GASTRIC ADENOCARCINOMA:

ASPECTS OF DEVELOPMENT AND PROGRESSION IN HIGH- AND LOW-RISK COUNTRIES

by:

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Preface

This study was carried out at the Gade Institute, University of Bergen between 2005 and 2009, in the frame of a collaboration between the University of Bergen, University of Costa Rica and Finsen Laboratory, Rigshospitalet, Copenhagen. This research was made possible through a four-year research grant from the Centre for International Health, University of Bergen. Perhaps, the best way I could summarize this period of time would be: "a time of cultural exchange and new experiences in three different countries, Norway, Costa Rica and Denmark".

My passion for science dates back to my childhood, when at 5 years old I used to tell my parents "… when I grow-up, I want to be scientist…" It was in the year 2000, when I read a scientific article published in *Nature*, reporting that polymorphisms of the IL-1B gene increased the risk of gastric cancer. That work gave rise to the idea that later on became my Master´s thesis. That article was given to me by the person who introduced me to gastric cancer biology, my *Mentor*, Professor Rafaela Sierra, a person to whom I am deeply thankful…

In 2004 Professor Rafaela Sierra told us that Professor Ole Didrik Laerum from Norway was visiting us in Costa Rica. His visit settled the basis of a new collaboration, which brought me to Norway, a country I had never thought I would go. Profesor Laerum gave me the great opportunity of coming and experiencing Norway. He introduced me to the field of molecular pathology. His impressive broad scientific knowledge and hard-working capacity were my inspiration. His always refreshing and encouraging attitude kept me positive and enthusiastic, even in stressful times. Thank you very much Professor Laerum, your wise advice has been invaluable!!

After some months of living in Norway, Professor Laerum urged me to visit Finsen Laboratory in Denmark, an institute where he had established a solid collaboration. There, I met Boye Schnack Nielsen, Martin Illemann and Leif R. Lund, Niels Behrendt and the former head, Keld Danø. From them, I learned important concepts of cancer biology, histology and immunohistochemistry. My gratitude is extended to all the personnel from the Finsen Laboratory, where I found friendliness and collaborative spirit.

I am especially thankful to all my coauthors and technicians in the laboratories from the three countries, who were crucial parts for achieving the results in this study. The Gade Institute, University of Bergen; the Health Research Institute, University of Costa Rica and Finsen Laboratory, Rigshospitalet, Copenhagen are thanked for excellent working facilities and economical support.

My deepest thanks to my family, Miguel Angel, Maria Elieth, Silvia, Karen and Fabián, who despite the long physical distance, have been close to my heart and mind at any moment. Finally, I thank all my friends for keeping on sending me positive wishes and especially to Veronika, my closest confidante…

Warner Alpízar Alpízar

Abbreviations used

APC	adenomatous poliposis coli gene
BabA	outer membrane protein BabA
CAG	chronic atrophic gastritis
CagA	cytotoxin-associated protein A
Cag-PAI	cytotoxin-associated pathogenicity island
CASP	caspase
CDH1	E-cadherin gene
CDX	caudal type homeobox
CEA	carcinoembrionic antigen
c-erbB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
c-met	hepatocyte growth factor receptor
COX	cyclooxygenase
DNA	deoxyribonucleic acid
EAI	enzyme immunosorbent assay
EBV	Epstein-Barr virus
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunosorbent assay
EPIC	European Prospective Investigation into Cancer and Nutrition
FGF	fibroblast growth factor
hTERT	human telomerase reverse transcriptase
IARC	International Agency for Research on Cancer
iceA	ulcer-associated gene restriction endonuclease (iceA)
IL-1Ra	interleukin-1 receptor antagonist
IL	interleukin
IM	intestinal metaplasia
INFG	interferon gamma
INFGR2	interferon gamma receptor 2
Lcn2	lipocalin two

LOH	loss of heterozygocity
MALT	mucosa-associated lymphoid tissue
MMP	matrix metalloproteinases
MUC	mucin
NF-κB	nuclear factor kappa-B, subunit 1
NGAL	neutrophil gelatinase-associated lipocalin
NOCs	*N*-nitroso compounds
PAI-1	plasminogen activator inhibitor one
PAI-2	plasminogen activator inhibitor two
PCR	polymerase chain reaction
RARβ	retinoic acid receptor beta
RFLP	restriction fragment length polymorphism
RUNX3	runt-related transcription factor 3
Scn	sideroccalin
SNP	single nucleotide polymorphism
TFF1	trefoil factor 1
TGF	tumor growth factor
TIMP	tissue inhibitor of metalloproteinase
TNF-α	tumor necrosis factor alpha
tPA	tissue-type plasminogen activator
uPA	urokinase-type plasminogen activator
uPAR	urokinase plasminogen activator receptor
VacA	vacuolating cytotoxin A
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VNTR	variable number of tandem repeats
WHO	World Health Organization

Background

INTRODUCTION

Cancer disease, in general, is considered as one of the world's major public health problems. In the year 2002, the different cancer sites accounted for approximately 10,9 million new cases and 6,7 million deaths globally.[1] Significant efforts are being made to elucidate risk factors and aetiological aspects underlying the disease, and to identify potential prognostic and diagnostic predictors that can be translated into prevention, early detection and cure of the malignancy. This, however, has been a difficult task due to the multifactorial causality and complexity of this group of diseases. Understanding of cancer thus represents one of the major challenges for the scientific community in the present century. Some types of cancer have greater impact on public health as they present higher incidence and mortality, among them gastric cancer. In the general introduction of this thesis, I present an overview of the current knowledge of gastric cancer, addressing epidemiological, clinico-pathological and biological aspects. Moreover, I give a general survey about mechanisms of extracellular matrix and tissue remodelling in neoplasia. Finally, I introduce the novel protein, NGAL, which I have studied during the course of my PhD studies as a molecule seemingly linked to inflammation, and cancer development and progression.

GASTRIC CANCER WORLDWIDE

- Incidence

Gastric cancer is the fourth most frequent type of cancer worldwide, preceded by lung, breast and colorectal cancers.[1] The incidence rates of this disease present considerable variation according to age, gender, socio-economical conditions and geographical location.[2, 3] Thus, most of the gastric cancer patients are older than 50 years at diagnosis [4], and the global incidence is twice as much in men as in women.[2] The most substantial variations in the incidence rates of this malignancy are, however, observed in relation to geographical regions. In general, the incidence of gastric cancer is high in East Asia, Eastern Europe, and parts of Central and South America, and low in Southern Asia, North and East Africa, Western and Northern Europe, North America and Australia (Figure 1).[5] Although nearly two thirds of the cases of this malignancy occur in developing countries, it cannot readily be categorized as a disease of less developed economies.[3, 6] The distribution of gastric cancer worldwide does not suggest any geographical pattern, and despite some of the highest risk populations are in Asian countries such as Japan, Korea and China, other Asian countries present relatively low rates. [4]

- Time trends in incidence

The incidence rates for gastric cancer have undergone a steady general decline during the past decades.[1, 5] This downward trend is equally observed among both sexes and in high- and low-risk areas, but has been more pronounced in developed countries (Figure 2).[3, 7] Interestingly, the fall in the incidence is particularly associated to non-cardia gastric carcinoma, in contrast to cardia cancer that seems to experience a permanent slight increase.[4, 8] Similarly, epidemiological studies have shown that the general decrease in incidence is mainly attributed to the fall in intestinal subtype of gastric cancer,

while the diffuse subtype shows a rather small change.[2] The reasons underlying the generalized decline in the incidence of this malignancy are not well understood, however it has been hypothesized that this may be associated to improvements in the storage and preservation of foods, better nutrition and reduced transmission of *H. pylori* in childhood.[1, 5]

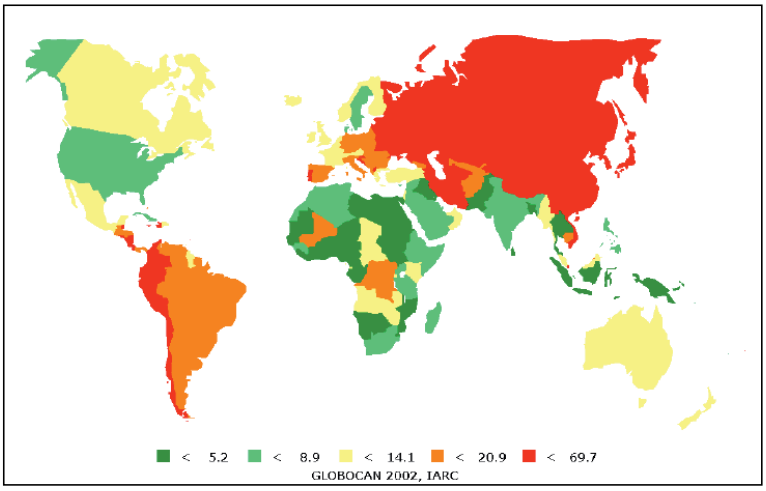

Figure 1. Global representation of the incidence rates of gastric cancer in males (age-standardized incidence per 100 000). From Globocan 2002 (available at: http://www-dep.iarc.fr/).

Despite the notable fall in the incidence rates, the absolute number of cases of gastric cancer continues to increase globally as a result of the population growth and ageing.[1, 3] In the year 1980 gastric cancer was the most common type of cancer globally, with approximately 669400 new cases diagnosed, representing 10,5% of the cancer burden.[9] Ten years later, in 1990, approximately 798000 new cases occurred.[10] For the year 2000, the number of new cases of gastric cancer reached 876000.[2] In

2002, the number of new cases was estimated to be 934 000, which meant 8,6% of the total number of cancer cases.[1] For 2010, the number of new cases of gastric cancer is expected to be 1,1 million.[1]

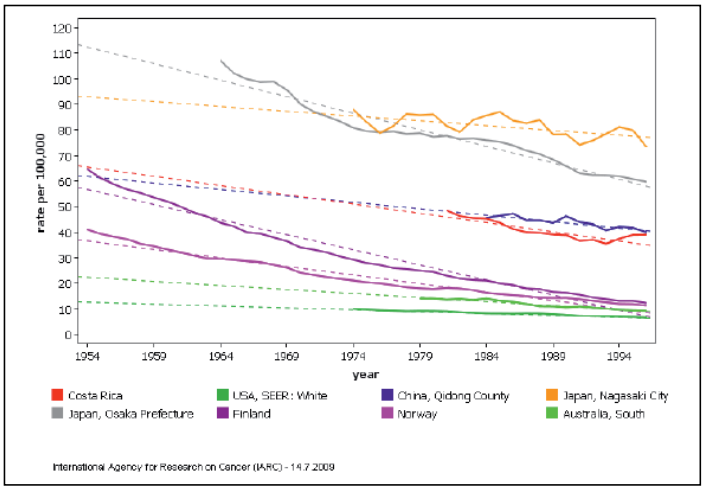

Figure 2. Time trends in the incidence rates of gastric cancer for males in selected countries. From IARC/WHO database (available at: http://www-dep.iarc.fr/).

- Mortality and survival

Gastric cancer is the second most common cause of death from cancer worldwide after lung cancer, accounting for nearly 700000 deaths in 2002.[1] Wide geographical variation in mortality rates exists throughout the world, being particularly high in the developing world.[5] Similar to the incidence, a constant decline in mortality rates in both sexes, and in low- and high-risk countries has occurred in the last decades.[5] The decline in mortality, however, seems to occur faster than with the incidence, and is particularly pronounced in certain populations/countries (Figure 3).[3, 11]

Mortality rates are notably high because, in most cases, the disease is diagnosed at advanced stages when the treatment is likely to fail. In general, the five-year survival for patients of gastric cancer is below 30% in most countries, despite some variations according to the country/geographical region.[3] It is noteworthy, however, the relatively high 5-year survival rates of gastric cancer in Japan, which have reached more than 50% in the last decades.[1] This is thought to be associated with the implementation of X-ray (photofluorography) based gastric cancer mass screening programs since early in 1960´s.[12, 13] Similar experience with X-ray based mass screening interventions in other high-risk countries have demonstrated a significant impact of early detection in the mortality of gastric cancer.[14] Nevertheless, studies in population groups with same ethnic background but dissimilar access to health care suggest that environmental and biological factors may also play an important role in explaining differences in mortality and survival of gastric cancer between high and low risk countries or developing versus developed economies [15].

HISTOLOGICAL AND ANATOMICAL CLASSIFICATION OF GASTRIC CANCER

Several classification systems have been proposed to aid the description of gastric cancer on the basis of macroscopic or histological features, which include Borrman, Japanese system, World Health Organization (WHO) system and Laurén.[16, 17] The Laurén classification system is most commonly used and describes the tumors in relation to microscopic configuration and growth pattern.[18] According to the Laurén system, gastric cancer is divided into intestinal and diffuse histological subtypes.[19] These two subtypes present marked differences in pathology, epidemiology, etiology and biological behavior.[16]

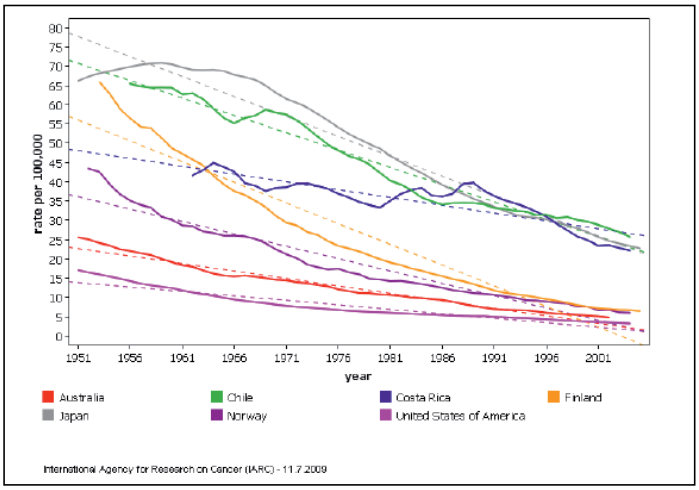

Figure 3. Time trends in the mortality rates of gastric cancer for males in selected countries. From IARC/WHO database (available at: http://www-dep.iarc.fr/).

Intestinal subtype gastric cancer is the most frequent globally and is particularly common in geographical regions with high-risk of the malignancy.[2] Intestinal subtype tumors are often localized in the lower part of the stomach (antrum), and are characterized by having well defined glandular formation, similar to the microscopic appearance of colonic mucosa.[18, 20] The development of intestinal subtype gastric cancer follows a stepwise sequence of precursor lesions starting with superficial gastritis, continuing through chronic atrophic gastritis, intestinal metaplasia, dysplasia to, ultimately, overt gastric cancer (this carcinogenic process is described below).[21] For unknown reasons, the multistep process often does not lead to neoplasia, as it stops at one of the stages and undergoes regression.[22, 23] It is hypothesized, however, that "a point of no return" exists where the process cannot be reverted. The etiology of intestinal subtype gastric cancer is mainly associated to environmental factors, the tumor frequently develops late in life (after 50 years of age), and is twice more common in

males than females.[24]

Diffuse subtype gastric cancer more commonly develops in the corpus of the stomach and is characterized by the lack of gland formation and cellular adhesion, with single/small clusters of neoplastic cells diffusely infiltrating the stroma of the stomach wall. No recognizable pre-neoplastic lesions have been observed during the development of diffuse cancers.[18, 20] Diffuse subtype tumors are associated with genetic predisposition, and presumably arise out of single-cell mutations in normal gastric glands.[25, 26] The diffuse subtype has a relatively constant or even slightly increase in incidence rates, more often occurs in young individuals, presents a similar prevalence in males and females, and is associated with a worse prognosis than the intestinal subtype.[24, 27]

The anatomical location of tumors in the stomach has also been considered as an important parameter for the classification of gastric cancer.[28] On the basis of anatomical location two subtypes of gastric cancer can be distinguished: tumors from the distal regions of the stomach (non-cardia cancer) and those arising at the most proximal part of this organ (cardia cancer).[28] These two anatomical subtypes of tumors present remarkable etiological differences. Non-cardia cancer is generally thought to develop as a result of the interaction between environmental, host and *H. pylori* factors (discussed below). In contrast, two distinct etiological mechanisms have been proposed for cardia gastric cancer. One is associated with atrophic gastritis and resembles the development of non-cardia malignancies. The second arises in similar fashion to esophageal carcinomas, as a result of frequent refluxing of acidic gastric juice into the distal esophageal mucosa, which leads to the transformation from squamous to columnar metaplastic epithelium to, ultimately, overt cancer.[28-30] Epidemiological dissimilarities also exist between these two anatomical subtypes of gastric tumors. Non-cardia gastric cancer accounts for the majority of the cases worldwide and is the predominant type in high-risk areas. In contrast, cardia cancer is more homogeneously distributed all over the world and its incidence tends to increase.[5, 28]

RISK FACTORS FOR GASTRIC CANCER

Several parameters have been suggested as risk factors for gastric cancer, which by establishing complex interactions may ultimately lead to development of this malignancy. Among the most recognized gastric cancer risk factors are dietary and nutritional aspects, genetic predisposition and sporadically-occurring mutations, and *Helicobacter pylori* infection.[16, 31] More recently, aspects related to the inflammatory response against the bacterial infection have emerged as important determinants for the risk of this malignancy.[32, 33]

- Dietary and nutritional aspects

Diet plays a dual role in gastric cancer etiology, providing a number of elements and vitamins that reduce the formation of carcinogens, but also as the source of well established carcinogenic molecules or precursors of them. Evidence indicates that diets high in fruits and vegetables may protect against gastric cancer while salted foods, consumption of processed foods and inappropriate preservation and storage of aliments could increase the risk of this malignancy. [34, 35]

The association between fruit and vegetable consumption and the risk of gastric cancer has been substantially evaluated. In general, epidemiological studies suggest an inverse association between the intake of fruits and vegetables and gastric cancer, which seems to be more pronounced in the case of citrus fruits and raw allium vegetables.[35-37] It has also been suggested that fruits may have stronger potential than vegetables to protect against gastric cancer development.[38, 39] These associations, however, differ according to anatomical and histological subtypes of gastric malignancies, sex and lifestyle behaviors (e.g. smoking and alcohol consumption).[36, 39] On the basis of the existent evidence, the International Agency for Research on Cancer (IARC/WHO) has considered that high intake of

fruits “probably” and high intake of vegetables “possibly” reduce the risk of gastric cancer.[40] Still, it remains unknown which constituents in fruit and vegetables specifically protect against the development of this malignancy.

Epidemiological studies have evaluated the association between specific antioxidant nutrients known to reduce the formation of carcinogenic molecules and the risk of gastric cancer. In general, antioxidant molecules such as lycopene, vitamin C and selenium seem to reduce the risk of gastric cancer.[38, 39, 41] In contrast, the association between β-carotene, vitamin A and vitamin E and gastric cancer is more controversial.[38, 41] As in the case of fruits and vegetables, the potential significance of antioxidant nutrients as protector factors varies substantially depending on the anatomical and histological subtype of gastric cancer, sex, lifestyle behaviors and interactions between antioxidant molecules.[39, 41]

Diets high in salt and preserved meats have been suggested to play a role in the etiology of gastric cancer.[21, 42] Salt may act as an irritant of the stomach wall and in connection to *H. pylori* infection may contribute to the damage of the mucosal layer, enhancing thus the susceptibility of epithelial cells to carcinogenic molecules that accumulate in this organ. Meat products like bacon, sausage, salami and ham are often rich in salt, nitrite, nitrosamines, and can also be the source of *N*-nitroso compounds, of established carcinogenic properties.[38, 42] A number of case-control and cohort studies have found that high uptake of salt and salted foods, and high consumption of red and processed meats are associated with higher risk of gastric cancer.[43-46] Nevertheless, these associations are not fully consistent and, therefore, the evidence is still inconclusive.[38, 46]

- Genetic predisposition and sporadically-occurring mutations

Genetic aspects play a fundamental role for the development and progression of gastric cancer. It is well established that a number of inherited germ-line mutations and genetic syndromes predispose to the development of this malignancy.[47, 48] Likewise, a diverse set of genetic and epigenetic *de novo* alterations are often found in gastric cancer, which probably occur at different stages during the development of the malignancy, and differ according to the histological subtype of the disease.[47, 49]

Familial aggregation of gastric cancer is observed in approximately 10% of the cases, in which two or more relatives from the same family are affected.[50] In general, the risk for developing gastric neoplasia among relatives of gastric cancer patients is estimated to be 2-3-fold higher than in persons with no familiar background of the disease. This, however, should be cautiously analyzed due to the fact that, besides the common genetic background, environmental and cultural factors (e.g. *H. pylori*, diet, lifestyle behaviors) may be similarly shared among the family members and in some cases are difficult to differentiate.[51] Nevertheless, the genetic susceptibility to develop this malignancy has been clearly established in a fraction of these familial-clustered gastric cancers.

Germ-line mutations of the E-cadherin gene (*CDH1*) are the most recognized genetic aberrations found in hereditary gastric cancer, accounting for approximately 1-3% of the cases.[26, 50] E-cadherin is a protein predominantly expressed in epithelial cells and exerts cell-cell adhesion and invasion suppression functions.[52] *CDH1*-associated familial gastric cancer follows an autosomal dominant pattern of inheritance, with more than 70% penetrance, and is caused by several alterations in the *CDH1* gene, mainly truncating mutations.[25, 53] Most of the gastric cancer cases attributed to *CDH1* aberrations are of diffuse subtype, particularly signed-ring cell adenocarcinomas, and are predominantly observed in young individuals.[25, 26, 50]

A considerable number of genetic and epigenetic alterations have been identified both in pre-neoplastic lesions leading to gastric cancer and neoplasia itself. These spontaneously occurring events can trigger aberrant effects at several molecular levels, including reactivation of telomerase, activation of oncogenes, inactivation of tumor suppressor genes, over-expression of growth factors and cytokines, altered expression of cell-cycle regulators and DNA-repairing enzymes, and increased microsatellite instability.[47, 54] Table 1 summarizes some of the genes that are aberrantly expressed in gastric pre-neoplastic and neoplastic lesions. It is worth noting that genetic and epigenetic events may alter the expression of known oncogenes (*c-met, K-sam, c-erbB2, K-ras*), tumor suppressor genes (*APC, p53*), DNA-repairing enzymes (*hMLH1*) and cell-adhesion molecules (E-caherin, β-catenin, γ-catenin) that are central for the cellular homeostasis. In addition, they are molecules that have been consistently linked with the development and progression of other types of cancer. These gene dysregulations are likely to occur during the course of the multistep gastric carcinogenesis as result of replication errors, mutations, gene amplifications, defective DNA-repair, aberrant metilation, loss of heterozygosity (LOH), or a combination of two alterations.[49]

Table I- Genes commonly altered in gastric pre-neoplastic lesions and gastric cancer *

Type of alteration	Gastric cancer	Pre-neoplastic lesions
Oncogene activation	*c-met, K-sam, c-erbB2 (HER2/NEU),* EGFR (HER1), *K-ras, bcl-2*	*c-met, K-ras,* EGFR (HER1)
Tumor suppressor inactivation	*APC, p53, p16, p73, pS2, RARβ, bcl-2, RUNX3, TFF1*	*APC, p53, RARβ, pS2, RUNX3, TFF1*
Growth factor and cytokines aberrant expression	EGF family, IL-1α, IL-6, IL-8, TGFβ, TGFβ receptor II, VEGF, bFGF	COX-2, TGFα
Cell-cycle regulators altered expression	Cyclin E, p27, E2F	Cyclin E, cyclin D2, p27
Microsatellite and chromosomal instability	DS191 locus, 17q21 LOH, 17q LOH, hTERT upregulation, DNA-repair errors	DS191 locus, D17S5 locus, hTERT upregulation,DNA-repair errors
DNA-repairing enzyme	*hMLH1* inactivation	*hMLH1* inactivation
Others	Histone H4 deacetylation, *CDH1*, β-catenin (CTNNB1), γ-catenin, CD44, VEGF, VEGFR, CASP10	*CDH1*, CDX-1, CDX-2, MUC2, MUC5AC

* : The information given on the table is based on Smith 2006, Vauhkonen 2006, Ebert 2002, Hamilton 2006, Lynch 2005, Yasui 2001, Werner 2001, Zhang 2008[20, 47-49, 54-57]

- *Helicobacter pylori* infection

Helicobacter pylori, formerly called *Campilobacter pyloridis*, is a gram-negative, spiral shaped bacterium, with a number of adaptations to colonize and inhabit in the acidic environment of the

stomach.[58-60] This microorganism was isolated from gastric biopsies of patients with gastritis and peptic ulcer disease, and first cultured in 1982 by Barry Marshall and Robin Warren.[61] In 1994, the International Agency for Research in Cancer (IARC-WHO) classified *H. pylori* as a type I carcinogenic agent in humans.[62] Currently, *H. pylori* is a clearly established risk factor for developing gastric cancer. In recognition of their discovery, Marshall and Warren were awarded the Nobel Prize in 2005.[63]

H. pylori causes one of the most extended chronic infections worldwide, infecting approximately one half of the world's population. In general, its prevalence is higher in developing countries, people of advanced age and groups of low socio-economical levels.[64, 65] The infection often occurs during childhood [66, 67], and family members are the main nucleus of transmission.[67, 68] The mode of spreading is not entirely clear, however, it is thought to occur in a person-to-person manner, via fecal/oral or gastric/oral routes.[69] In most cases infected people do not experience any symptoms, however those who are infected present a 2-5fold increased risk of developing gastric cancer than non-infected individuals [70]. In addition, the risk is higher for individuals who acquire the infection earlier in life.[71] *H. pylori* infection is associated with the risk of both intestinal and diffuse histological subtypes of gastric cancer.[72] This bacterial infection is also associated with the risk of gastric MALT lymphomas. In fact, it has been shown that *H. pylori* eradication can induce regression of gastric MALT lymphomas in 70 to 80% of cases.[64, 71]

Several *H. pylori* strains have been characterized and some of them are particularly associated with the risk of gastric cancer, including those carrying the virulence factors VacA and CagA.[73, 74] VacA is a cytotoxic protein, coded by *vacA* gene, which induces vacuole formation in epithelial cells of the gastric mucosa that results in impairment of these cells. Other intracellular effects in gastric mucosa have also been unraveled for VacA, including mitochondrial damage and cytochrome c release that

leads induction of apoptosis, inhibition of T-lymphocyte activation and activation of cell-signaling pathways.[75, 76] All *H. pylori* strains produce this protein, however it is differentially expressed depending on the genotype of polymorphisms in specific sites of the *vacA* gene, namely signal (*s*) and mid (*m*) regions, as well as in the recently described intermediate (*i*) region.[75, 77] Particular haplotypes of these three polymorphic sites are associated with increased risk of gastric cancer.[77]

H. pylori CagA-positive strains express the protein CagA, coded by the *cagA* gene. This gene is part of a genomic region called Cag Pathogenicity Island (PAI-Cag), which is only found in approximately 60% of the strains. PAI-Cag positive strains possess a Type IV secretion system that resembles a needle and injects the CagA protein into the gastric epithelial cells. Once translocated to the cytoplasm, CagA can be phosphorylated leading to a number of cellular outcomes, including rearrangements of the cytoskeleton, induction of inflammatory mediators and induction of proliferative and oncogenic proteins.[78, 79] The phosphorylation of CagA takes place at specific sites of the C-terminal region of the protein known as EPIYA motifs, which can vary in number. Slight variations inside EPIYA motifs also exist, which result in four different subtypes: A, B, C and D.[80] An increasing number of EPIYA-C motifs translates into more phosphorylation sites, thus enhancing the CagA-induced cellular effects. This is suggested to increase considerably the risk of gastric cancer.[81]

Although *vacA* and *cagA* genes have no physical or functional relation they are often coexpressed. Interestingly, VacA/CagA-positive strains are more prevalent in countries with high-risk of gastric cancer [82], and clinical and epidemiological studies have shown that individuals infected with strains carrying certain genotypic combinations of those two genes have the highest risk of developing gastric cancer.[74, 77, 81]

Other less characterized *H. pylori* virulence factors have also been linked with gastric cancer development.[83] Some strains express the protein BabA, an outer-membrane protein that binds the Lewis B blood group antigen in gastric epithelial cells. Accumulating evidence suggest that BabA-expressing strains adhere more tightly to these cells, which may influence the disease severity.[83] Similarly, a virulence factor called *iceA*, which is upregulated by direct contact with gastric epithelial cells has also been described. Two distinct variants exist, *iceA1* and *iceA2,* and the first one is associated with peptic ulcer disease in some populations.[83]

The underlying pathogenic mechanisms of *H. pylori* in gastric cancer development are discussed below. Nevertheless, the specific mechanisms by which the *H. pylori* virulence factors interfere with the physiological and molecular processes of the host are far from being clear.

- Other risk factors

In the past, studies assessing the relation between tobacco smoking and gastric cancer found a positive association between these parameters.[84] More recently, a large prospective investigation conducted in European countries (EPIC study) found a causative relation between smoking and gastric cancer, with particularly higher risk of cardia than distal gastric malignancy.[85] Accordingly, one of the most recent meta-analyses reported a causal association between smoking and gastric cancer.[86] Thus, the existent evidence suggests tobacco smoking as a behavioral risk factor for gastric cancer.

Humans are exposed to *N*-nitroso compounds (NOCs) from diet, tobacco smoke and other environmental sources, as well as from endogenous synthesis.[87] Several of these nitrogen-derived molecules have proven to be carcinogenic.[87] It has been shown that NOCs can be formed

intragastrically in *H. pylori*-infected individuals.[88] The relation between NOCs and the risk of gastric cancer was the focus of intense investigation in past decades, and some studies have associated them with increased risk of gastric cancer.[46, 89] Other studies, however, have found no association between NOCs and gastric malignancy.[46, 90] Interestingly, endogenous-synthesized NOCs may confer enhanced risk of gastric cancer, while dietary NOCs may not and this relation can also be dependent on *H. pylori* status and vitamin C levels in plasma.[89, 90] Thus, the causal relation between NOCs and gastric cancer still remains unclear.

Epstein-Barr virus (EBV) is a ubiquitous virus with carcinogenic properties, which has been linked to the development of several malignancies.[91] A considerable number of studies have suggested the association between EBV and gastric cancer.[92, 93] Nonetheless, the clinico-pathological significance of EBV in gastric carcinogenesis remains controversial, and the pathogenic mechanisms are far from being clear.[91, 92] In general, EBV has been related to adenocarcinomas that develop in cardia and body of the stomach, diffuse subtype cancers, and the degree of association substantially varies according to ethnicity.[93]

- **The inflammatory response against *H. pylori* infection**

H. pylori infection triggers a chronic inflammation that is characterized by the recruitment of several types of immune cells in the gastric mucosa, including neutrophils, macrophages, dendritic cells and lymphocytes.[94, 95] This response is driven by a broad group of mediators of inflammation that are secreted by infiltrating immune and gastric epithelial cells. One of the central mediators of the *H. pylori*-induced inflammation is the transcription factor NF-κB, which becomes activated in response to stimuli triggered by the bacteria, leading to the induction of cytokines and other inflammatory

mediators.[54, 96] NF-κB has been consistently linked to the development of cancer.[97] As part of the host response against *H. pylori*, mutagenic molecules such as reactive nitrogen- and oxygen-derived species are released to the gastric mucosa. These molecules can exert oncogenic effects, including DNA and protein damage and inhibition of apoptosis.[54, 98] A third group of inflammatory mediators induced by this bacterial infection are pro-inflammatory, chemotactic and immunoregulatory cytokines, which can potentiate cancer development and progression in several ways. [95]

H. pylori infection predominantly induces a Th-1polarized immune response, which results ineffective in clearing the bacterial infection. The magnitude of this inflammatory response is greatly influenced by bacterial and host genetic factors.[95] In absence of treatment, the infection persists lifelong in the host and the inflammation prolongs.[94, 99] Hence, an excessive and prolonged secretion of mediators of inflammation into the gastric mucosa may have profound outcomes to the gastric physiology, which result in increased risk of gastric cancer.[54]

Functional polymorphisms in genes encoding key cytokines of the host response against *H. pylori* can increase their expression levels, which potentiate the inflammatory response. The pro-inflammatory cytokines interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNFα) are well established mediators of the inflammatory response induced by *H. pylori*.[94] Both of them are potent inhibitors of gastric acid secretion, and in particular IL-1β.[100] Polymorphisms of the genes IL-1B, IL-RN and TNF-A, encoding IL-1β, the antagonist receptor of IL-1(IL-1Ra) and TNF-α, respectively, have been associated with higher risk of gastric cancer and some gastric pre-neoplastic lesions.[33, 101-104] Enhanced expression of IL-1β and TNF-α, and down-regulation of IL-1Ra may have important outcomes for both the gastric physiology and host response against *H. pylori,* which then increase the risk of gastric cancer. It has also been shown that carriers of certain genotypes on IL-1B, IL-RN

polymorphisms when infected with specific *H. pylori* strains may present an 87-fold higher risk of gastric cancer.[105]

Interleukin-8 (IL-8) is one of the central mediators of the host response against *H. pylori*. [94] This chemokine plays an important role in the migration and activation of lymphocytes and neutrophils, thus amplifying the inflammatory response.[106, 107] Pro-angiogenic properties have also been revealed for IL-8.[108, 109] Several single nucleotide polymorphisms (SNPs) found in the gene are thought to increase IL-8 levels in blood, and one of these SNPs has been associated with an increased risk of gastric cancer, atrophic gastritis and gastric ulcer disease.[110-112] Overexpression of IL-8 may not only enhance the inflammatory response against *H. pylori*, but also promote tumor progression due to its pro-angiogenic potential.

Interlukin-10 (IL-10) is an immunoregulatory cytokine that modulates the inflammatory response by down-regulating the expression of pro-inflammatory cytokines, including IL-1β and TNFα.[113] Several polymorphisms located on the promoter region the IL-10 gene confer differential expression of this protein, and some of them have also been associated with higher risk of gastric cancer and gastric precancerous lesions.[114-116] Interestingly, the combination of genotypes on polymorphisms of the IL-1B, TNF-A and IL-10 genes increases the risk of developing gastric cancer by 27-fold.[114] Down-regulated expression of IL-10 may result in poor control over the production of pro-inflammatory cytokines. This leads to an excessive inflammatory response, which is associated with higher risk to develop this malignancy.

Other cytokines have been less consistently linked to the risk of developing gastric cancer and gastric precancerous lesions, including interleukins 2, 4, 6, 12 (IL-2, IL-4, IL-6, IL-12), interferon-γ and its receptor (IFNG and INFGR2, respectively).[117, 118] These are important mediators of immune

responses against infections and act in concert with the above-mentioned cytokines to trigger inflammation.

PATHOGENESIS OF GASTRIC CANCER

The pathogenesis of gastric cancer is a complex and multifactorial process, which is a classical example of gene-environment interactions. The precise mechanisms underlying gastric carcinogenesis are not yet fully understood and vary according to the histological type of the malignancy (Figure 4).

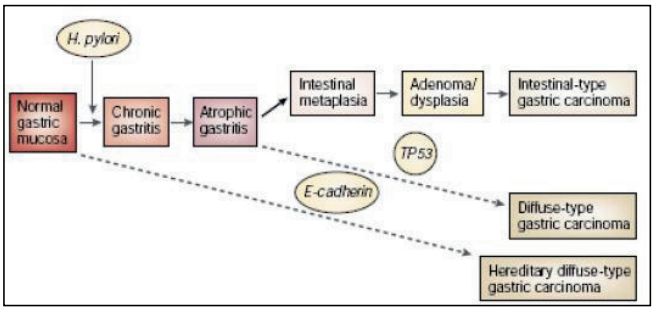

Figure 4. Model of gastric carcinogenesis according to histological subtypes. Adapted from Yuasa.[119]

There are three major driving forces in the etiology of gastric cancer: 1) environmental factors, including dietary aspects and lifestyle behaviors, 2) *H. pylori* infection and 3) host genetic factors.[120, 121] The combination of environmental insults and *H. pylori* infection leads to the development of superficial gastritis that subsequently becomes a chronic inflammation (chronic non-atrophic

gastritis).[21, 121] Intriguingly, most of the infected individuals will never develop any pathology (Figure 5). Thus, the factors determining who develops gastric cancer remain largely unknown, although this is hypothesized to be related to *H. pylori* strain differences, host genetic factors underlying the inflammatory response, and the extent and anatomical location of the chronic gastritis.[64, 119]

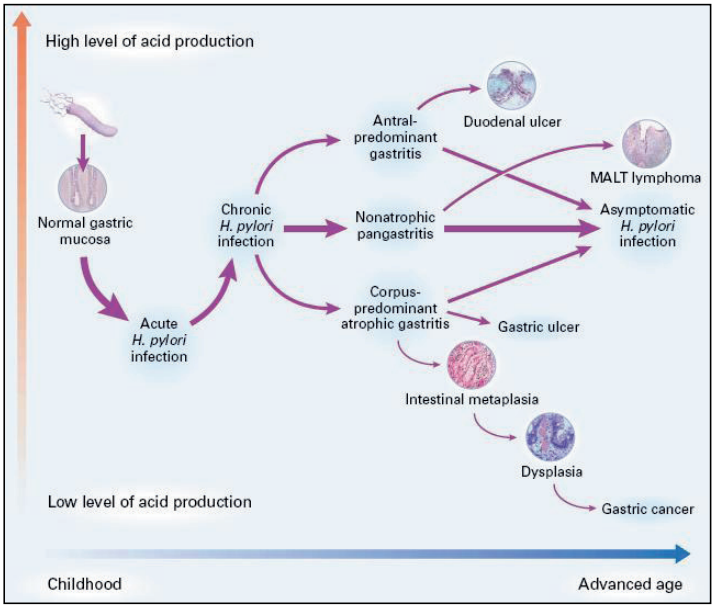

Figure 5. Gastric clinico-pathological outcomes associated to *H. pylori* infection. From: Suerbaum and Michetti.[64]

A very severe and persistent inflammatory response will result in chronic atrophic gastritis (CAG). CAG is characterized by the focal loss of glands and specialized cells of the gastric mucosa [21, 121], and is the hallmark in the development of both intestinal and diffuse histological subtypes of gastric

cancer (Figure 4). CAG commonly begins in the lower part of the stomach (antrum) and subsequently extends upwards to the body of the stomach (corpus), leading to the reduction in gastric acid secretion (hypochlorhydria).[120] The less acidic stomach is more favorable for *H. pylori* growth, but also allows the colonization by other microorganisms.[122] Bacterial overgrowth and inflammation may, therefore, lead to the accumulation of carcinogenic molecules in the stomach, increasing the probability of cellular and genomic alterations in gastric epithelial cells.

The subsequent step in the gastric precancerous process leading to intestinal type of gastric cancer is the transformation of the stomach mucosa into an intestinal-like epithelium, known as intestinal metaplasia (IM).[21, 121] A number of histological, physiological and molecular changes occur during this transformation, including the appearance of mucin-filled goblet cells.[121, 123] These changes are not well tolerated by *H. pylori* and often results in its spontaneous disappearance from the transformed epithelium.[124, 125] The further progression through the multistep cascade leads to dysplasia, which is characterized by several histological alterations of the epithelium, including nuclear atypia, irregular shape and loss of polarity of the epithelial cells. In advanced stages of dysplasia, these atypical cells may start migrating through the basal membrane, as early invasive carcinomas.[21, 121]

EXTRACELLULAR MATRIX DEGRADATION AND TISSUE REMODELING IN CANCER

Among the key events in cancer development and progression are the spread of malignant cells from its primary site to the surrounding stroma (invasion) and, in advanced stages, to distant locations (metastasis). Invasion and metastasis are thought to be the final of six fundamental alterations in cell physiology underlying malignant transformation and growth.[126] The invasion and metastasis of neoplastic cells occur in a sequence of interrelated steps, which include detachment from its primary

tumor, passing through the surrounding tissue and entering into circulation by penetrating lymphatic and/or vascular vessels. When reaching distant organs, metastasizing cells must be able to settle and grow in the new microenvironment of the host tissue.[127] It is well recognized that invasion and metastasis are associated with degradation of the extracellular matrix (ECM). This EMC breakdown mainly involves two families of extracellular proteases: matrix metalloproteinases (MMPs) and the plasminogen activation system.[128, 129]

- The matrix metalloproteinases

The MMPs encompass a family of at least 23 zinc-dependent endopeptidases, divided into eight distinct structural subclasses, which are capable of virtually cleaving all components of the ECM. MMPs are synthesized as inactive zymogens (pro-MMPs) by several types of cells, and are activated upon reaching the extracellular space by other MMPs or serine proteases, including plasmin.[129, 130] MMPs participate in a number of remodeling processes, in normal and pathological conditions, including carcinogenesis.[129, 131] Their proteolytic activity is mainly regulated by four endogenous tissue inhibitors of metalloproteinases (TIMPs 1-4).[131]

Some MMPs have been linked to key events in cancer development, including neoplastic cell growth, apoptosis, angiogenesis and metastasis.[129] Increased levels of MMPs in body fluids of patients with cancer have been associated with certain clinico-pathological outcomes such as advanced tumor stage and poor prognosis in several types of malignancies, including gastric cancer.[129, 130, 132, 133] The attributed role of MMPs in cancer development and progression is mainly related to their proteolytic activity, which facilitates the migration of malignant cells. Given that cells have receptors for structural ECM components, cleavage of ECM proteins by MMPs also generates fragments that can activate cell-

signaling pathways, leading to enhanced tumor cell migration. In addition, MMPs mediate the release of cell-membrane-bound precursor forms of growth factors, including TGF-α and FGF-2 and thus promoting neoplastic progression.[129] It is noteworthy that not all the MMPs contribute to tumor progression, as some of them (e.g. MMP-8, MMP-12 and MMP26) seemingly inhibit tumor growth and malignant transformation.[134-137]

- The plasminogen activation system

This is a proteolytic cascade system leading to the activation of plasminogen. The activated form of this zymogen is the serine protease plasmin, which can degrade several ECM proteins, including fibrin, fibronectin, laminins and vitronectin.[138] Plasmin is also a very efficient enzyme degrading fibrin deposits. Other substrates for plasmin include latent transforming growth factor-β (TGF-β), pro-uPA and some pro-forms of MMPs.[139, 140]

Plasminogen is activated after proteolytic cleavage, by either of the two well characterized serine proteases, urokinase-type and tissue-type plasminogen activators (uPA and tPA, respectively). uPA plays a crucial role in tissue remodeling, while tPA is particularly important in generating plasmin for vascular fibrinolysis.[141-143] Plasma kallikrein was recently unraveled as a third activator of plasminogen.[144, 145]

uPA is responsible for generating active plasmin on cell surfaces and is, therefore, a central mediator in the degradation of ECM during both normal tissue remodeling and cancer invasion.[146] Initiation of plasminogen activation follows the binding of the inactive form of uPA (pro-uPA) with high affinity to the glycolipid-anchored uPA receptor (PAR), then triggering its activation (Figure 6).[147]

Active uPA, subsequently, catalyzes the conversion of plasminogen into plasmin. In a positive feedback amplification step, plasmin converts the receptor-bound pro-uPA to active uPA, which accelerates plasminogen activation.[147] The activity of tPA and uPA is regulated by two naturally occurring inhibitors (PAI-1and -2), being PAI-1 the primary physiological regulator of plasminogen activation in the ECM.[143]

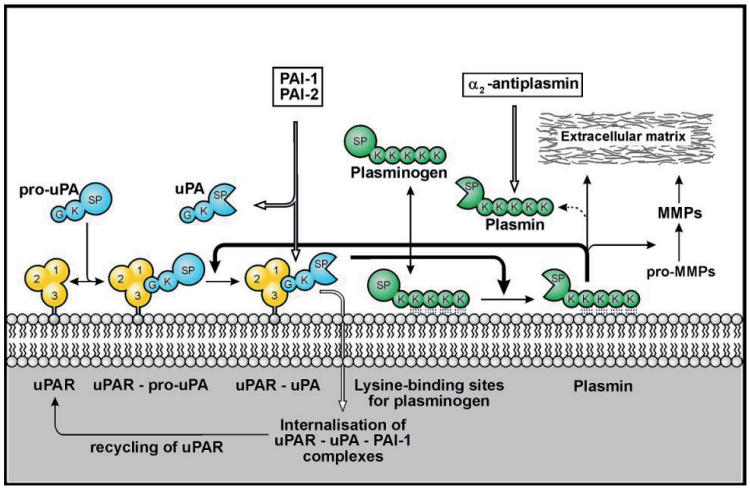

Figure 6. Pericellular proteolytic cascade that leads to the activation of plasminogen. From Ploug 2003.[147]

The plasminogen activation system has been consistently associated with cancer development and progression. Several studies have shown that plasminogen is involved in tumor growth, vacularizarion and metastasis.[148-150] Likewise, the components of the system directly involved in the activation of plasminogen on cell surfaces (uPA, uPAR and PAI-1) have been consistently linked to cancer invasion and metastasis.[151-155] Some members of this system may also elicit important cellular

and physiological effects, independent of their role in the activation of plasminogen.[156, 157] uPAR induces the activation of cell-signaling pathways via interaction with integrins on membrane-cell surface, leading to increased proliferative, pro-invasive and pro-metastatic potential.[158, 159] PAI-1 plays a seemingly important role in cell detachment by binding to ECM proteins such as vitronectin and type-1 collagen, which may lead to enhanced dissemination of tumor cells.[160] In general, uPA, uPAR and PAI-1 are mainly expressed by neoplastic or stromal cells at the invasive front of tumors [161-163], thereby localizing the proteolytical, pro-invasive and pro-metastatic effects of these molecules at the leading edge of the neoplastic growth.

The levels of uPA, uPAR and PAI-1 in the tumor tissue and circulation are of prognostic significance in cancer. It has been convincingly shown that high levels of the three components are associated with poor prognosis in a number of cancers, including breast, colon, lung, ovarian and prostate malignancies.[164-174] Using a semi-quantitative immunohistochemical approach, we recently found that uPAR is an independent prognostic predictor for overall survival in gastro-esophageal adenocarcinoma.[175] Other studies have also suggested the prognostic value of uPAR as immunohistochemical parameter in oral squamous cell carcinoma.[176, 177] Likewise, studies in bone marrow aspirates from gastric cancer patients revealed that the expression of uPAR in neoplastic cells disseminating into the bone marrow is an independent prognostic parameter.[178, 179] Therefore, the components of the plasminogen activation system are potentially interesting candidates for further consideration as diagnostic or prognostic predictors in cancer.

NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN (NGAL)

NGAL, also known as Lipocalin-2 (Lcn2) or siderocalin (Scn), is a protein of relatively recent discovery that is over-expressed during infection and inflammation. NGAL was initially described as a protein of neutrophil's specific granules [180, 181], and subsequently shown to be a siderophore binding protein.[182] Siderophores are the strongest iron chelators known, and are produced by a variety of bacteria and fungi as a mechanism for obtaining this element. When complexed with iron, siderophores are taken up by the microorganisms to sustain their growth.[183-185] NGAL can interrupt this metabolic pathway by binding the siderophores and thus prevent their acquisition by microorganisms.[182, 186] The significance of this has been demonstrated *in vivo,* where NGAL knock-out mice rapidly succumb to infections when challenged by siderophore producing *E.coli* and *Klebsesiella pneumoniae*.[187, 188]

in vitro studies have shown that the expression of NGAL in epithelial cells is crucially dependent on mediators of inflammation induced by some pro-inflammatory cytokines, and in particular the NF-κB transcription factor.[189, 190] Further studies in humans have revealed that under normal physiological conditions NGAL expression is low, if present, in a variety of tissues, including gastric mucosa [191, 192]. NGAL mRNA and protein levels are, however, greatly enhanced in epithelial cells during infection and inflammation, both in animal models and humans.[193-195] Therefore, on the basis of the *in vivo* and *in vitro* evidence, it has been proposed that NGAL is a component of the innate immune system with an important role as bacteriostatic agent.[182, 187, 196]

In addition to its antimicrobial role, several studies have associated NGAL with cancer, but none have definitively demonstrated its direct involvement. NGAL is highly expressed by tumor cells in several types of cancers, including breast, pancreas, ovarian, colon and rectal malignancies.[192, 197-201] Moreover, increased levels of this molecule have been associated with poor prognosis in some of these

neoplasias.[200, 201] Recently, it was also shown that NGAL levels in tissue and urine from breast cancer patients are associated with invasion and metastasis and that NGAL promotes breast cancer progression by inducing epithelial to mesenchymal transition.[202] Nonetheless, the biochemical mechanisms underlying the potential effects NGAL in cancer are far from being clear. Based on the current evidence, it is speculated that NGAL may serve as an important iron transporter, similar to the transferrin receptor system. Human endogenous siderophores have been hypothesized to exist, although not entirely proven, and it is possible that expression of NGAL provides the carcinoma cells with the ability to acquire iron at the expense of normal tissues, and thus to support their growth.[203, 204]

Aims of the study

The discovery of *Helicobacter pylori* in 1982 opened a new avenue for the understanding of the etiology of gastric cancer. It is currently accepted that gastric cancer is a final consequence of the interaction between *H. pylori*, host and environmental factors. However, the biological mechanisms underlying the development and progression of this malignancy are far from being unraveled. Thus, the main purpose of the studies included as part of this thesis was to investigate the role of certain aspects of *H. pylori*, the inflammatory response against the bacterial infection and extracellular matrix remodeling processes in the development and progression of gastric cancer, both in countries with high- and low-risk of gastric cancer.

The following objectives were formulated to achieve this purpose:

1. To determine the association of *H. pylori* CagA-positive infection and polymorphisms of the genes IL-1B and IL-RN with the risk of developing gastric atrophy and peptic ulcers in dyspeptic patients from a high-risk country.

2. To characterize the expression pattern of the protein NGAL in gastric non-neoplastic and neoplastic mucosa, focusing on the potential induction of this molecule by *H. pylori*.

3. To compare the expression pattern of uPAR in gastric cancer, and explore the connection between uPAR expression and *H. pylori* infection in non-neoplastic mucosa, both in cases from high- and low-risk countries.

4. To evaluate the prognostic significance of uPAR expression in neoplastic and accessory cells in gastric cancer.

List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

Paper I

Relation of atrophic gastritis with *Helicobacter pylori*-CagA^{+} and interleukin-1 gene polymorphisms.

Sierra R, Une C, Ramírez V, **Alpízar-Alpízar W**, González MI, Ramírez JA, de Mascarel A, Cuenca P, Pérez-Pérez G, Mégraud F.

World Journal of Gastroenterology 2008; 14(42):6481-6487.

Paper II

Neutrophil gelatinase-associated lipocalin (NGAL/Lcn2) is upregulated in gastric mucosa infected with *Helicobacter pylori*.

Alpízar-Alpízar W, Laerum OD, Illemann M, Ramírez JA, Arias A, Malespín-Bendaña W, Ramírez V, Lund LR, Borregaard N, Nielsen BS.

Virchows Archiv 2009; 455(3):225-233. Online First (DOI: 10.1007/s00428-009-0825-8)

Paper III

Urokinase plasminogen activator receptor is expressed in invasive cells in gastric carcinomas from high- and low-risk countries.

Alpízar-Alpízar W, Nielsen BS, Sierra R, Illemann M, Ramírez JA, Arias A, Durán S, Skarstein A, Ovrebo K, Lund LR, Laerum OD.

International Journal of Cancer 2009; Published on-line (DOI: 10.1002/ijc.24755)

Paper IV

Urokinase plasminogen activator receptor on invasive tumor cells is an immunohistochemical prognostic predictor in non-cardia gastric adecarcinoma.

Alpízar-Alpízar W, Christensen IJ, Skarstein A, Ovrebo K, Illemann M, Laerum OD.

Ready for submission: *International Journal of Cancer*

Summary of papers

Paper I

In this paper we have determined the association of Helicobacter pylori CagA+ infection and pro-inflammatory polymorphisms of the genes interleukin (IL)-1RN and IL-1B with the risk of gastric atrophy and peptic ulcers in a dyspeptic population of 501 patients from Costa Rica. Infection with *H. pylori* CagA+ was determined by serology and polymerase chain reaction (PCR). IL-1B and IL-1RN polymorphisms genotyping was performed by PCR. Pepsinogen concentrations were analyzed by enzyme linked immunosorbent assay (ELISA). Information concerning nutritional and socio-demographic factors was obtained from all patients. 86% of the dyspeptic patients were *H. pylori*-positive and of these, 67.8% were positive for CagA. Atrophic antral gastritis (AAG) was associated with CagA+ status and low fruit consumption. Atrophic body gastritis (ABG) was associated with pepsinogen PGI/PGII < 3.4 and frequent alcohol consumption. Duodenal ulcer was associated with CagA+ and smoking. The pro-inflammatory alleles IL-1B+3954 and IL-1RN*2 did not confer an enhanced risk for any type of atrophy. We thus concluded that *H. pylori* CagA+ infection is not associated with ABG, but it is a risk factor for AAG in this dyspeptic population.

Paper II

The expression pattern of NGAL/Lcn2 has been characterized in gastric mucosa (45 non-neoplastic and 38 neoplastic tissue samples) and the connection between NGAL/Lcn2 expression and *H. pylori* infection explored. Immunohistochemical analysis showed high NGAL/Lcn2 expression in normal and gastritis affected mucosa compared to low expression in intestinal metaplasia, dysplasia and gastric cancer. In normal and gastritis affected mucosa (n=36 tissue samples), NGAL/Lcn2 was more frequently seen in epithelial cells located at the neck and base of the glands in *H. pylori*-positive cases than in similar epithelial cells of non-infected cases. In conclusion, the high expression of NGAL/Lcn2 in normal and gastritis affected mucosa infected with *H. pylori* suggests that NGAL/Lcn2 is upregulated locally in response to this bacterial infection. It is discussed whether this may have a causal relation to the development of gastric cancer.

Paper III

Gastric cancer incidence and mortality rates vary according to geographical regions. The receptor for urokinase plasminogen activator (uPAR) is involved in extracellular matrix degradation by mediating cell surface associated plasminogen activation and its presence on gastric cancer cells is linked to micro-metastasis and poor prognosis. Immunohistochemical analyses of a set of 44 gastric cancer lesions from Costa Rica showed expression of uPAR in cancer cells in both intestinal subtype and diffuse subtype. We compared the expression pattern of uPAR in gastric cancers from a high risk country (Costa Rica) and a low risk country (Norway). We found uPAR on gastric cancer cells in 24 of 44 cases (54%) from Costa Rica and in 13 of 23 cases (56%) from Norway. uPAR was also seen in macrophages and neutrophils in all cases. We also examined the non-neoplastic mucosa and found that uPAR was more frequently seen in epithelial cells located at the luminal edge of the crypts in cases with *Helicobacter pylori* infection than in similar epithelial cells in non-infected mucosa. In conclusion, the expression of uPAR in cancer cells in more than one half of the gastric cancer cases suggests that their uPAR-positivity do not contribute to explain the different mortality rates between the two countries. However, the actual prevalence of uPAR-positive cancer cells in the gastric cancers may still provide prognostic information.

Paper VI

The prognostic significance of uPAR as immunohistochemical parameter was evaluated in tissue samples from a retrospective series of 95 gastric cancer patients. uPAR was expressed by neoplastic cells, macrophages, myofibroblasts and neutrophils in both intestinal and diffuse subtypes. No association was demonstrated between the expression of uPAR on cancer cells and histological subtype or tumor stage. Univariate analysis revealed a significant association between the expression of uPAR on invasive tumor cells and overall survival of gastric cancer patients. Multivariate analysis confirmed uPAR immunoreactivity in invasive tumor cells as an independent prognostic factor for overall survival in gastric cancer. The results, therefore, suggest the prognostic relevance of uPAR expression on invasive cells in gastric cancer.

Discussion

Considerations related to the biological material and methodological aspects

The studies presented in this thesis were carried out with biological material from dyspeptic and gastric cancer patients, collected in two different hospitals from Costa Rica (Dr. Calderón Guardia and Dr. Max Peralta) and Haukeland University Hospital, Bergen, Norway. The biological samples consisted of serum and leucocytes (paper I), and formalin-fixed, paraffin-embedded tissue (papers II, III and IV). Serum and leucocytes used in paper I were collected between January and July 2000 and stored at -70˚C since then.[205] The good quality and suitability of these biological samples for experimental purposes was ensured by proper collection, manipulation and optimal storage conditions, but also by the fact that two studies had previously been conducted on this material with minimal methodological problems.[101, 205] Similarly, the formalin-fixed and paraffin-embedded gastric tissue used in papers II, III and IV were taken from gastric cancer patients, as part of the clinical routine procedures, between the years 1990 to 2007 and stored at the pathology archives of the above mentioned hospitals. If properly fixated and stored, the long time of storage of this type of tissue has been shown to not to compromise in large extent its suitability for experimental analyses.[206, 207]

The methodological approaches employed in the studies that are part of this thesis were chosen based on the suitability to the type of biological material available. Thus, enzyme linked immunosorbent assay (ELISA) and enzyme immunosorbent assay (EAI), polymerase chain reaction (PCR) and immunohistochemistry were the main techniques used. Certain technical considerations, inherent to the use of these methodological approaches, are then fundamental to ensure the reliability of the results obtained.

Validation of protocols and determination of cut-off points are crucial when performing ELISA and EAI quantifications.[208-210] Accordingly, in the serology-based assessments of *H. pylori* status and pepsinogens levels performed in paper I had been validated and the cut-off points determined in a previous study, which was conducted with the same set of samples.[205] Moreover, the determination of *H. pylori* status in serum was confirmed by alternative approaches, which included histological observation in tissue sections from paraffin-embedded biopsies stained with toluidine blue, and *H. pylori* culture from fresh biopsies that were taken on different anatomical sites of the stomach. The serological assessment of CagA status was also confirmed by PCR, using DNA from isolated strains as template, with sets of primers that generate an amplification product only if the *H. pylori* strains carry the *cagA* gene.[211] The primers used in paper I were, *cag*A_1-A_2 and, if negative, a second PCR was done with another set of primers (*cag*A_3-A_4). [211]

Genotyping of IL-B and IL-1RN polymorphisms in paper I was carried out by PCR, using DNA isolated from leucocytes as template. PCR-RFLP (restriction fragment length polymorphism) was employed for the allelic designation of the IL-1B+3954 polymorphism, which is based on the use of a restriction enzyme that differentially cleaves the PCR products according to the nucleotide sequence of the locus where the polymorphism is.[33] On the other hand, IL-1RN polymorphism consists of a variable number of tandem repeats (VNTR) of 86 base-pairs at the second intron of the gene, which generates then PCR products of different size according to the number of repeats.[33] Both genotyping approaches were optimized in a similar setting in my previous study.[101] Given the great sensitivity of the PCR technique, a careful manipulation of the DNA and PCR products must be ensured in order to minimize the possibility of contamination by DNA from other samples or from other exogenous sources.

The sensitivity and reproducibility of immunohistochemical staining crucially depend on aspects related to the fixation, tissue processing, and the specificity of the antibody for the target

epitope.[212] Thus, a thorough optimization that takes into account the adequate choice of retrieval method (enzymatic or heat-induced retrieval) and antigen-retrieval solution, optimal time of pre-treatment, testing of antibody´s specificity, determination of the optimal antibody concentration and use of positive/ negative controls is of fundamental importance when performing immunohistochemistry. [212]

As mentioned above, the formalin-fixed paraffin-embedded gastric tissue samples used for the studies of papers II, III and V were from different countries and, therefore, some degree of variation in tissue fixation and processing should be expected. In order to minimize the possibility of variation in the staining associated to these aspects, the immunohistochemical stainings of the papers II, III and IV were carried out with antibodies of well established specificity.[163, 213-215] Moreover, the sensitivity and reproducibility of immunohistochemical staining was confirmed by repeating the staining with at least one alternative antibody in each of the cases. Thus, in paper II, an in-house affinity purified mouse monoclonal antibody (mAb)[216], and a commercial rat mAb against NGAL were alternatively used. Accordingly, three anti-uPAR antibodies were used in papers III and IV, R2 and R4 mAbs that target different domains on uPAR protein [213, 215], and a preparation of affinity purified pAbs.[215] Furthermore, a mAb directed against an irrelevant molecule (trinitrophenyl hapten)[217], and non-immune rabbit IgG were used to test for the specificity (negative controls) of the NGAL and uPAR antibodies in papers II, III and IV. Finally, neutrophils served as internal controls for the staining on each of these three papers, as they are known to express both NGAL [180, 181] and uPAR.[218]

Geographical variations in incidence and mortality of gastric cancer

Gastric cancer presents marked variations in incidence and mortality rates according to geographical location (between and within countries). The factors underlying these differences are far

from being unraveled given the complexity and multifactorial nature of this malignancy. Costa Rica is among the countries with highest incidence and mortality rates for gastric cancer worldwide ([219] and Globocan 2002; available at: http://www-dep.iarc.fr/). In contrast, Norway presents a low incidence and mortality rates for this malignancy ([220] and Globocan 2002). It is likely that aspects connected to ethnical, environmental, cultural and socioeconomic differences between Costa Rica and Norway explain these differences.

H. pylori is a well recognized risk factor for gastric cancer and, in general, the prevalence of infection, particularly of strains carrying specific virulence factors, is correlated with the incidence of the malignancy.[82] In paper I, it was found that the prevalence of *H. pylori* infection among 501 dyspeptic patients from Costa Rica was approximately 86%. A slightly lower prevalence of infection (77%) was observed in Costa Rican gastric cancer patients (paper III). This is expected, given the different methodological approaches used for assessing the infection (serology and immunohistochemistry, respectively). In contrast, paper III revealed that among Norwegian gastric cancer patients, the prevalence of *H. pylori* infection was 30%. Although the number of patients from Norway was small (23 patients), this prevalence of infection is similar to what has been reported by Asfelt *et al*.[221] in 912 Norwegian individuals (34% in men and 41% in women), using a stool-based ELISA kit. Another study carried out in 944 Norwegian individuals found a general prevalence of infection of 37%, using a serological approach.[222] In marked contrast, Lindetmo *et al*.[223] reported a prevalence of more than 70% in 244 Norwegian individuals with peptic ulcer disease and their controls, by using urea breath test and serological procedures. This study, however, was based on patients diagnosed between 1967 and 1990. It was recently suggested that the prevalence of this bacterial infection in Norway has decreased markedly.[221] Taken together, the results presented in this thesis and those of studies conducted in the Norwegian population suggest a marked difference in the prevalence

of *H. pylori* infection between Costa Rica and Norway. This may contribute to explain the substantial differences in gastric cancer incidence between these two countries.

The mortality in gastric cancer is generally high worldwide because, in most cases, the tumors are diagnosed at an advanced stage of the disease when treatment is likely to fail. According to the IARC/WHO, the mortality rates in Costa Rica are approximately 30.1 in males and 17.0 in females, while in Norway they are estimated to be 9.4 in males and 5.0 in females (Globocan 2002, available at: http://www-dep.iarc.fr/). Five-year survival in Costa Rica is less than 15% [224], and in Norway it is approximately 21.9%.[225] A gastric cancer, X-ray-based mass screening intervention was conducted in the region of Costa Rica with highest incidence and mortality rates for this malignancy. An evaluation of its impact concluded that this intervention substantially reduced stomach cancer mortality (around 50%) and improved the 5-year survival (up to 85%) in this particular region.[14] It is, therefore, likely that the early detection has an impact on gastric cancer mortality but it may not be the only influencing variable. A more complex scenario that probably involves the interaction of a number of factors, including biological and natural aspects, determines the mortality for this malignancy. Interesting examples on that aspect are the unexplained difference of incidence and mortality rates between men and women in both low- and high-risk countries worldwide, and the general steady decrease of both rates in low- and high-risk countries in the last decades.

uPAR plays a central role in the activation of plasminogen, which is a key protease for extracellular matrix breakdown during cancer invasion and metastasis.[226] uPAR has been consistently associated with a poor prognosis.[155, 165, 168, 169] In an attempt to evaluate whether this molecule can partially explain the differences in mortality, the expression pattern of uPAR was compared in gastric cancers from Costa Rica and Norway in paper III. However, no differences were found in either the types of cells expressing this protein or the prevalence of uPAR-positive tumor cells between the two

countries. Thus, the expression of uPAR may not contribute to explain the differences in mortality between high and low-risk countries.

On the basis of the work in this thesis, it is tempting to speculate that the differences in cancer incidence and mortality between geographical regions might in particular be related to factors that play a role in the initiation of the gastric carcinogenesis process. However, once the carcinogenic process goes further, the biology of the malignancy may be rather homogeneous in high- and low-risk countries. It should, however, be emphasized that early detection does have an impact on gastric cancer mortality as such.

The role of *H. pylori*-induced inflammatory response in gastric neoplasia

Aspects related to the *H. pylori*-induced inflammation are the focus of papers I and II. It is well recognized that *H. pylori* triggers a chronic inflammatory response, which may significantly alter the gastric physiology.[83, 227] The magnitude of this response depends to a great extent on bacterial virulence factors and the host genetic inflammatory profile.[83, 95, 228] Thus, bacterial virulence factors and the host response against the infection are thought to be critical aspects for determining the final outcome resulting from the gastric precancerous multi-step process.[64, 83, 229] The cellular and molecular mechanisms through which the *H. pylori*-induced inflammation drives gastric carcinogenesis are, however, not fully understood.

In the year 2000 was first described the association between cytokine polymorphisms that directly influence the magnitude of the inflammatory response and the risk of gastric cancer and precancerous lesions.[33] Since then, a considerable number of studies have confirmed such an association.[227, 230, 231] Interestingly, host genetic pro-inflammatory polymorphisms, combined to infection with more virulent *H. pylori* strains have shown to confer the highest risk for developing

gastric cancer.[105, 232] The association of two polymorphisms on IL-1B and IL-1RN genes with the risk of atrophic gastritis was evaluated in paper I. This was based on studies that found association between these two polymorphisms and the risk of gastric cancer in Costa Rica [101, 233], as well as in other geographical locations.[234]

The investigations in paper I, however, did not demonstrate an association between the IL-1 polymorphisms and the risk of atrophic gastritis. This may be related to the fact that the control groups were non-atrophic dyspeptic patients, which are likely to present an enhanced inflammatory activity as compared to asymptomatic normal individuals. Moreover, the prevalence of alleles that enhance the expression levels of the pro-inflammatory cytokine IL-1β seems to be high in the Costa Rican population, as suggested by paper I and a previous study [101], as well as it is the prevalence of *H. pylori* infection.[205, 235] It has been suggested that the effect of pro-inflammatory polymorphisms may be more evident and easier to determine in populations with low risk of gastric cancer, low prevalence of risk alleles and low prevalence of *H. pylori* virulent strains [117, 230, 236, 237]

The possible association of the *H. pylori* virulence factor CagA with the risk of developing atrophic gastritis was also investigated in paper I. It was found that the prevalence of this virulence factor was very high in this dyspeptic population. No association was demonstrated between CagA-positive strains and atrophic gastritis in the body of the stomach. This virulence factor was, however, associated with atrophic gastritis in antrum. Although *H. pylori* predisposes to both pathologies [64, 238], atrophic body gastritis is clearly established as a pre-neoplastic condition that may eventually lead to gastric cancer, while atrophic gastritis occurring at the antral region of the stomach does not.[23, 64] It is likely that the high prevalence of *H. pylori* CagA-positive infection and the fact that the control groups used for direct comparison were not entirely normal may have masked the link between this bacterial virulence factor and atrophic body gastritis. Taken together, the high prevalence of *H. pylori* infection,

particularly of more aggressive strains, and high frequency of genotypes conferring increased expression pro-inflammatory cytokines might contribute to explain the high incidence of gastric cancer in the Costa Rican population.

In paper II it was revealed a high expression of NGAL in gastric epithelial cells during early stages of gastric carcinogenesis, which are characterized by chronic inflammation, and suggested to be induced by *H. pylori* infection. Induction of NGAL in epithelial cells crucially depends on the inflammatory mediators IL-1β and NF-κB.[189, 190] Both molecules are upregulated in *H. pylori*-induced inflammatory response [83], and the virulence factor CagA has been demonstrated to potentiate NF-κB activation.[96] NF-κB has been consistently linked to cancer, as its activation leads to the production of molecules that enhance the growth, survival, vascularization and invasion of carcinoma cells.[97, 239] Although not directly shown, accumulating evidence suggests a causative role of NGAL in cancer development and progression.[202, 204, 240] Thus, from the work of this thesis, NGAL emerges as a potentially novel causative participant in the gastric carcinogenic process. Given the proposed oncogenic functions of NGAL [202], it becomes indeed an interesting candidate to further investigate in future studies. NGAL may be among the pro-neoplastic proteins that are induced upon activation of NF-κB, thus contributing to explain the attributed role of inflammation in gastric carcinogenesis.

Another interesting finding of this thesis was the upregulation of uPAR in epithelial cells of normal-appearing and gastritis affected tissue, which seems to be linked to *H. pylori* infection (paper III). uPAR expression is generally considered as a late phenomenon, related to the invasion and metastasis. However, its expression seems to be also induced at early stages of gastric carcinogenesis (this is further discussed below).

uPAR expression during malignant invasion and its prognostic relevance

uPAR is a central component of the proteolytic cascade leading to plasminogen activation at cell-surface membranes, which is a key step for the extracellular matrix remodeling during malignancy.[226] A number of biochemical functions, independent of its role in plasminogen activation, have also been unraveled for this molecule, including the activation of cell-signaling pathways that result in enhanced malignant cell migration and proliferation.[156] uPAR is thus an obvious candidate to study in connection to cancer development and progression. Strong body of evidence has indeed showed the relevance of this protein in invasion and metastasis, as well as its association with some clinico-pathological outcomes, including poor survival, in a number of malignancies.[155, 241]

The expression of uPAR and its prognostic significance has been studied in malignancies of the gastrointestinal (GI) tract. A particularly vast number of studies has been conducted in colorectal carcinomas. In fact, some of the studies that revealed important aspects about the expression of uPAR in malignancy and demonstrated its clinico-pathological relevance were conducted in colon cancer.[168, 242-244] In contrast, the expression of uPAR in tumors of esophagus and stomach, have been investigated to much lesser extent.

In general, the expression of uPAR in GI tract malignancies follows a similar pattern, with neutrophils, macrophages, myofibroblasts and neoplastic cells as the main uPAR-positive cell populations (papers III, IV and [163, 175]). Neutrophils generally express uPAR, as it is synthesized during neutrophils differentiation in the bone marrow and is, therefore, present on all circulating neutrophils.[245] For instance, the expression of uPAR by neutrophils is not particularly connected to invasive phenotypes or pathological outcomes in carcinomas of the GI tract. In contrast, the expression of uPAR is clearly upregulated in macrophages and myofibroblasts that are located at the invasive front of the tumors, in close proximity to cancer cells, in GI tumors (papers III, IV and [163, 175]). This is

speculated to be induced by cancer cells through the secretion of specific cytokines/growth factors. Thus, the expression of uPAR by macrophages and myofibroblasts is thought to contribute to the invasive and metastatic potential in GI tract malignancies, particularly in colon cancer.[161]

Neoplastic cells also contribute to the expression of uPAR in adenocarcinomas of the GI tract. uPAR expression is particularly evident in tumor cells located at the invasive edges of the malignant growth (papers III, IV and [163, 175]). However, the prevalence of uPAR-positive cancer cells seems to be the major difference between carcinomas of the GI tract, being more prevalent in esophageal and gastric carcinomas than in colon carcinomas (Papers III, IV [163, 175]). This is, in fact, interesting when considering that the global five-year survival estimates for esophageal, gastric and colon carcinomas are, on average, 10%, 25% and 50%, respectively.[1, 225] Thus, it is tempting to speculate that the prevalence of uPAR-positive cancer cells may be an important determinant of the aggressiveness in cancers from the GI tract, which then would argue in favor of the prognostic significance of uPAR in these tumors.

It is showed in paper IV that the expression of uPAR in invasive cancer cells is an independent predictor of survival in gastric cancer. Given the biochemical functions of uPAR, the expression of this molecule in tumor cells may confer neoplastic cells enhanced ability to invade, metastasize and proliferate. This may contribute to explain the potential link between uPAR expression in these cells and survival. Thus the findings of paper IV suggest that uPAR could be an interesting and potentially relevant candidate to further investigate in connection to the prognosis of gastric cancer patients. This is important, given the fact that the mortality for gastric cancer is high, few parameters have proven to be of prognostic value for this malignancy and even less number of suggested prognostic predictors that may be directly involved in the cellular and molecular biology of the disease, which include E-cadherin, p53, carcinoembrionic antigen (CEA) and c-erbB-2.[16, 18, 246]

Expression of uPAR in gastric cancer development

Given the possible clinical significance of uPAR in gastric cancer, it is then very important to unravel when, during gastric carcinogenesis, is uPAR expressed and what induces its expression in gastric mucosa. *in vitro* studies have found that uPAR is significantly induced in gastric cancer cell lines co-cultured with *H. pylori*.[247] It has also have been shown that when gastric cancer cell lines are challenged with *H. pylori* CagA-positive strains the expression of uPAR is significantly enhanced as compared to neoplastic cells facing CagA-negative bacterial strains.[248] In paper III it is described the expression of uPAR in gastric epithelial cells of normal-appearing and gastritis affected mucosa, *in vivo*, and it is shown to be associated with the presence of *H. pylori* infection. This suggests that uPAR is expressed since very early stages of the pre-neoplastic process leading to gastric cancer. A recent *in vivo* study have also showed increased levels of uPAR mRNA and protein in epithelial cells of non-neoplastic mucosa infected with this bacterium.[249] Thus, it is relevant to investigate more systematically *in vivo* whether the possible induction of uPAR by *H. pylori* is particularly associated with specific bacterial virulence factors, including CagA, VacA and sub-variants of then (e.g. EPIYA motifs in CagA and the *s*, *m* and *i* regions of VacA).

The study conducted in paper III does not take into account the characterization of *H. pylori* of the mentioned virulence factors, as it was found difficult to optimize the appropriate methodological approach for their genotyping in formalin-fixed, paraffin-embedded material. Isolation of DNA and PCR using specific primers was tried on this material, but it was not successful probably because most of the DNA isolated was predominantly genomic DNA. It is likely that the amount of bacterial DNA was little and diluted, making difficult the subsequent amplification by PCR. Laser-capture microdissection technique could have improved the obtaining of bacterial DNA.

The pathogenicity island (PAI)-CagA is an obvious candidate to further pursue on the possible induction of uPAR by *H. pylori.* This virulence factor has well known pathogenic effects on gastric epithelial cells, including induction inflammatory, oncogenic and proliferative proteins.[78] Alternative experimental and methodological approaches, using fresh human gastric tissue and animal models suitable for infecting with *H. pylori* and other *Helicobacter* species (e.g. *Helicobacter felis*), is necessary to further investigate of the role of *H. pylori* and its virulence factors in the induction of uPAR.

Conclusions

On the basis of the work conducted in this thesis, the following conclusions may be formulated:

1. In a high risk population of Costa Rica, *H. pylori* CagA-positive infection is associated with atrophic gastritis of the antral region of the stomach, but no association could be demonstrated for atrophic body gastritis. No association was demonstrated between IL-1B and IL-1RN polymorphisms and any type of atrophic gastritis. Nevertheless, the prevalence of *H. pylori* infection was more than 86%, and that of CagA-positive strains is nearly 68% in this population. The prevalence of alleles predisposing to gastric cancer is also high. The high prevalence of these bacterial and host factors in the general population may contribute to explain why Costa Rica has one of highest incidence rates for this malignancy worldwide.

2. The expression of NGAL is, in general, high in epithelial cells of normal-appearing and gastritis affected mucosa and low in intestinal metaplasia, dysplasia and cancer. NGAL expression in gastric epithelial cells is significantly higher in *H. pylori*-infected individuals than in those non-infected. Thus, NGAL seems to be upregulated in gastric mucosa in early stages of the gastric carcinogenic stepwise process that are characterized by robust inflammatory activity, and this upregulation may be induced in response to *H. pylori* infection. NGAl upregulation may have a causal relation to gastric cancer development.

3. uPAR is expressed by macrophages and cancer cells in gastric cancer. The prevalence of uPAR-positive tumor cells in similar in gastric cancers from high- and low-risk countries (Costa Rica and Norway, respectively). In non-neoplastic gastric mucosa, uPAR is more frequently seem in epithelial cells of *H. pylori*-infected cases than in similar cells in non-infected mucosa. Thus, uPAR may not contribute to explain the different mortality rates that exist according to geographical locations.

4. No association was demonstrated between uPAR immunoreactivity in myofibroblasts or macrophages and the survival of gastric cancer patients. In contrast, the expression of uPAR in invasive tumor cells was revealed as an independent prognostic predictor for overall survival of gastric cancer.

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