associations with gastric cancer	No associations with gastric cancer

OR, Odds ratio; HR, Hazard ratio

## 2.6 Interleukin 6

IL6 is a cytokine, which has been shown to play a significant role in inflammation associated cancer. IL6 signals through the type I cytokine receptor and glycoprotein 130 (gp130) subunit. The phosphorylation of gp130 leads to the activation of the JAK/STAT3 pathway, which might be the main carcinogenetic link for IL6 (Neurath & Finotto 2011). *H. pylori* infections and peptic ulcers have been documented as associating with an increased IL6 expression (Furukawa *et al.* 1998, Tsai & Hsu 2010). Increased IL6 serum levels have also been associated with gastric cancer (Ashizawa *et al.* 2005, Ikeguchi *et al.* 2009, Kabir & Daar 1995, Kai *et al.* 2005, Lukaszewicz-Zajac *et al.* 2011). Both IL6 and IL6 receptor expression have been documented in gastric cancer and it has been suggested that IL6 promotes tumor growth through autocrine and paracrine pathways (Ito *et al.* 1997,

Lee *et al.* 2010, Matsuo *et al.* 2003), but the exact pathways and mechanisms are unknown. High IL6 serum levels have been connected to changes in lipid metabolism, *e.g.* low HDL serum levels, and coronary heart disease (Danesh *et al.* 2008, Zuliani *et al.* 2007).

### **2.6.1 Genetic polymorphisms**

The IL6 gene is located on chromosome locus 7p21 and a single nucleotide (G/C) polymorphism has been documented in the IL6 gene promoter at location -174 in the 5' flanking region (rs1800795). Allele G at position -174 has been associated with higher serum levels of IL6 (Fishman *et al.* 1998) and the effect has been documented also in a Finnish population (Raunio *et al.* 2007). Based on other partly conflicting results, the relationship between IL6 -174 polymorphism and IL6 serum levels seems to be modified by sex, exercise, inflammatory state and immune challenge (Bennermo *et al.* 2004, Brull *et al.* 2001, Kristiansen *et al.* 2003, Oberbach *et al.* 2008).

The IL6 -174 polymorphism has not been associated with increased susceptibility to *H. pylori* infection, the severity of gastric inflammation or the risk of duodenal ulcers (Hwang *et al.* 2003, Ramis *et al.* 2015). A single case-control study has documented an association with IL6 -174 allele G and gastric carcinoma: Gatti *et al.* (2007) demonstrated an association with IL6 -174 allele G and gastric cancer in a Brazilian population with chronic gastritis patients as a control group, but no significant associations were found with the histologic subtypes (31 diffuse type cancers, 25 intestinal type cancers). Other studies on the subject have not found any associations (Table 4). A meta-analysis of six original articles has documented a risk increase of non-cardia gastric cancer with an odds-ratio of 2.02 associated with the IL6 -174 genotype GG when compared to the CC genotype, but no conclusions about the genotypes and risk of the histologic subtypes of gastric adenocarcinoma could be made. The same study also associated allele G with the risk of getting gastric ulcers (Sugimoto *et al.* 2010). Two other meta-analyses, which were based mostly on the same original studies reported no associations with IL6 -174 genotypes and gastric cancer (Wang *et al.* 2012b, Yin *et al.* 2012), but these studies have been criticized for the lack of subgroup analyses and for their data extraction methodology (Sand 2013, Wang *et al.* 2012a).

The IL6 -174 polymorphism has also been associated with lipid abnormalities (Fernandez-Real *et al.* 2000) and serum lipid changes during lifestyle interventions (Curti *et al.* 2012, Halverstadt *et al.* 2005). IL6 -174 has been also associated with

the risk of coronary heart disease, but this association seems to vary between different subject groups (Basso *et al.* 2002, Humphries *et al.* 2001).

**Table 4. Original studies on the relationship between interleukin 6 polymorphism -174 and gastric cancer.**

Reference	IL6 -174
El-Omar <i>et al.</i> 2003	No associations with gastric cancer
Hwang <i>et al.</i> 2003	No associations with gastric cancer
Kamangar <i>et al.</i> 2006	No associations with gastric cancer
Gatti <i>et al.</i> 2007	Allele G was associated with non-cardia gastric cancer. No associations with histologic subtypes.
Kang <i>et al.</i> 2009	No result as almost all of the study patients had GG genotype.
Ramis <i>et al.</i> 2015	No associations with gastric cancer



### **3 Aims of the study**

The present work focused on the role of genetic polymorphisms of TLR4 and IL6 in the development of *H. pylori* related diseases. The specific objectives were to determine the answers to the following research questions:

1. Do the TLR4 +896 and +1196 polymorphisms associate with any outcome of *H. pylori* related gastroduodenal disease?
2. Do the TLR4 +896 and +1196 polymorphisms associate with *H. pylori* related dyslipidemia?
3. Does the IL6 -174 polymorphism associate with any outcome of *H. pylori* related gastroduodenal disease?
4. Does the IL6 -174 polymorphism associate with *H. pylori* related dyslipidemia?



## 4 Patients and methods

### 4.1 Description of the study and patients

This study is a part of a more extensive study planned for the comparison of several host genetic, histological inflammation related and endocrine host factors, *H. pylori* bacterial and clinical factors. Therefore, three patient series were collected consisting of a control group, a cancer group and a dyspeptic patient group (Table 5). The study setting in genetic parameters was a case-control study, where the cases comprised dyspeptic and cancer patients. Genetic host factors included several cytokine and other inflammatory marker-related polymorphism measurements, of which TLR4 and IL6 polymorphisms were analyzed in this smaller study. There are earlier publications on the data of some additional parameters tested in the larger study (Karhukorpi *et al.* 2002, Koivurova *et al.* 2003). All study subjects originated from the ethnically homogeneous Finnish population. The study followed the guidelines of the declaration of Helsinki and was approved by the regional ethics committee of the Northern Ostrobothnia Hospital District.

**Table 5. The patient series.**

Variable	Control group	Dyspepsia group	Cancer group
N	179	216	61
Male	56 (31.2 %)	89 (41.2 %)	32 (52.5 %)
Age, years (SD)	39.2 (13.4)	53.8 (13.4)	65.9 (12.6)

SD, standard deviation

#### 4.1.1 Control group (I, II)

The purpose of the control group was for comparison of genetic factors between the groups. The control group consisted of university staff and students, from whom no data were collected concerning dyspeptic symptoms or visits to gastroenterologists. Blood samples were collected for DNA analysis. No serum samples were taken and *H. pylori* status was not assessed.

#### 4.1.2 Dyspepsia group (I–III)

The dyspepsia group consisted of 216 patients suffering from dyspepsia symptoms. The group was assembled from unselected consecutive adult dyspeptic patients

fulfilling Rome criteria between the years 1996 and 2000 from three hospitals in the city of Oulu, Finland, all performing outpatient endoscopies. The patients with immunosuppressive or malignant diseases, or who had been treated for *H. pylori* infections, or who had previous or current immunosuppressive medication, or who underwent current anticoagulant or antibiotic treatment, and patients with previous gastric surgery were excluded. Upper gastrointestinal endoscopies were performed for all the patients by experienced endoscopists. Endoscopy findings, including the presence or absence of gastric or duodenal ulcers, were registered. Biopsies were taken from the descending part of the duodenum (n=206), gastric antrum (n=213) and gastric corpus (n=207) for histological analysis.

Consecutive patients were enrolled until a certain number of ulcer patients and *H. pylori* negative and positive non-ulcer patients were achieved (Tables 6 and 7). The ulcer group consisted of patients with active duodenal or gastric ulcers. Patients with previously diagnosed ulcers or gastric or duodenal scarring but without an active ulcer are described separately.

There was no significant difference in sex distribution between the groups. The *H. pylori* positive non-ulcer patients and the active ulcer patients were older than the *H. pylori* negative non-ulcer patients ( $p < 0.001$ ). There were proportionately more males in the ulcer group than in the *H. pylori* positive non-ulcer group ( $p = 0.033$ ; OR=2.304; CI: 1.069-4.966). The patients were asked about their smoking habits (163 non-smokers) and the use of antacids (n=4), sucralfate (n=10), histamine 2 receptor antagonists (n=28) or proton pump inhibitors (n=16). Data on the use of statin medication and NSAID medication was not collected.

**Table 6. Characteristics of the non-ulcer patients.**

Variable	H. pylori negative	H. pylori positive	P-value for difference
N	78	51	-
Age, mean, years (SD)	48.2 (13.3)	57.8 (13.1)	<b>&lt;0.001</b>
Male	34 (43.6%)	14 (27.5%)	0.093
Smokers	14 (17.9%)	7 (13.7%)	0.629
On dyspepsia medication	9 (11.5%)	12 (23.5%)	0.089

SD, standard deviation

**Table 7. Characteristics of the ulcer patients.**

Variable	Active ulcer	Inactive ulcer	P-value for difference
N	73	14	-
Age, mean, years (SD)	56.6 (13.6)	55.2 (16.0)	0.744
Male	34 (46.6%)	7 (50.0%)	1.000
Smokers	29 (39.7%)	3 (21.4%)	0.239
On dyspepsia medication	33 (45.2%)	4 (30.8%)	0.379
Gastric ulcers	23 (31.5%)	9 (64.3%)	<b>0.032</b>
<i>H. pylori</i> positive	71 (97.3%)	8 (57.1%)	<b>&lt;0.001</b>

SD, standard deviation.

#### 4.1.3 Cancer group (I, II)

The gastric cancer group consisted of 61 patients from the years 1996–2000. The cancer patient data and tissue samples were obtained from the archives of Oulu University Hospital.

#### 4.2 Histopathological analyses (I–III)

Histopathologic analyses were performed on the gastroscopy biopsies of the dyspepsia group and on surgical specimens of the cancer group. The histological analyses were performed on hematoxylin and eosin stained slides from formalin fixed and paraffin embedded tissue materials.

The histopathologic types of the gastric cancers were re-classified by two pathologists according to the Lauren classification. After independent classifications, a consensus meeting was arranged and the classification of those cases with any discrepancy was determined.

The histopathologic changes of the duodenal, antral and stomach corpus mucosal biopsies were analyzed according to the Sydney system (Misiewicz 1991) by one pathologist. All assessments were done blinded for the genetic data. Dichotomial variables for active gastritis (neutrophil score >0 in either antrum or corpus), antrum dominant gastritis (neutrophil score higher in antrum than corpus), atrophy (atrophy score >0 in either antrum or corpus) and metaplasia (metaplasia score >0 in either antrum or corpus) were formed from the Sydney system based variables.

### **4.3 Detection of genetic polymorphisms (I–III)**

The control and dyspeptic patients' DNA was extracted from the blood leucocytes. The gastric cancer patients' DNA was extracted from a fresh frozen gastric tissue specimen representing tissue not containing neoplastic cells. Extraction was performed as previously described by (Koivurova *et al.* 2003). Polymerase chain reaction tests from the DNA samples were performed to detect the IL6 -174 gene polymorphism and the TLR4 +896 and +1196 polymorphisms using previously described primers and conditions (Fishman *et al.* 1998, Lorenz *et al.* 2001). The investigators who performed the genetic analyses were blinded to the clinical data.

### **4.4 Detection of *Helicobacter pylori* (I–III)**

Positive *H. pylori* status was based on a positive serology and either a positive bacterial culture, PCR test or histological assessment. The bacterial culture and PCR were performed as previously described (Koivurova *et al.* 2003). The presence of the *H. pylori* gene variants of *cagA* and *vacA* genes were detected as previously described (Karhukorpi *et al.* 2000).

### **4.5 Serum analyses (I, III)**

G17 (n=213), PGI (n=210) and PGII (n=216) measurements were performed with GastroPanel™ assays by Biohit (Helsinki, Finland) laboratory from the dyspepsia patients' serum samples. Of 199 dyspepsia patients, serum total cholesterol, HDL, LDL and triglyceride serum concentrations were measured by routine methods in the Oulu University Hospital clinical laboratory.

### **4.6 Immunohistochemistry (II)**

Formalin fixed and paraffin embedded sections representing normal stomach and duodenal mucosa were pretreated by heating with microwaves in ethylenediaminetetraacetic acid (for single stains) or sodium citrate (pH 6, for double stains) at 850W for 10 minutes for antigen retrieval. The immunohistochemical stainings were performed with mouse monoclonal antibodies against human TLR4 (1:1000, H00007099-M02, Abnova, Taipei, Taiwan), rabbit polyclonal antibodies against human G17 (1:250, A 0568, Dako, Glostrup, Denmark) and rabbit polyclonal antibodies against human somatostatin

(1:600, A0566, Dako). The incubation time was 60 minutes for TLR4 and somatostatin antibodies and 30 minutes for gastrin antibodies at room temperature. For the detection of the antibody reaction we used the EnVision™ detection kit and the double stains of TLR4, gastrin and somatostatin were performed utilizing the EnVision™ G|2 Doublestain kit (Dako) with related protocols. The validation of immunohistochemical analysis was performed with negative controls including a buffer solution or irrelevant antibodies instead of gastrin and TLR4 antibodies. Duodenal epithelial cells were used as a positive control for TLR4 (Eiro *et al.* 2012). The expression intensity of TLR4 in different cell populations of gastric mucosa was assessed by using single stained sections and a scale from a negative to weak, moderate and strong expression.

#### **4.7 Statistical analyses (I–III)**

Means and standard deviations (SD) are presented for continuous variables and medians and interquartile ranges (IQR) for skewed variables. The significance of the differences between two groups of normally distributed continuous variables was analyzed using the independent samples t-test. The significance of differences between two groups of skewed continuous variables was analyzed using the Mann-Whitney U test. The significance of differences between three groups of normally distributed continuous variables was analyzed using a one-way variance analysis. The difference between three groups of skewed continuous variables was analyzed using the Kruskal-Wallis test. The differences between discrete variables were analyzed using the chi-squared test, Fisher's exact test and binary logarithmic regression models. Forward stepwise models with likelihood criteria were used in multivariate regression analyses. Odds ratios (OR) and 95% confidence intervals (CI) were calculated from cross tabulations or the regression models. The correlations between continuous variables were analyzed with Spearman's rank correlation test. A p-value of less than 0.05 was considered statistically significant. Missing data were excluded pairwise from the analyses. The data were analyzed using the SPSS software version 19 (IBM, Armonk, New York, United States).



## 5 Results

### 5.1 *Helicobacter pylori*

*H. pylori* genotyping was successful in 47 (92.2%) of the non-ulcer patients and in 52 (73.2%) of the ulcer patients. There were 39 (83.0%) *cagA* positive non-ulcer patients and 50 (96.2%) *cagA* positive ulcer patients. Thus *cagA* positivity associated with the ulcer group ( $p=0.046$ ; OR=5.128; CI: 1.030-25.529).

The *vacA* genotypes showed very similar distributions between the non-ulcer and ulcer groups: the non-ulcer group comprised of 24 (51.1%) s1m1, 18 (38.3%) s1m2, and 5 (10.6%) other genotypes, and the ulcer group comprised of 27 (51.9%) s1m1, 19 (41.3%) s1m2, and 6 (11.5%) other genotypes.

#### 5.1.1 Mucosal histopathology

The histological variables are compiled in Table 8. Of the *H. pylori* positive non-ulcer group, 60.0% had antrum predominant gastritis and 40.0% had body predominant or pan-gastritis. The location of active gastritis did not associate significantly with the presence of atrophy or metaplasia. Inside the *H. pylori* positive non-ulcer group, *cagA* positivity associated significantly with higher scores of antral lymphocytes ( $p=0.022$ ) and neutrophils ( $p=0.043$ ). In a stepwise regression analyses with sex, age, smoking and *cagA* as covariates, *cagA* positivity associated with atrophy (OR=9.000; CI: 1.398–57.944;  $p=0.021$ ) and males with metaplasia (OR=4.500; CI: 1.274–15.898;  $p=0.037$ ).

Within the *H. pylori* positive active ulcer group, *cagA* positivity associated significantly with higher scores of antral neutrophils ( $p=0.009$ ) but lower scores of corpus atrophy ( $p=0.043$ ). The active gastric ulcer group had a similar proportion of antrum predominant gastritides (71.4%) as the duodenal ulcer group (72.1%). The gastric ulcer patients had atrophy more often (93.3%) than the duodenal ulcer patients (58.5%;  $p=0.021$ ) and a trend for a higher proportion of metaplasia (47.4% versus 25.0%,  $p=0.088$ ). In a stepwise regression analyses with sex, age, smoking and *cagA* as covariates, *cagA* positivity associated with active gastritis (OR=44.000; CI: 1.457-1328.467;  $p=0.030$ ) and age (per year OR=1.105; CI: 1.022–1.196;  $p=0.012$ ) and smoking (OR=21.863; CI: 2.769–172.637;  $p=0.003$ ) associated with metaplasia.

**Table 8. Histological scores.**

Variable	<i>H. pylori</i> negative non-ulcer group (n=75-78)	<i>H. pylori</i> positive non-ulcer group (n=46-50)	<i>H. pylori</i> positive ulcer group (n=56-68)
Duodenal villus atrophy score >0 (%)	7.8%	0%	4.6%
Mean antrum lymphocyte score (0-3)	0.2	2.2*	2.5*
Mean antrum neutrophil score (0-3)	0.04	1.2*	1.6*
Antrum atrophy score >0 (%)	4.0%	60.9%*	94.2%*
Antrum metaplasia score >0 (%)	3.8%	24.5%*	30.9%*
Mean corpus lymphocyte score (0-3)	0.2	2.0*	1.8*
Mean corpus neutrophil score (0-3)	0.0	0.7*	0.7*
Corpus atrophy score >0 (%)	2.6%	22.4%*	6.5%
Corpus metaplasia score >0 (%)	1.3%	8.3%	0.0%

\*P<0.05 when compared to the *H. pylori* negative non-ulcer group. P-values calculated by Mann-Whitney U test or chi-squared test.

### 5.1.2 Gastrin and pepsinogen concentrations

Serum G17 concentrations were higher in the *H. pylori* positive non-ulcer patients than in the *H. pylori* negative non-ulcer patients (Table 9, p=0.001). Furthermore, PGI (p<0.001) and PGII (p<0.001) concentrations were higher in the *H. pylori* positive patients than in the negative patients. The PGI/PGII-ratio was also significantly lower in the *H. pylori* positive patients (p<0.001). The *H. pylori* positive active ulcer patients had higher G17 (p<0.001), PGI (p<0.001) and PGII (p<0.001) serum concentrations and a lower PGI/PGII-ratio (p<0.001) than *H. pylori* negative non-ulcer patients, and higher PGI (p=0.007) and PGII (p=0.036) serum concentrations than the *H. pylori* positive non-ulcer patients.

In the *H. pylori* positive non-ulcer group, patients with antrum predominant gastritis displayed lower PGII levels (12.0 µg/l; IQR=5.0 versus 15.5 µg/l; IQR=12.0; p=0.041) and a higher PGI/PGII-ratio (8.0 µg/l; IQR=2.7 versus 5.4 µg/l; IQR=5.0; p=0.001) than the patients with corpus predominant or pan-gastritis.

Age, sex, smoking, use of dyspepsia medication or cagA positivity did not associate with G17 in any group.

**Table 9. Median gastrin and pepsinogen concentrations and interquartile ranges.**

Serum concentration	<i>H. pylori</i> negative non-ulcer group (n=76–78)	<i>H. pylori</i> positive non-ulcer group (n=49–51)	<i>H. pylori</i> positive ulcer group (n=68–71)
Gastrin-17, pmol/l (IQR)	3.3 (3.0)	6.8 (5.1)*	5.5 (4.6)*
Pepsinogen I, µg/l (IQR)	72.0 (32.0)	94.0 (37.0)*	111.4 (24.7)*
Pepsinogen II, µg/l (IQR)	5.5 (3.2)	12.3 (7.6)*	15.0 (9.8)*
PGI/PGII-ratio (IQR)	12.5 (4.0)	7.2 (3.6)*	7.0 (3.2)*

\*P<0.05 when compared to the *H. pylori* negative non-ulcer group. P-values calculated using the Mann-Whitney U test.

### 5.1.3 Dyslipidemia

Of the 199 study subjects with lipid serum level data, 78 were *H. pylori* negative non-ulcer patients, 51 were *H. pylori* positive non-ulcer patients, 57 were active ulcer patients (of which 56 *H. pylori* positive) and 14 were inactive ulcer patients (of which 8 *H. pylori* positive). The HDL serum level was significantly lower in the *H. pylori* positive group than in the *H. pylori* negative non-ulcer group (p=0.007; Table 10). The *H. pylori* positive active ulcer group demonstrated lower HDL concentrations than both the *H. pylori* negative and positive non-ulcer groups (p<0.001 and p=0.026 respectively). There were no significant differences in total cholesterol, LDL or triglyceride serum levels between the patient groups.

Females had significantly higher HDL levels than males in the *H. pylori* positive non-ulcer group (0.88 mmol/L; IQR=0.37 versus 0.72 mmol/l; IQR=0.37; p=0.022). Age correlated significantly with HDL in the *H. pylori* positive ulcer group (r=-0.295; p=0.038). Smoking or cagA positivity did not associate with HDL in any group. A multifactorial regression model (*H. pylori* negative non-ulcer as a reference group, age, sex and smoking as cofactors) associated the *H. pylori* positive non-ulcer group (OR=3.478 (CI: 1.520–7.960; p=0.003), the *H. pylori* positive ulcer group (OR=5.373; CI: 2.195–13.151; p<0.001) and sex (male OR=3.111; CI: 1.467–6.599; p=0.003; other cofactors not-significant) with low HDL (<1.00 mmol/l).

**Table 10. Serum lipids concentrations.**

Serum concentration	<i>H. pylori</i> negative	<i>H. pylori</i> positive	<i>H. pylori</i> positive
	non-ulcer (n=78)	non-ulcer (n=51)	active ulcer (n=56)
Total cholesterol mmol/L, mean (SD)	5.54 (1.11)	5.45 (1.11)	5.50 (1.18)
HDL mmol/L, median (IQR)	0.97 (0.39)	0.85 (0.31)*	0.75 (0.26)*
LDL mmol/L, mean (SD)	4.12 (1.00)	3.84 (0.99)	4.01 (0.88)
Triglycerides mmol/L, median (IQR)	1.13 (0.71)	1.15 (0.66)	1.13 (0.69)

SD, standard deviation; IQR, interquartile range; \*P<0.05 when compared to the *H. pylori* negative non-ulcer group. P-values were calculated using the Mann-Whitney U test.

## 5.2 TLR4 in *Helicobacter pylori* related diseases

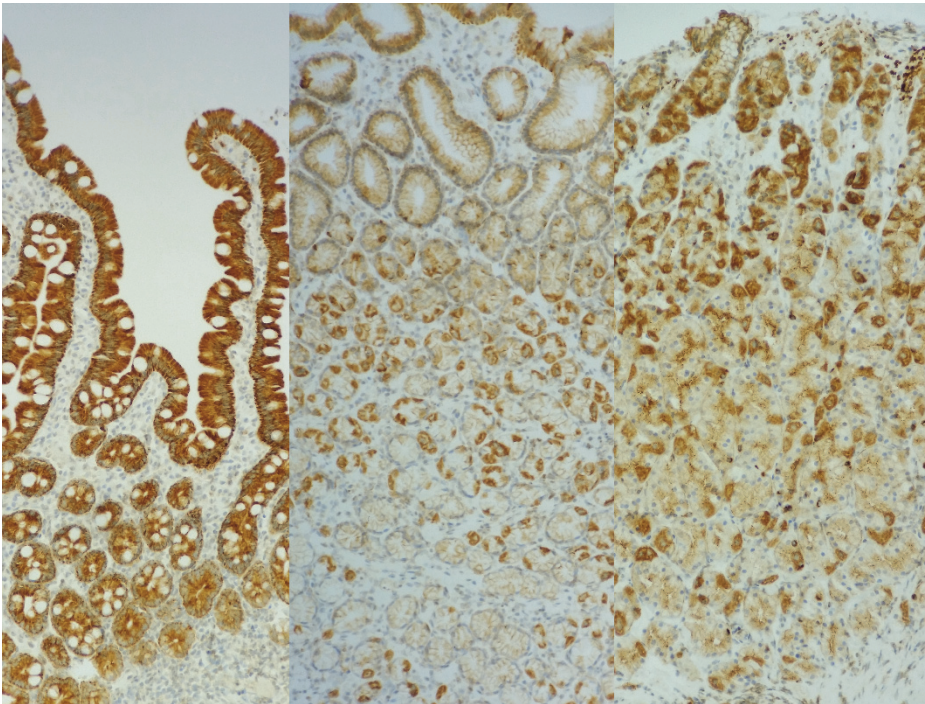
TLR4 expression patterns on gastroduodenal mucosa were tested immunohistochemically on tissue samples representing histologically normal mucosa. The distribution of TLR4 +896 and +1196 polymorphisms was analyzed in all subject groups.

### 5.2.1 TLR4 expression in gastroduodenal mucosa

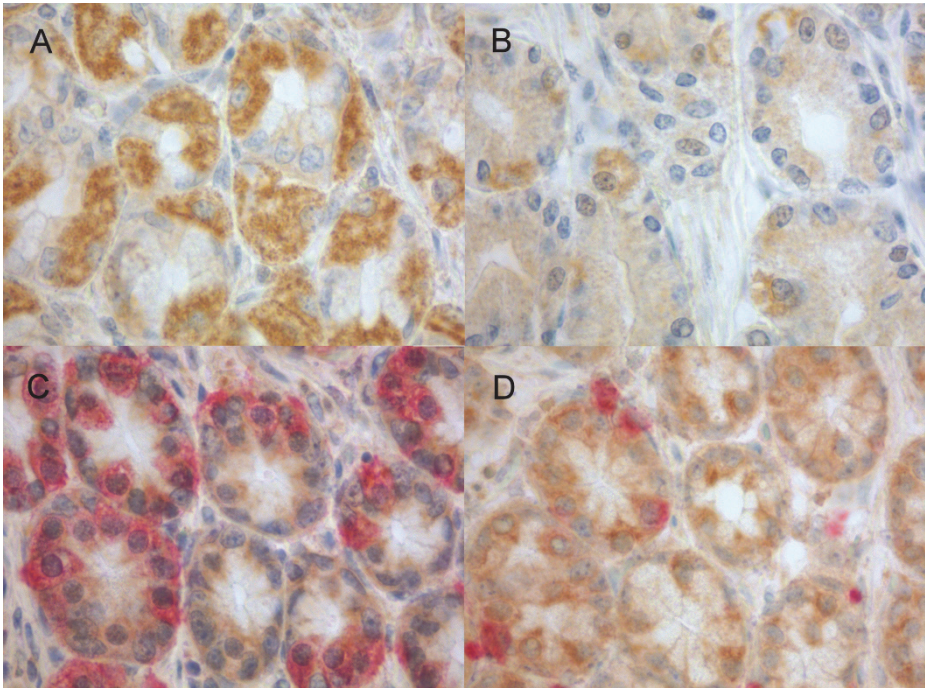
To assess the expression patterns of TLR4 in normal gastroduodenal mucosa, we used immunohistochemical single and double stainings for biopsies from histologically normal human duodenum and stomach (Fig. 3) samples. In the duodenum, strong cytoplasmic TLR4 expression was seen throughout the epithelia and weak to moderate expression in lamina propria lymphocytes. In the gastric mucosa, strong TLR4 expression was seen in the cytoplasm of epithelial cells in gastric surface and the upper parts of the foveolar epithelium. In the glandular neck zone, the epithelial cells were positive with weak to moderate expression levels. However, some cells were moderately to strongly positive and some cells were weakly stained as were the majority of cells in the antral glands. In the corpus glands TLR4 immunopositivity was present in the parietal cells, where the expression varied from mild to moderate but some individual cells were strongly positive.

To clarify the identity of the strongly stained cells in the glandular neck regions, double stains with TLR4 and either gastrin or somatostatin antibodies were performed. The majority of the gastrin expressing G cells present in the glandular neck were TLR4 positive, and the majority of somatostatin expressing D cells were similarly TLR4 positive (Fig. 4). These expression patterns suggest that the

majority of moderately or strongly TLR4 positive cells in the glandular neck region are G cells and D cells.



**Fig. 3. Microphotographs demonstrating TLR4 expression in duodenum (left), antrum (middle) and corpus (right).**



**Fig. 4. Microphotographs demonstrating TLR4 expression in gastric mucosal cells (1, published by permission of Public Library of Science). The immunohistochemical staining of TLR4 expression in the corpus glands indicates strong expression mainly in the parietal cells and weak expression in other cells (A). In the glandular neck zone of the antrum (B) of the stomach occasional cells are strongly positive, while other cells are weakly positive. Double stainings (C, D) show TLR4 positivity (brown) in gastrin positive cells (red, C) and in somatostatin positive cells (red, D) in the antrum.**

### **5.2.2 TLR4 polymorphisms, *Helicobacter pylori* infection and mucosal histopathology**

The TLR4 +896 allele A and +1196 allele C were in total linkage disequilibrium in all of our study populations; All subjects are divided into groups of wild type homozygotes (genotype +896 AA combined with +1196 CC), heterozygotes (+896 AG with +1196 CT) and mutant homozygotes (+896 GG with +1196 TT). The genotype frequencies in the control group (wild type homozygotes 79.9%; heterozygotes 19.6%; mutant homozygotes 0.6%) were consistent with the Hardy-Weinberg equilibrium. The distributions of TLR4 genotypes were also similar in the *H. pylori* negative non-ulcer patients (75.6%; 23.1%; 1.3% respectively) and *H.*

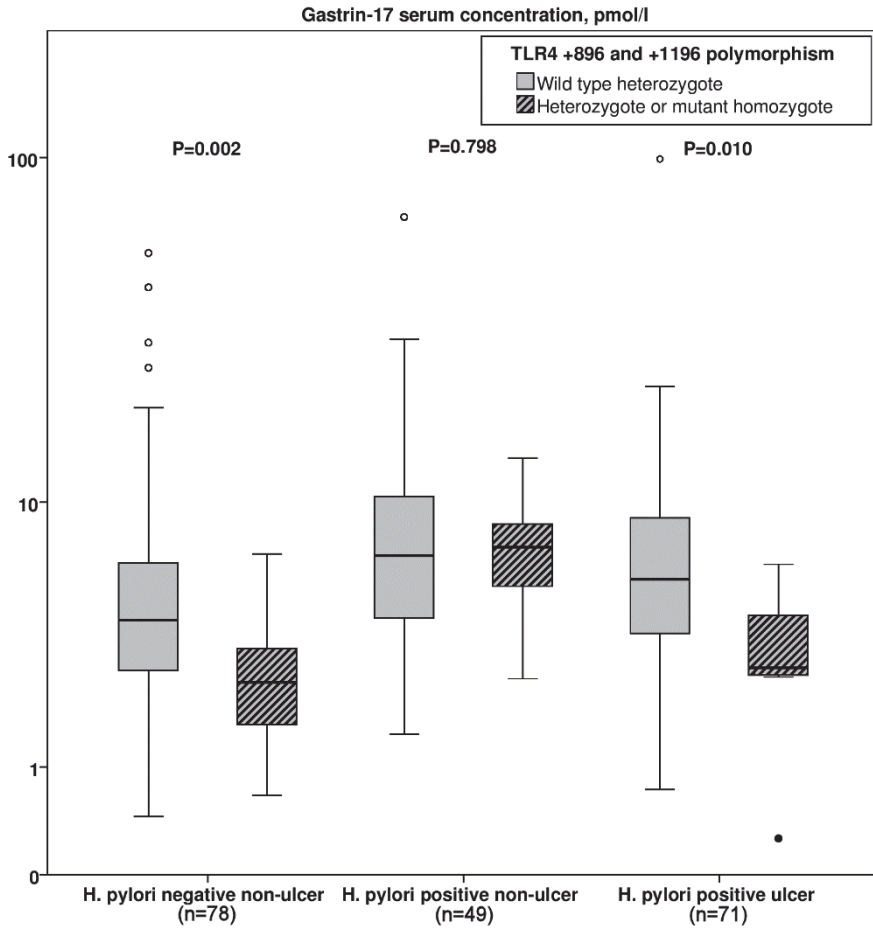
*pylori* positive non-ulcer patients (80.4%; 19.6%; 0.0% respectively). In the *H. pylori* positive non-ulcer patients, the TLR4 polymorphisms did not display significant differences in the Sydney system based scores of antral or corpus lymphocytes, neutrophils, atrophy, intestinal metaplasia or *H. pylori*.

### **5.2.3 TLR4 polymorphisms and gastrin and pepsinogen concentrations**

We compared G17 serum levels between TLR4 wild type homozygotes and a combined group of heterozygotes and mutant homozygotes separately in the *H. pylori* negative and positive non-ulcer groups and *H. pylori* positive active ulcer group. Serum G17 serum levels were higher in the TLR4 wild type homozygotes compared to the heterozygotes and mutant homozygotes in *H. pylori* negative non-ulcer patients and in *H. pylori* positive active ulcer patients, but not in the *H. pylori* positive non-ulcer group (Fig. 5).

TLR4 wild type homozygotes demonstrated higher G17 concentrations also when the ulcer group was restricted to gastric ulcers (respectively 5.6 pmol/l; IQR=4.2 versus 2.8 pmol/l; IQR=2.3;  $p=0.031$ ). In the *H. pylori* negative group, TLR4 wild type homozygotes also had higher PGII levels (respectively: 5.9  $\mu\text{g/l}$ ; IQR=3.2;  $n=57$  versus 4.9  $\mu\text{g/l}$ ; IQR=2.0;  $n=19$ ;  $p=0.003$ ).

To increase the sensitivity, we pooled all the patients with G17, PGI and PGII data and compared the concentrations between TLR4 wild type homozygotes and mutant allele carriers in the whole group (Table 11). Serum G17 serum levels were higher in the TLR4 wild type homozygotes compared to the heterozygotes and mutant homozygotes and the TLR4 wild types had also higher serum levels of PGI and PGII. To take account of the effects of multiple variables on the G17 levels, we performed a stepwise logistic regression model. In the model, the TLR4 wild type genotypes were more common in the patients who surpassed the upper reference limit for the serum gastrin concentration provided by the test manufacturer of 7.0 pmol/l (27.2%; 58/213) with an OR of 2.835 (CI: 1.040-7.728;  $p=0.042$ ). This model also associated *H. pylori* positivity with high gastrin levels (OR=2.297; CI: 1.170–4.510;  $p=0.016$ ), but indicated the absence of a role for age, use of medication, smoking and sex.



**Fig. 5. Boxplot figure of gastrin-17 serum concentrations in subgroups based on Helicobacter pylori, ulcers and Toll-like receptor 4 +896 and +1196 genotypes. The p-values were calculated using the Mann-Whitney U test.**

**Table 11. Median gastrin and pepsinogen concentrations and interquartile ranges in pooled Toll-like receptor 4 genotype groups.**

Serum concentration	TLR4 wild type homozygotes	TLR4 heterozygotes and mutant	P-value
	(n=171-177)	homozygotes (n=39)	
Gastrin-17, pmol/l (IQR)	5.0 (5.3)	3.1 (3.2)	<0.001
Pepsinogen I, µg/l (IQR)	93.0 (43.3)	80.2 (45.8)	0.035
Pepsinogen II, µg/l (IQR)	11.2 (9.8)	7.8 (7.8)	0.005
PGI/PGII-ratio (IQR)	8.7 (5.6)	10.3 (4.9)	0.079

The p-values were calculated using the Mann-Whitney U test.

### 5.2.4 TLR4 polymorphisms and peptic ulcers

The ulcer groups displayed tendencies towards wild type homozygote dominance in TLR4 genotypes compared to the controls, but the difference was non-significant (Table 12). To further clarify this indefinite result, we also compared the TLR4 genotype distribution of the ulcer groups to *H. pylori* negative non-ulcer patients: the wild type homozygotes (versus heterozygotes and mutant homozygotes) associated significantly with the ulcer groups (OR=2.480; CI: 1.074–5.729; p=0.034 for all ulcers; OR=2.536; CI: 1.032–6.223; p=0.043 for active ulcers). To take other ulcer risk factors (TLR4 polymorphisms, cagA, age, sex and smoking) into account, we performed a stepwise regression model where we compared the *H. pylori* positive active ulcer group to the *H. pylori* positive non-ulcer dyspepsia group. TLR4 wild type homozygotes associated with the peptic ulcer group (OR=4.390; CI: 1.134-16.998; p=0.032) as did cagA positivity (OR=6.221; CI: 1.117–34.644; p=0.037) and smoking (OR=5.491; CI: 1.959–15.388; p=0.001).

**Table 12. Distribution of Toll-like receptor 4 +896 and +1196 genotypes in control, non-ulcer and ulcer groups.**

Group	Distribution of TLR4 +896 and +1196 genotypes			P-value for difference	
	Wild type homozygote	Heterozygote	Mutant homozygote	Distribution	WT vs mutant allele carrier
Control	79.9% (143)	19.6% (35)	0.6% (1)	Reference	Reference
HP- non-ulcer	75.6% (59)	23.1% (18)	1.3% (1)	0.509	0.501
HP+ non-ulcer	80.4% (41)	19.6% (10)	0.0% (0)	1.000	1.000
All ulcers	88.5% (77)	10.3% (9)	1.1% (1)	0.087	0.109
Active ulcers	89.0% (65)	9.6% (7)	1.4% (1)	0.100	0.094

TLR4, Toll-like receptor 4; HP, *Helicobacter pylori*; WT, Wild type; p-values were calculated using a chi-squared test.

### 5.2.5 TLR4 polymorphisms and gastric carcinoma

Of the 61 carcinomas, 56 (91.8%) were non-cardia carcinomas. Of the non-cardia carcinomas, 29 (51.8%) were intestinal type, 23 (41.1%) diffuse type; 2 (3.6%) mixed type and 2 (3.6%) of indeterminate types. The intestinal type group displayed more males than the diffuse and mixed type group (65.5% versus 32.0%;  $p=0.028$ ) and the patients were also older in the intestinal type group than in the mixed and diffuse type carcinoma group (72.6 years;  $SD=8.1$  versus 59.5 years;  $SD=12.8$ ;  $p<0.001$ ).

The whole carcinoma group and non-cardia carcinoma group displayed similar distributions of the TLR4 +896 and +1196 genotypes as controls (Table 13). We also compared the histological subgroups to the control group. The intestinal carcinoma group displayed different distributions of TLR4 genotypes than the control group, but did not show a significant difference in the wild type homozygote versus heterozygote and mutant homozygote comparison. The distribution of TLR4 genotypes of the diffuse and mixed type carcinoma group did not differ from the controls.

**Table 13. Distribution of Toll-like receptor 4 +896 and +1196 genotypes in control and carcinoma groups.**

Group	Distribution of TLR4 +896 and +1196 genotypes			P-value for difference	
	Wild type homozygote	Heterozygote	Mutant homozygote	Distribution	WT vs mutant allele carriers
Control	79.9% (143)	19.6% (35)	0.6% (1)	Reference	Reference
All carcinomas	77.0% (47)	19.7% (12)	3.3% (2)	0.277	0.715
Non-cardia carcinomas	76.8% (43)	19.6% (11)	3.6% (2)	0.253	0.706
Intestinal non-cardia carcinomas	65.5% (19)	27.6% (8)	6.9% (2)	<b>0.023</b>	0.094
Diffuse and mixed non-cardia carcinomas	92.0% (23)	8.0% (2)	0.0% (0)	0.282	0.179

TLR4, Toll-like receptor 4; WT, wild type; p-values were calculated using a chi-squared test.

### 5.2.6 TLR4 polymorphisms and *Helicobacter pylori* related dyslipidemia

The TLR4 +896 and +1196 genotypes did not display significantly different total cholesterol, LDL, HDL or triglyceride serum levels in any of the patient groups (*H. pylori* negative non-ulcer, *H. pylori* positive non-ulcer, active ulcer).

### 5.3 IL6 -174 polymorphism in *Helicobacter pylori* related diseases

The IL6 -174 genotype frequencies of the control group were consistent with the Hardy–Weinberg equilibrium (Table 14). The non-ulcer groups and the ulcer groups displayed similar IL6 -174 genotype distributions as the control group. The IL6 -174 genotypes did not display significant differences in the Sydney system based scores of antral or corpus lymphocytes, neutrophils, atrophy, intestinal metaplasia or *H. pylori* in any of the subject groups (*H. pylori* negative non-ulcer, *H. pylori* positive non-ulcer, active ulcer).

**Table 14. Distribution of interleukin 6 -174 genotypes in control, non-ulcer and ulcer groups.**

Group	Distribution of IL6 -174 genotypes			P-value for difference	
	GG	GC	CC	Distribution	GG+GC vs CC
Control	20.7% (37)	48.0% (86)	31.3% (56)	Reference	Reference
<i>H. pylori</i> negative non-ulcer	23.1% (18)	42.3% (33)	34.6% (27)	0.712	0.664
<i>H. pylori</i> positive non-ulcer	23.5% (12)	49.0% (25)	27.5% (14)	0.818	0.612
All ulcers	23.0% (20)	50.6% (44)	26.4% (23)	0.726	0.476
Active ulcers	21.9% (16)	49.3% (36)	28.8% (21)	0.934	0.764

P-values were calculated using a chi-squared test.

#### 5.3.1 IL6 -174 polymorphism and gastric carcinoma

The gastric carcinoma patients showed a different distribution of IL6 -174 genotypes than the controls (Table 15). In histological subgroup analyses the diffuse and mixed subtypes included significantly more GG and GC genotypes than the controls, while the genotype distribution of intestinal type carcinoma patients did not differ significantly from controls. Both genotypes GG and GC (CC as the reference genotype) associated with the diffuse and mixed type carcinomas when

compared to the controls in regression analysis (GG OR=5.535; CI: 1.231–24.888; p=0.026; GC OR=6.054; CI: 1.217–30.111; p=0.028).

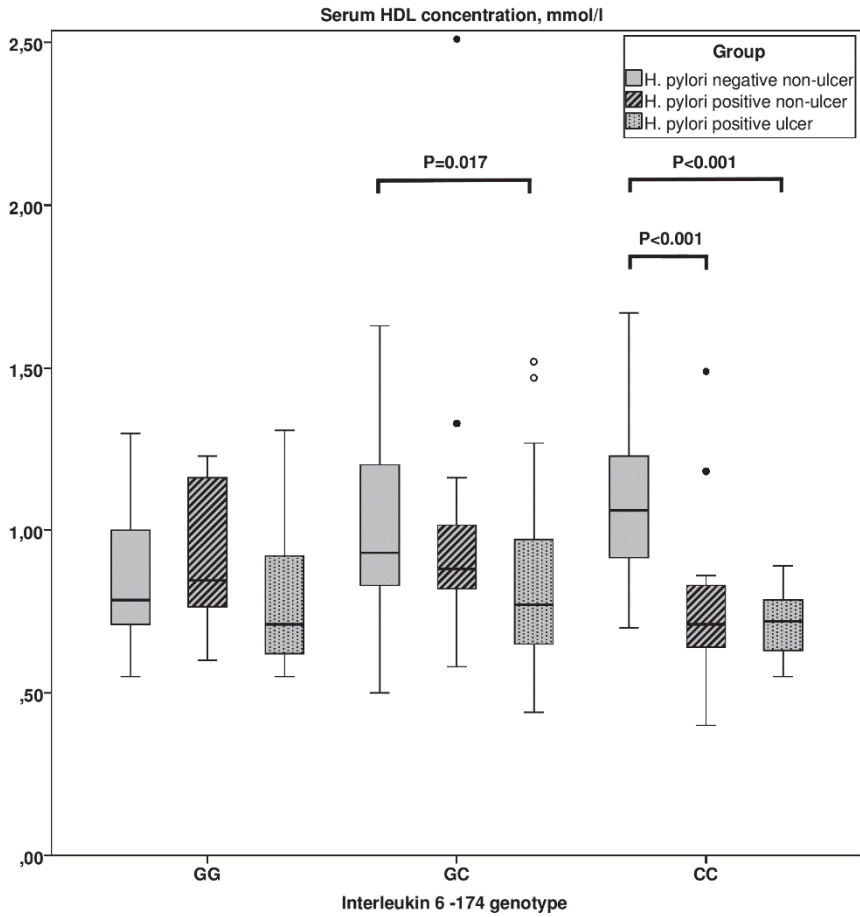
**Table 15. Distribution of interleukin 6 -174 genotypes in control and carcinoma groups.**

Group	Distribution of IL6 -174 genotypes			P-value for difference	
	GG	GC	CC	Distribution	GG+GC vs CC
Control	20.7% (37)	48.0% (86)	31.3% (56)	Reference	Reference
All carcinomas	24.6% (15)	62.3% (38)	13.2% (8)	<b>0.022</b>	<b>0.007</b>
Intestinal	21.9% (7)	59.4% (19)	18.8% (6)	0.357	0.206
Diffuse and mixed	29.6% (8)	63.0% (17)	7.4% (2)	<b>0.033</b>	<b>0.010</b>
Diffuse	29.2% (7)	62.5% (15)	8.3% (2)	0.063	<b>0.028</b>
Non-cardia	21.4% (12)	64.3% (36)	14.3% (8)	<b>0.034</b>	<b>0.015</b>
Non-cardia intestinal	17.2% (5)	62.1% (18)	20.7% (6)	0.377	0.282
Non-cardia diffuse and mixed	28.0% (7)	64.0% (16)	8.0% (2)	0.052	<b>0.017</b>
Non-cardia diffuse	26.1% (6)	65.2% (15)	8.7% (2)	0.081	<b>0.027</b>

P-values were calculated using a chi-squared test.

### **5.3.2 IL6 -174 polymorphism and *Helicobacter pylori* related dyslipidemia**

To assess whether the IL6 -174 genotype modulates the association between *H. pylori* and HDL, we analyzed the HDL concentration between the subject groups (*H. pylori* positive and negative non-ulcer and *H. pylori* positive ulcer) separately in subgroups based on IL6 -174 genotypes (Fig. 6). The previously seen difference in HDL between *H. pylori* negative and positive non-ulcer groups was only seen in CC genotype patients (respectively 1.06 mmol/l versus 0.71 mmol/l; p<0.001). The previously seen difference in HDL between the *H. pylori* negative non-ulcer group and the *H. pylori* positive ulcer group was seen in the GC (respectively 0.93 mmol/l versus 0.77 mmol/l; p=0.017) and CC genotype patients (respectively 1.06 mmol/l versus 0.72 mmol/l; p<0.001) but not in the GG genotype patients. The *H. pylori* positive non-ulcer group associated significantly with low HDL (<1.00 mmol/l) when compared to the *H. pylori* negative non-ulcer group in a regression model (OR=10.200; CI: 1.885–55.195; p=0.007; age, sex and smoking non-significant) in the CC genotype patients, but not in the GC or GG genotype patients.



**Fig. 6. Boxplot figure of high density lipoprotein serum concentrations in subgroups based on Helicobacter pylori, presence of ulcer and interleukin 6 -174 genotype. The p-values were calculated using the Mann-Whitney U test.**



## 6 Discussion

### 6.1 TLR4 and gastrin in *Helicobacter pylori* related diseases

The TLR4 +896 and +1196 wild type homozygosity associated with a modestly increased risk of peptic ulcers. In addition, the same genotype associated with higher G17, PGI and PGII serum concentrations. The TLR4 +896 and +1196 polymorphisms were not associated with the severity or the distribution of gastric inflammation.

Hypergastrinemia and increased gastric acid secretion are well known abnormalities associated with the development of peptic ulcers. The specific mechanisms by which an *H. pylori* infection causes hypergastrinemia are not known (Chu & Schubert 2013, Ernst & Gold 2000, McColl *et al.* 2000). The activation of innate immunity has been proposed to be a stimulator for gastrin secretion based on results from a mouse study (Fukui *et al.* 2006). In another animal model, murine G cells were documented as expressing TLR4, and in vitro experiments with these cells have shown that LPS induces gastrin secretion from G cells, presumably through TLR4 mediated mechanisms (Kidd *et al.* 2009). The regulation of gastrin secretion by *H. pylori* could also function through somatostatin, which is the primary inhibitor of gastrin stimulated acid secretion. Gastric infection has been associated with the suppression of somatostatin secretion suggesting that either direct or indirect recognition of bacterial products is important in the regulation somatostatin secreting D cells (Zavros & Merchant 2005).

In our studies, the wild type form of TLR4, which has been associated with more activity in LPS recognition than the mutant +896/+1196 type (Kutikhin 2011), was associated with higher levels of gastrin. This suggests that TLR4 activation is also linked to gastrin secretion in humans. In addition, the PGI and PGII levels were higher in the TLR4 wild types indicating functional gastric secretion in relation to the increased gastrin levels both in the corpus and antrum of the stomach. This implies that the wild type TLR4 activation also leads to increased gastric acid secretion. Based on our results, we propose TLR4 activation dependent hypergastrinemia and the subsequent increased gastric acid output as a new possible pathogenic mechanism for *H. pylori* related peptic ulcer development (Fig. 7). Enhanced gastrin secretion in response to TLR4 activation can be considered physiologically appropriate because TLR4 is essential for the innate immunity

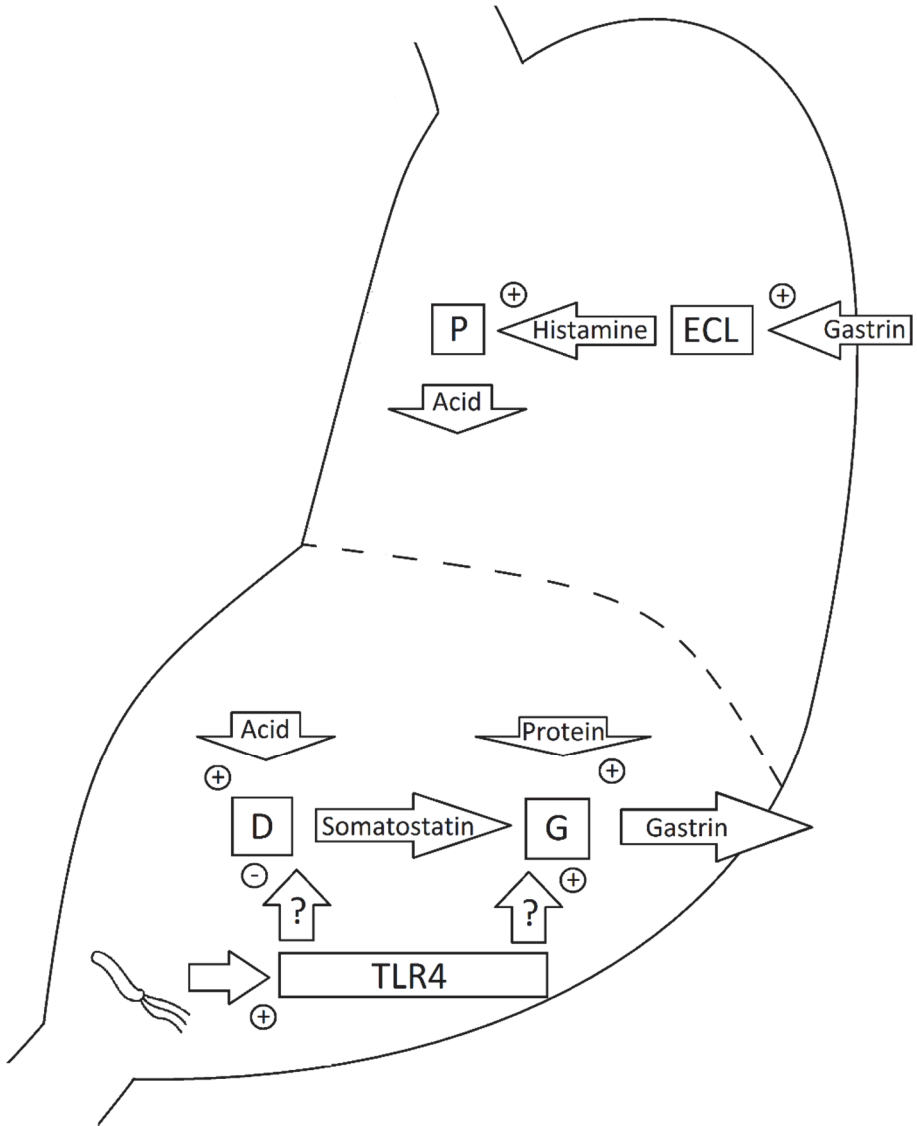
against gram negative bacteria and gastric acid secretion is an important part of the immunological surveillance mechanism in the gastrointestinal tract. In cases where *H. pylori* is able to avoid or locally neutralize the otherwise bactericidal gastric acid, the constant activation of wild type TLR4 +896/+1196 receptors could lead to hypergastrinemia and an increased acid load leading to an ulcer.

The TLR4 +896 and +1196 wild types and high G17 levels were also linked in the *H. pylori* negative subjects. This might be due to TLR4 recognizing the LPS of other gram negative microbial flora present within the gastric contents. The previously mentioned murine G cell model showed higher gastrin secretion stimulating potency for *Salmonella enteritidis* and *Escherichia coli* LPS than for *H. pylori* LPS (Kidd *et al.* 2009). The association between TLR4 genotypes and G17 was also seen in the *H. pylori* positive ulcer patients, but surprisingly not in the *H. pylori* positive non-ulcer group. The lack of association might be due to the small number of subjects in that group. Additionally, this analysis did not take other mechanisms of ulcer pathogenesis than hypergastrinemia into account. There is also the possibility that the group may have been too homogeneous to observe differences in G17, because ulcer patients, who typically have hypergastrinemia, had been already separated into another group.

Our immunohistochemical studies of gastric antral mucosa indicate, that in addition to the expression in the surface and foveolar epithelium, TLR4 positivity is seen in the glandular neck zone and it is located in the gastrin and somatostatin expressing cells, *i.e.* G and D cells. We speculate that TLR4–ligand interaction in G and D cells could have an effect on gastrin and somatostatin secretion and potentially explain the associations between the TLR4 polymorphisms and increased serum gastrin and the peptic ulcer risk. There are of course multiple factors which might influence the interaction between TLR4 and acid secretion. For example, the cytokine IL1 $\beta$ , which is induced by *H. pylori* and TLR4 activation, has been documented as suppressing gastrin and gastric acid secretion (Datta De *et al.* 2013, Lochhead & El-Omar 2008). *H. pylori* might also effect gastrin secretion via other bacterial proteins (Wiedemann *et al.* 2012). Further studies are needed to assess the potential role of TLR4 in gastrin and gastric acid regulation. The differences in both basal and induced gastrin and gastric acid secretion between the TLR4 genotypes should be analyzed.

The TLR4 +896 and +1196 mutant alleles have been previously documented as being associated with a modest risk increase of gastric cancer (Castano-Rodriguez *et al.* 2013, El-Omar *et al.* 2008, Zhang *et al.* 2013, Zhou *et al.* 2014, Zhu *et al.* 2013, Zou *et al.* 2013). In our studies, the TLR4 polymorphisms did not

associate with the whole group of gastric cancers, but the intestinal gastric cancer group had a slightly different distribution of the TLR4 +896/+1196 genotypes than the controls: the mutant alleles showed a non-significant predominance in intestinal cancer patients. The lack of robust associations in our study might be due to the small number of cancer patients. Nevertheless, the association between TLR and gastrin also provides a speculative mechanism for gastric cancer susceptibility; the hypoactive mutant TLR4 receptor may not stimulate gastrin and gastric acid secretion sufficiently and could lead to a hypochlorhydric disease phenotype. The hypochlorhydric disease phenotype associates with atrophy and metaplasia, which are considered to be precursor conditions for intestinal type carcinoma. Additionally, this hypothesis is influenced by several additional factors in the physiology of gastric acid regulation and needs further study.



**Fig. 7. Schematic drawing of the proposed mechanisms in which Toll-like receptor 4 activation by bacterial lipopolysaccharide could regulate gastric acid secretion. We propose that TLR4 activation could stimulate gastrin secretion in G cells (G) directly or suppress somatostatin secretion in D cells (D). The increased gastrin secretion would stimulate histamine secretion from enterochromaffin-like cells (ECL) and finally result in increased hydrochloric acid secretion from parietal cells (P).**

## 6.2 IL6 -174 and the diffuse type gastric carcinoma

The IL6 -174 allele G was associated with diffuse and mixed type carcinoma, but not with intestinal type carcinoma, prevalence of *H. pylori*, inflammation, atrophy or metaplasia. The lack of association between IL6 -174 polymorphisms and inflammation suggest that the carcinogenetic effect of IL6 in the diffuse type gastric carcinoma is non-inflammatory.

Previous studies on the interaction of IL6 -174 and gastric cancer have not found a similar association as our study did. This might be due to the fact that many studies did not group their cancers on a histopathological basis or if they did, the subgroups were small. Additionally, the ethnicity of the study group might have a significant effect as the distribution of IL6 -174 genotypes has been documented as being highly variable in different populations.

The pathophysiological link between IL6 and the diffuse type gastric cancer remains speculative. The histologic hallmarks of the diffuse type cancer are the poor cohesion of the cancer cells and mesenchymal or signet cell like morphology (Lauren 1965). The main trigger for developing diffuse type gastric adenocarcinoma is thought to be the loss of the E-cadherin function through mutations, transcriptional repressors or promoter hypermethylation (Carneiro *et al.* 2012, Chan & Wong 2001). E-cadherin is a cell junction protein which functions in the maintenance of epithelial cell cohesion and in the regulation of proliferation (Graziano *et al.* 2003). A similar kind of E-cadherin dysfunction is seen in infiltrating lobular breast carcinoma, which histologically demonstrates loss of cell cohesion (Chan & Wong 2001).

The IL6 -174 allele G, which was associated with diffuse and mixed type carcinomas, has been documented as correlating with high serum concentrations of IL6 (Fishman *et al.* 1998). IL6 has been documented as having a downregulating effect on the E-cadherin expression in several studies with breast cancer cell lines, mammary epithelial cell lines and head and neck tumor cell lines (Asgeirsson *et al.* 1998, De Luca *et al.* 2012, Leslie *et al.* 2010, Sullivan *et al.* 2009, Yadav *et al.* 2011). In these cell culture studies IL6 has been reported to cause anti-adhesive effects (Asgeirsson *et al.* 1998), increased migration (De Luca *et al.* 2012, Leslie *et al.* 2010) and mesenchymal morphological changes, which are typical of infiltrating cells (Asgeirsson *et al.* 1998, Yadav *et al.* 2011). The biochemical pathway through which IL6 downregulates E-cadherin seems to work through the JAK/STAT3 pathway (Leslie *et al.* 2010, Sullivan *et al.* 2009, Yadav *et al.* 2011). The induction of STAT3 by IL6 has been demonstrated also in gastric cell lines

(Okamoto *et al.* 2011). Thus E-cadherin downregulation by increased IL6 expression is a potential carcinogenic route for the risk association of IL6 -174 G allele and diffuse type of gastric cancer.

### **6.3 The interaction between *Helicobacter pylori*, IL6 -174 and HDL**

Our results reproduced the previously seen association between *H. pylori* and low HDL serum levels and also documented even lower HDL levels in the peptic ulcer patients. When the association was tested in subgroups based on the IL6 -174 genotypes, we demonstrated that *H. pylori* positivity associates with low serum HDL only in the IL6 -174 CC genotype patients. There was a similar but non-significant trend in the GC genotype patients and no perceivable trend in the GG genotypes.

*H. pylori* infections and peptic ulcers have been associated with an increased IL6 expression (Furukawa *et al.* 1998, Tsai & Hsu 2010) and high IL6 serum levels have been associated with low HDL serum levels (Zuliani *et al.* 2007). IL6 has been documented as reducing the activity of lipoprotein lipase (Greenberg *et al.* 1992) and decreased lipoprotein activity lipase has been associated with a decrease in HDL (Tsutsumi 2003), which offers a possible mechanism for the interaction between IL6 and HDL.

Previous studies have shown, that exercise lowers IL6 serum levels only in IL6 -174 C allele carriers (Oberbach *et al.* 2008). Similarly, exercise and lifestyle interventions have been documented as increasing HDL levels significantly more in the IL6 -174 C allele carriers than in the G allele carriers (Curti *et al.* 2012, Halverstadt *et al.* 2005). In line with these studies, our results suggest that the HDL levels are more affected by *H. pylori* infections in the IL6 -174 allele C carriers than in the GG genotype. Based on these results and on the previously documented inverse association between IL6 and HDL concentrations (Zuliani *et al.* 2007), we hypothesize that the allele IL6 -174 C carriers demonstrate variable IL6 and HDL levels which are more dependent on external factors such as *H. pylori* infection, exercise or diet, and that allele G carriers demonstrate more constant and higher levels of IL6 and lower levels of HDL.

Although the effect of *H. pylori* on serum cholesterol has been well documented, it is still not clear if *H. pylori* is also a risk factor for coronary heart disease or other atherosclerotic diseases. There is epidemiological evidence and also a few eradication studies in support of the pathogenicity of *H. pylori* in

coronary heart disease, but the issue needs further study (Sharma & Aggarwal 2015).

Additionally, recent observations have suggested that improvements in diet by eating whole grain supplements may induce concurrent intestinal microbiota changes, decrease IL6 levels and may associate with changes indicating improvement of metabolic dysfunction (Martinez *et al.* 2013). These studies favor the role of IL6 in systemic responses caused by dietary changes and their associated changes in the gastrointestinal mucosal flora. Based on our current findings, which indicate that IL6 -174 polymorphisms modify systemic effects induced by changes of mucosal bacterial flora, we suggest that the role of IL6 polymorphisms should be investigated as a possible mechanism explaining associations between intestinal microbiome and cardiovascular disease risk factors.

#### **6.4 Limitations of the study**

The subject group sizes were fairly small, especially in the carcinoma groups. Thus, the results need to be reproduced in other studies. As many of the results seem to demonstrate risks of modest magnitude, the confirmatory studies should be performed with larger study populations where adequately sized groups of patients with gastric and duodenal ulcers can be formed. Additionally, the carcinoma groups should be adequately sized and always grouped histopathologically.

We did not have data on the use of NSAIDs on the patients and thus we cannot rule out the NSAID etiology of the ulcers. In the analyses, the ulcer group is restricted to *H. pylori* positive patients. Thus, the possible NSAID use is at most a cofactor in the ulcers and we do not suspect NSAID use to be a relevant confounding factor. As NSAID use and *H. pylori* infections are considered to be cumulative risk factors for gastric ulcers and gastric acid secretion is also a major factor in NSAID related ulcers, the interpretation of our results in relation to TLR4 and gastrin seems applicable also in situations where NSAID use is a cofactor with *H. pylori* related gastric ulcers.

We did not have data on the use of cholesterol reducing medicine, but we deem it unlikely that the use of statin medication would confound our results, because the prevalence of statin medication in Finland in 1996-2000 was very low (1.3–4.2% respectively) (Ruokoniemi *et al.* 2008). We also lack data on lifestyle factors which might influence serum cholesterol levels. As we did not have IL6 serum measurements of our patients, we can only hypothesize that the association between *H. pylori* infection and low HDL levels is transmitted through IL6. Thus we propose

a study with HDL and IL6 measurements with IL6 polymorphism analyses to clarify the issue.

## 7 Conclusions

The present studies enlighten the significance of the role of host immune system genetic polymorphisms in *H. pylori* related diseases. Based on the results, the following conclusions were made:

1. The TLR4 +896 and +1196 wild type homozygous patients associated with a risk of peptic ulcers. Because the same genotype patients also associated with higher G17 serum levels, and TLR4 expression was seen in G and D cells, we propose that TLR4 activation might stimulate gastrin and gastric acid secretion and thus function as a possible mechanism for this association.
2. The TLR4 +896 and +1196 genotypes did not associate with serum lipids.
3. The IL6 -174 allele G was associated with a risk of the diffuse type gastric adenocarcinoma.
4. The association between *H. pylori* and HDL serum levels was dependent on IL6 -174 polymorphism. *H. pylori* associated with lower HDL levels only in the IL6 -174 CC genotype patients, which suggests that the detrimental effect of an *H. pylori* infection on HDL levels is transmitted through IL6.



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## Original publications

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:

- I Pohjanen VM, Koivurova OP, Huhta H, Helminen O, Mäkinen JM, Karhukorpi JM, Joensuu T, Koistinen PO, Valtonen JM, Niemelä SE, Karttunen RA & Karttunen TJ (2015) Toll-like receptor 4 wild type homozygosity of polymorphisms +896 and +1196 is associated with high gastrin serum levels and peptic ulcer risk. *PLoS One* 10(7): e0131553.
- II Pohjanen VM, Koivurova OP, Mäkinen JM, Karhukorpi JM, Joensuu T, Koistinen PO, Valtonen JM, Niemelä SE, Karttunen RA & Karttunen TJ (2013) Interleukin 6 gene polymorphism -174 is associated with the diffuse type gastric carcinoma. *Genes Chromosomes Cancer* 52(10): 976–982.
- III Pohjanen VM, Koivurova OP, Niemelä SE, Karttunen RA & Karttunen TJ (2016) The role of *Helicobacter pylori* and interleukin 6 -174 gene polymorphism in dyslipidemia: a case-control study. *BMJ Open* 6(1): e009987.

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Original publications are not included in the electronic version of the dissertation.



1356. Ohukainen, Pauli (2016) Molecular profiling of calcific aortic valve disease
1357. Oikarinen, Anne (2016) Effects of risk factor targeted lifestyle counselling intervention on quality of lifestyle counselling and on adherence to lifestyle change in stroke patients
1358. Rannikko, Irina (2016) Change in cognitive performance and its predictors in general population and schizophrenia in early midlife : the Northern Finland Birth Cohort 1966 Study
1359. Lantto, Ilkka (2016) Acute Achilles tendon rupture : epidemiology and treatment
1360. Räsänen, Päivi (2016) Kotona asuvien ikääntyvien itsestä huolenpito : hoitotieteen keskitason teorian ydinrakenteen testaaminen
1361. Hannila, Ilkka (2016) T2 relaxation of articular cartilage : normal variation, repeatability and detection of patellar cartilage lesions
1362. Pihlaja, Juha (2016) Treatment outcome of zirconia single crowns and fixed dental prostheses
1363. Moilanen, Jani (2016) The use of antipsychotic medication and its association with outcomes and brain morphometry in schizophrenia – the Northern Finland Birth Cohort 1966 Study
1364. Heikkilä, Vesa-Pekka (2016) New techniques and methods for decreasing healthy tissue dose in prostate cancer radiotherapy, with special reference to rectal doses
1365. Aro, Jani (2016) Novel load-inducible factors in cardiac hypertrophy
1366. Myllymäki, Mikko (2016) Hypoxia-inducible factor prolyl 4-hydroxylase-2 in Tibetan high-altitude adaptation, extramedullary erythropoiesis and skeletal muscle ischemia
1367. Rubino, Antonino S. (2016) Efficacy of the Perceval sutureless aortic valve bioprosthesis in the treatment of aortic valve stenosis
1368. Krökki, Olga (2016) Multiple sclerosis in Northern Finland : Epidemiological characteristics and comorbidities
1369. Mosorin, Matti-Alexi (2016) Prognostic impact of preoperative and postoperative critical conditions on the outcome of coronary artery bypass surgery
1370. Pelkonen, Sari (2016) Frozen embryo transfer : Early pregnancy, perinatal outcomes, and the health of singleton children

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