Helicobacter pylori associated effects on inflammatory radical formation and angiotensin II receptors in the stomach

Anders Elfvin

Göteborg 2007

The Department of Gastrosurgical Research
Institute of Clinical Sciences

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GÖTEBORG UNIVERSITY

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Göteborg University
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ABSTRACT

Helicobacter pylori infection of the stomach always results in mucosal inflammation and a marked systemic immune response. Despite the profound host defence reactions the bacterium avoids elimination and persists in the mucosa. This results in chronic inflammation and an increased risk for peptic ulcers and adenocarcinoma. Still a majority of infected individuals never develop any symptomatic disease. Previous results from our laboratory indicate that *H. pylori* reduces the power of host defence by restricting gastric NO production by pathogen-derived competitive iNOS inhibitors. It was considered of interest to further investigate not only the *H. pylori* associated inhibition of nitro-radical formation, but also interactions with the oxy-radical formation in gastric carcinogenesis. Furthermore, because the renin angiotensin system (RAS) recently was ascribed immunomodulatory actions, it was considered of interest to also explore if *H. pylori* influences the presence and location of this regulatory system in the gastric mucosa. The *H. pylori* infected Mongolian gerbil was used as the experimental model and was followed up to 18 months after infection. A first aim of this thesis was to by use of histopathology validate the model's suitability for studies of *H. pylori* (strains SS1 and TN2GF4) induced gastric mucosal pathology. The results indicate that the *H. pylori* infected Mongolian gerbil cannot be confirmed as being a cancer model, but it is suitable for studies of acute and chronic mucosal inflammation. The Mongolian gerbil model was then used to elucidate *H. pylori* strain dependency on the expression of the oxy- and nitro-radical forming enzymes, and to investigate whether *H. pylori* infection results in inhibition of either or both of the nitro- and oxy-radical formation. Western blotting was used to assess iNOS and MPO expressions as representatives for nitro- and oxy radical forming pathways, respectively. Radical formation was assessed as presence of nitrotyrosine or by use of NO or H_2O_2 sensitive microelectrodes. The results confirm that *H. pylori* infection in Mongolian gerbils despite an up-regulation of nitro- and oxy-radical forming enzymes results in inhibition of radical formation. Response patterns differed over time in relation to the *H. pylori* strain under study. The results were confirmed in human gastric specimens using similar western blot assessing expression of nitro-and oxy-radical forming enzymes as well as nitrotyrosine. Finally, gene transcripts and immunoreactivity to the angiotensin II receptors AT1R and AT2R were found present in the antral wall of the Mongolian gerbil. The investigation indicated a possible *H. pylori* strain dependent influence on the AT1R expression.

The present studies on experimentally infected Mongolian gerbils and asymptomatic human tissues support strongly that *H. pylori* avoids to be eliminated from the gastric mucosa by interfering with the nitro- and oxy-radical formation. In addition the investigations also suggest the presence of a *H. pylori* strain dependent influence on the AT1R expression constituting a novel immunomodulatory principle.

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LIST OF PUBLICATIONS

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

I. Elfvin A, Bölin I, Von Bothmer C, Stolte M, Watanabe H, Fändriks L, Vieth M. *Helicobacter pylori* induces gastritis and intestinal metaplasia but no gastric adenocarcinoma in Mongolian gerbils. Scand J Gastroenterol. 2005;40(11):1313-20.

II. Elfvin A, Bölin I, Lönroth H, Fändriks L. Gastric expression of inducible nitric oxide synthase and myeloperoxidase in relation to nitrotyrosine in *Helicobacter pylori*-infected Mongolian gerbils. Scand J Gastroenterol. 2006;41(9):1013-8

III. Elfvin A, Edebo A, Bölin I, Fändriks L Quantitative measurement of nitric oxide and hydrogen peroxide in *Helicobacter pylori* infected Mongolian gerbils *in vivo.* Scand J Gastroenterol. 2007; 42(25):1-7

IV. Elfvin A, Edebo A, Hallersund P, Casselbrant A, Fändriks L. Gastric expression of NADPH oxidase, inducible nitric oxide synthase and myeloperoxidase in relation to nitrotyrosine in *Helicobacter pylori*-infected humans. In manuscript

V. Hallersund P, Ewert S, Casselbrant A, Helander H F, Edebo A, Fändriks L, Elfvin A. Angiotensin II receptor expression and location in normal and *Helicobacter pylori*-infected stomach of the Mongolian gerbil. Submitted

CONTENTS

LIST OF ABBREVIATIONS

ADMA	asymmetrical dimethyl arginine
ACE	angiotensin converting enzyme
Ang II	angiotensin II
AT1R	angiotensin II type 1 receptor
AT2R	angiotensin II type 2 receptor
cagA	cytotoxin associated gene A
H_2O_2	hydrogen peroxide
HOCl	hypochlorus acid
H. pylori	*Helicobacter pylori*
IL	interleukin
IFN-γ	interferon gamma
iNOS	inducible nitric oxide synthase
MPO	myeloperoxidase
NADPH-oxidase	nicotinamide adenine dinucleotide phosphate oxidase
NF-κB	nuclear factor kappa B
NO	nitric oxide
O_2^-	superoxide
$ONOO^-$	peroxynitrite
PCR	polymerase chain reaction
RAS	renin angiotensin system
RNI	reactive nitrogen intermediates
ROS	reactive oxygen species
SOD	superoxide dismutase
SS1	the Sydney strain 1 of *Helicobacter pylori*
TGF-β	transforming growth factor beta
Th cell	T helper cell
TNF-α	tumour necrosis factor alpha
Treg	regulatory T cell
TN2GF4	the TN2GF4 strain of *Helicobacter pylori*
VacA	vacuolating cytotoxin A

In remembrance of my father

FOREWORD

The Nobel Prize in Physiology or Medicine 2005 was awarded the Australian physicians Barry J. Marshall and J. Robin Warren "for their discovery of the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease"(1). This remarkable event was the culmination of an extraordinary scientific process that had turned the widespread peptic ulcer-disease from a condition with only symptomatic treatment options into a curable one. That paradigm shift is one the greatest revolutions in modern medicine and was dependent on a preceding detailed understanding of the production of hydrochloric acid by the stomach. Although the peptic ulcer disease nowadays is not of any great clinical concern *H. pylori* is still a cause of significant morbidity. Being one of the most common infections worldwide and with a strong association to the development of stomach cancer there is still a need for understanding the pathophysiological processes following *H. pylori* infection. One of the keys for *H. pylori* to become a successful pathogen is its outstanding ability to survive in the gastric mucosa. It has to withstand not only the hostile intragastric acidic and peptic activity but also the host defences induced following infection. The reasons why the bacterium can persist in the stomach despite a strong immune response are obscure. The host response to *H. pylori* infection results in up-regulation of several enzymes involved in production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI). As several nitro- and oxy-radicals have potent bactericidal effects, inhibition of radical production would be beneficial for bacterial survival.

My thesis is based on 5 papers in which I have tested the hypothesis that *H. pylori* has the ability to inhibit the activity of the up-regulated nitro- and oxy-radical producing enzymes. Such an inhibition would cause a reduced or imbalanced radical production of possible importance for the pathological processes leading to chronic inflammation and malignant transformation. Furthermore as the renin angiotensin system (RAS) is involved in both immunomodulatory actions and has a strong relationship to radical formation I found it of interest to also challenge the hypothesis that *H. pylori* influences the presence and location of this regulatory system in the gastric mucosa.

Because the scientific literature is huge concerning both the *H. pylori* associated effects on inflammatory radical formation and the RAS a brief review of parts relevant for this thesis project is given below. Then the scientific approach is considered after which novel results are reviewed and eventually discussed in relation to the existing paradigms. My wish is that the research summarised in this thesis may contribute to the understanding of the obscure development of *H. pylori* related disease.

A.E.

BACKGROUND

HELICOBACTER PYLORI

Epidemiology

H. pylori have been found in the stomachs of humans in all parts of the world. It is one of the most common bacterial infections worldwide, infecting more than half of the world's population. There is a strong correlation between prevalence of infection and socioeconomic status (2). In some low-income countries 70-90 % of the population is infected with *H. pylori*, whereas in high-income countries the prevalence is 25-50%. Most infections are acquired in childhood (3, 4). However, the incidence of *H. pylori* infection is declining, and today only 10 % of the children in high income countries are infected (5, 6). The mode of bacterial transmission of *H. pylori* is still not fully understood. Faecal-oral transmission is probably the most important (7, 8).

Bacteriology

H. pylori is a spiral shaped micro-aerophil gram-negative bacterium, 2,5-5 μm long and 0.5-1.0 μm wide. It has four to six unipolar flagella that are essential for bacterial motility (9).The bacteria produce a number of factors that are important to the organism's survival, virulence and induction of pathophysiological effects in the host. The virulence factors mentioned below are known to be important for bacterial colonization and development of disease. *H. pylori* produces large amounts of ***urease***, which represents about 10-15% of the total bacterial protein synthesis. Urease exists both on the bacterial external surface and internally in the cytoplasm. It acts by converting gastric urea into ammonia (NH_3) and carbon dioxide (CO_2) that converts to bicarbonate (HCO_3^-) by carbonic anhydrase (10). The produced NH_3 and HCO_3^- acts as a buffer optimizing pH conditions in the medium surrounding the microbe. The external urease used to be regarded as the most important in this aspect, as it generates a cloud of ammonia around the bacteria that protect it against the acidity of the stomach. However, it is now thought that in the conditions found in the stomach low pH, cytoplasmatic urease rather than surface bound urease is essential for the survival of the organism (10). At acidic pH an urea channel is activated and the urea diffusion in to the cytoplasm can increase 300 fold, allowing maximal production of NH_3 and CO_2 (11). NH_3 efflux from the cytoplasm then buffers the bacterial periplasm to a pH consistent with bacterial viability. Bacterial urease is important for bacterial colonization and virulence. The urease produced

by *H. pylori* also acts as a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production (12).
A well-established cluster of virulence is the ***cytotoxin associated pathogeneicity island (cag PAI)*** being a 27-gene locus that is present in a majority of the clinical strains found in Europe and the USA. *H. pylori* strains with the cag PAI have been shown to be more virulent, with an increased risk of development of duodenal ulcers and gastric adenocarcinoma, than strains lacking the gene complex (13). ***The cytotoxin associated gene A (cagA)*** is located in the cag PAI, and encodes for the CagA protein. In the *H. pylori* infected gastric mucosa the CagA protein is inserted into the host epithelial cells (14). Inside the host cell the CagA interferes with cell signalling pathways and induces cytoskeletal rearrangements (15). It has been suggested that cagPAI positive strains are involved in the activation of transcription factor nuclear factor -κB (NF-κB), resulting in production of inflammatory mediators such as interleukin-8 (Il-8) (16, 17). ***The cytotoxin associated gene E (cagE)*** is another of the functional genes in the cag-PAI. The gene encodes for the protein CagE and has been ascribed an important role in carcinogenesis following *H. pylori* infection (18, 19). The *in vitro* observation of large vacuoles in the cytoplasm of cells incubated with *H. pylori* led to the discovery of the ***Vacuolating cytotoxin A (VacA)*** (20). VacA induces apoptosis in epithelial cells, but it is still not clarified why vacuolation is required for this type of apoptosis (21).The VacA gene is present in all isolates of *H. pylori*, but not all strains express the 95-kD VacA protein. The VacA protein inserts itself into the epithelial cell membrane and forms a channel through which bicarbonate and organic anions can be released (22). Possibly this is a way to provide the bacterium with nutrients.
The gene ***babA2*** encodes for the protein BabA, which is an outer-membrane protein. *H. pylori* strains that possess the bab2 gene are associated with an increased incidence of gastric adenocarcinoma. BabA-expressing strains adhere more tightly to epithelial cells, which might promote pathogenesis (23).

Histopathology

The *H. pylori* infection is always associated with an inflammation in the gastric mucosa. Classically “inflammation” is the somatic reaction to a noxious influence e.g. an infection. The inflammatory reaction to *H. pylori* relates primarily to the histopathologic picture of the inflamed stomach mucosa, collectively termed ***gastritis***. Experimental studies show that an acute gastritis, characterized by the appearance of acute inflammatory cells including neutrophil polymorphonuclear infiltration, appears shortly after colonization with *H. pylori* (24-26). According to the Updated Sydney system gastritis can be divided into three broad categories: acute, chronic, and special forms (the latter for example NSAID- associated chemical gastritis and bile reflux gastritis) (27). Regardless of the regional distribution of chronic gastritis, the inflammatory pictures consist of a lymphocytic and plasma cell infiltrate in the lamina propria, occasionally

accompanied by neutrophilic inflammation of the neck region of the mucosal pits (28). Chronic inflammation may be accompanied by mucosal atrophy defined as loss of the glandular structures. Chronic gastritis is separated into two major categories based on the presence or absence and topographic distribution of atrophy. *H. pylori* may cause chronic non-atrophic gastritis or a multifocal atrophic gastritis (27). Most individuals with *H. pylori* infection have a more prominent gastritis in the antrum compared to the corpus (27). Atrophic gastritis is often associated with replacement of gastric epithelium with columnar and goblet cells with the morphology of intestinal epithelial cells; ***intestinal metaplasia*** (29). On the basis of morphology and enzyme histochemistry intestinal metaplasia is divided into three main sub-types and into small intestinal or colonic types (30-33). Presence of intestinal metaplasia is believed to increase the risk of development of dysplastic changes in the gastric mucosa. The term ***dysplasia*** is used to describe disorderly but non-neoplastic proliferation. It means loss of uniformity of the individual cells, as well as a loss in their architectural orientation. Dysplastic cells exhibit a considerable variation in size and shape, and their nuclei are often hyperchromatic (28).

Approximately 15-20 % of the *H. pylori* infected population develop ***duodenal ulcers*** (DU) (34-36). A *H. pylori* induced inflammation of the antral mucosa in the presence of an intact oxyntic (acid producing) mucosa will result in acid hypersecretion due to a blockage of mechanisms normally inhibiting the gastric acid secretion (36, 37). *H. pylori* infection of the antrum results in an increase of the release of gastrin. Gastrin has a stimulatory effect on the acid secreting mucosa, which will result in a markedly increased maximal acid secretion capacity (38, 39). Under normal conditions antral somatostatin acts as a physiological inhibitor of gastrin release.The increase in gastrin release may be due to a *H. pylori* induced inhibiton of somastostatin release in the antrum (37, 40). In the uninfected stomach acidification of the duodenal bulb will immediately activate bicarbonate secretion from the duodenal mucosa neutralizing the acid. Nitric oxide (NO) produced by iNOS is an activator of bicarbonate secretion in the duodenum (41, 42). *H. pylori* infected DU patients have markedly reduced bicarbonate secretion in response to acidification of the duodenal bulb (43). The mechanism by which *H. pylori* infection is reducing bicarbonate secretion may be by inhibiting iNOS activity which results in reduced NO production (44). The increased acid load might lead to development of gastric metaplasia in the duodenum (36). *H. pylori* colonize the metaplasias that subsequently become inflamed and are considered to be the initial sites of ulceration (45).
Individuals with DU following antrum-predominated gastritis and high acid production seem less likely to develop gastric adenocarcinoma (46). However, if the *H. pylori* inflammation also includes the oxyntic mucosa, the acid secretion will instead be reduced due to *H. pylori* induced inhibition on the parietal cell level e.g. via enhanced Il-1β expression. This may start the development of atrophic gastritis and increase the risk of gastric adenocarcinoma (45, 47).

Many studies have demonstrated the close relationship between *H. pylori* infection and the development of ***gastric adenocarcinoma*** (48, 49). In 1994 *H. pylori* became classified as a class I carcinogen by WHO (49).
The two main sites of cancer in the stomach are the cardia region (the esophagogastric junction) and the distal portion of the stomach. The adenocarcionomas of the esophagogastric junction, and the more distal adenocarcionomas (below termed gastric adenocarcinomas) have many differences, and probably they represent two distinct diseases with different etiologies (50)

An inverse relationship between *H. pylori* infection and the risk of development of adenocarcinoma of the esophagus and the esophagogastric junction has been proposed, but remains to be confirmed, and is outside the scope of this thesis (51). Approximately 90% of gastric cancers are adenocarcinomas, and the majority are located along the lesser curvature of the antro-pyloric region. The remaining 10% are mostly made up of non-Hodgkin's marginal B-cell lymphomas (MALT–lymphomas) and leiomyosarcomas with a more diffuse localisation. An interesting finding regarding MALT lymphomas is that a high percentage of cases regress after antibiotic treatment (52). Being relatively rare the lymphomas are not further discussed in this thesis. Gastric adenocarcinomas can be divided into two groups, the well-differented (intestinal) type and the un-differented (diffuse) type. The intestinal type of adenocarcinoma usually occurs at high age, and is more common among men. The diffuse type more commonly affects younger people, and occurs equally among men and women (53). *H. pylori* significantly increase the risk of development of both subtypes of gastric adenocarcinoma. The mechanisms of development of the intestinal type of cancer are best characterized and is proposed to be related to corpus-dominant gastritis, mucosal atrophy and intestinal metaplasia.

Carcinogenesis

In 1992 Correa and colleagues presented a multi-step model for the development of gastric cancer (54). Correa postulated that there is a temporal sequence of precancerous changes that eventually leads to the development of gastric adenocarcinoma. The so-called Correa´s cascade starts with a chronic active gastritis, most commonly caused by *H. pylori* infection. This chronic gastritis can develop to an atrophic gastritis, which allows transformation to intestinal metaplasia, dysplasia and eventually to the development of adenocarcinoma. This is now widely accepted as the route of cancer development in the stomach. The Correa´s cascade is a good starting point for research, but probably the truth is more complex than previously known. Metaplasia does not obligately follow atrophy, it may actually be the opposite: intestinal metaplasia can lead to the development of atrophy (55). Furthermore some types of intestinal metaplasia might be a paracancerous phenomenon, and not necessarily precancerous (55).

Regarding risk factors for cancer development, *H. pylori* is the primary cause of gastric inflammation and the leading etiologic agent of gastric adenocarcinoma. Virulence factors expressed by the bacteria such as CagA, VacA and BabA are related to a higher incidence of gastric pathology (56, 57). Another factor that is clearly related to increased risk of gastric adenocarcinoma is high salt consumption (58). On the other hand lifestyle factors as smoking and obesity that seem to be important for cancer in the esophagogastric junction are not so for the *H. pylori* related gastric tumours (59).
Plasma levels of GI hormones and growth factors e.g. gastrin, as well as cytokines such as IL-8, have been shown to be elevated in gastric cancer tissue compared to controls (60). The cyclooxygenas-prostaglandin system (COX-2-PGE_2) is involved in gastric carcinogenesis by playing a role in cell proliferation, apoptosis and angiogenesis (61, 62) .

In summary infections with virulent *H. pylori* strains are associated with a markedly increased risk of development of gastric adenocarcinoma. Still a majority of infected individuals never develop any neoplastic changes. Probably it is a combination of bacterial factors, environmental insults and the competence of the host immune response that spurs the initiation of mucosal atrophy and gastric cancer. Hence, a deeper understanding of the host response to *H. pylori* is important in understanding the carcinogenesis.

Immunology

Immunity is usually divided into innate and adaptive immunity. Innate immunity refers to responses that do not require previous exposure to the immune stimulus, whereas adaptive immunity involves immunologic memory and is the response to a previously identified immunologic stimulus. However, the border between the innate and the adaptive immunity is not always clear. Infection with *H. pylori* causes a strong immune response and local infiltration by both polymorphonuclear and mononuclear cells giving the characteristic picture of mucosal inflammation. The cellular damage and grade of inflammation are strongly associated to the presence of virulence factors in the *H. pylori* strain infecting the mucosa. The most important virulence factors in this aspect are the previously mentioned cag PAI and vacA. Activated macrophages are central players in the first line of defence against infection. They are important coordinators of both the innate and adaptive immune response (63). As shown in Figure 1 macrophages also produce cytokines such as IL-12 that stimulate T-helper 1 (Th1) cells resulting in production of other cytokines like interferon-γ (IFN-γ) (63). The role of T-cells in the immune response to *H. pylori* infection has been thoroughly studied and involves complex interactions with activation of both Th and T regulatory cells (Treg) (64). One of the most important pro-inflammatory cytokines in the defence against *H. pylori* infection is IL-1β.

Increased production of IL-1β induces expression of many other pro-inflammatory cytokine genes such as TNF-α, IL-2, IL-6 and IL-12 (65).
The *H. pylori* activation of the host cellular immune response includes an increased expression of nitro- and oxy-radical forming enzymes as a part of the bactericidal defence. Despite a strong host immune response to the *H. pylori* infection the microbe is not eradicated. The *H. pylori* induced immune response is apparently ineffective and the bacterium remains in the stomach.

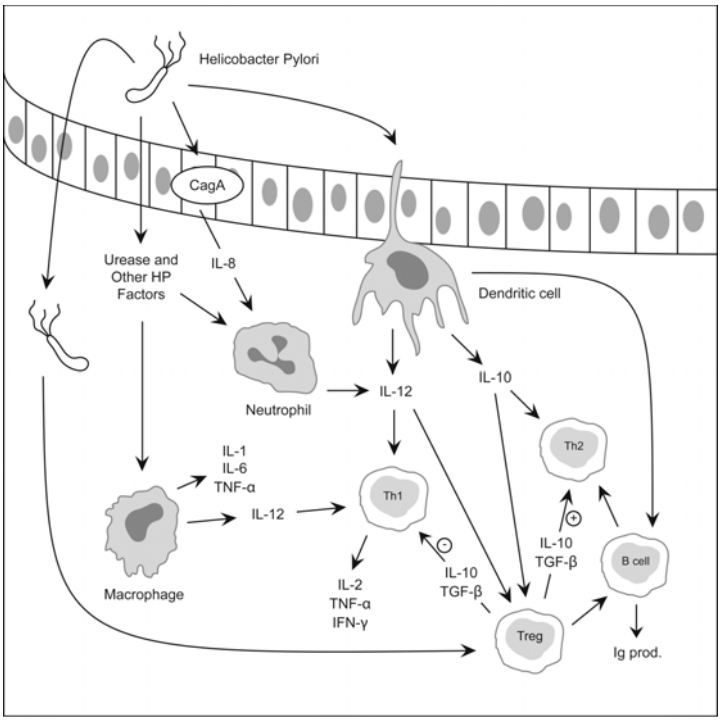

Fig 1*: Schematic figure of the immune response to H. pylori. The infection activates neutrophils and macrophages as well as T- and B-cells. A number of interleukins and other cytokines are involved in the interplay between the cells.*

The enigma

The reasons why the bacterium can persist in the stomach despite a strong immune response are multifaceted. The inability to clear the infection may for example be related to insufficient activation of dendritic cells (DC). It may be that in chronic *H. pylori* infection DC´s become inhibited by the prolonged antigen exposure leading to suboptimal Th1 development (66). Furthermore *H. pylori* persistence may be related to the presence of *H. pylori*-specific regulatory

T cells that actively suppress $CD4^+$ T-cell responses resulting in an ineffective immune response (Fig1) (64).
In this thesis focus is on the possible ability of the microbe to inhibit the nitro- and oxy-radical systems. To understand the interaction between the microbe, and the radical forming systems a brief description of the latter is given below.

RADICALS

What is a radical?

Oxidation is gain of oxygen or loss of an electron by a substance. *Reduction* is loss of oxygen, the gain of an electron or hydrogen by a substance.
An *oxidizing agent* takes an electron or hydrogen from another chemical, or adds oxygen. A *reducing agent* supplies electrons or hydrogen to another chemical, or removes oxygen.
Electrons are most stable when they are paired in their orbits. Unpaired electrons are more reactive as they are attracted to magnetic fields. The definition of a free radical is a substance that has unpaired electrons and is capable of independent existence (67).
By this definition atomic hydrogen (H) is a free radical because it only has one electron, and O_2 is a radical because it has two unpaired electrons in its outer orbital. Although O_2 is reactive it is a quite stable state of oxygen. Superoxide (O_2^-) has an additional electron, and is more reactive than O_2.

The oxy-radical system

An often-used term is ***reactive oxygen species (ROS***). This term includes true radicals that have unpaired electrons as well as chemicals that do not have unpaired electrons but still can take part in radical type reactions (gain or loose electrons) (67). Examples of non-radical ROS are hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and ozone (O_3).
Oxidative stress is a reality in most living organisms. Oxidative stress is a general term used to describe the steady state of oxidative damage in a cell, tissue, or organ, caused by ROS. The oxidative stress is caused by an imbalance between the production of ROS and the system's ability to detoxify the reactive species or easily repair the resulting damage. In humans, oxidative stress is involved in the development of many diseases e.g. atherosclerosis and cancer. However, ROS are also beneficial as they are used in the immune response to attack and eliminate pathogens. There are many different sources by which ROS are generated. Reduction of molecular oxygen generates a mixture of O_2^- and H_2O_2, which represents the major sources of intracellular ROS (68). Among the large number of enzymes and molecules involved in oxidative stress there are a few enzymes and products playing a central role. As shown in Figure 2 the most

important enzymes are nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), superoxide dismutase (SOD), myeloperoxidase (MPO) and nitric oxide syntase (NOS). These enzymes give rise to four products; O_2^-, H_2O_2, nitric oxide (NO) and hypocloric acid (HOCl). All the large number of oxidants produced from phagocytes arises from reactions involving these four compounds (69).

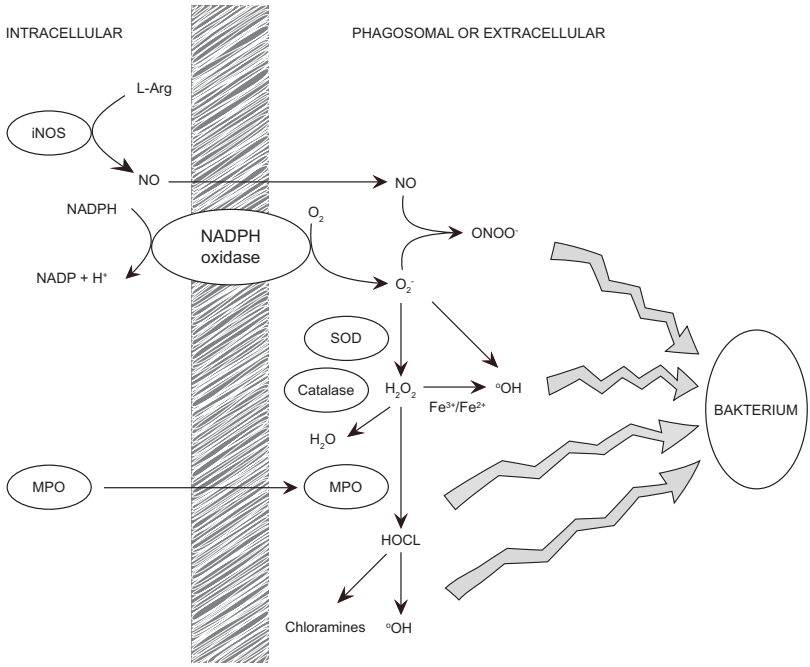

Figure 2: *Schematic of the most important players in the nitro- and oxy-radical systems. Several ROS and RNI have potent bactericidal effects.*

The role of phagocytes in host defence was discovered and described by Elie Metchnikoff in 1883 (70). The role of oxygen in phagocytosis was first described by Baldridge and Gerrard as the "extra respiration of phagocytosis" in 1933 (71). They found that neutrophils demonstrated a dramatic increase in oxygen uptake, a so-called "respiratory burst", when phagocyting bacteria. In 1964 Rossi and Zatti proposed that ***NADPH oxidase*** was responsible for the respiratory burst of phagocyte (72). In 1973 it was reported that the initial product of the respiratory burst was O_2^- (73). We now know that ROS are produced as a consequence of NADPH oxidase activity (74). NADPH oxidase is a transmembrane electron transport chain. It is found in phagocytes including neutrophils, eosinophils, monocytes and macrophages. It was originally thought that this system was restricted only to phagocytes and used solely in host

defence (75). However it is now known that different members in the NADPH oxidase family are present in a variety of nonphagocytic cells of leukocyte and nonleukocyte origin (76). The most studied form is the phagocyte NADPH oxidase. The enzyme is built up of five components $P40^{phox}$, $p47^{phox}$, $p67^{phox}$, $p22^{phox}$ and $gp91^{phox}$ (Nox2). When inactive three of the components including $p47^{phox}$ exist in the cytosol as a complex. When the cell is exposed to stimuli, the cytosolic components become phosphorylated and migrate to the membrane and form the active enzyme (77). The active NADPH oxidase catalyzes the production of O_2^- by the one-electron reduction of oxygen, using NADPH as the electron donor. The O_2^- produced serves as starting material for the production of different ROS. O_2^- reacts with one of the most fundamental antioxidant enzymes ***superoxidedismutase (SOD)***. SOD catalyzes the reaction between two O_2^- and two H^+ to H_2O_2 and O_2 (78, 79). The reaction is called dismutation, because O_2^- reacts with itself to give an oxidised product O_2 and a reduced product H_2O_2. At high concentrations O_2^- can dismute spontaneously, but the presence of SOD results in a much lower steady state intracellular concentration of O_2^- (69). The product H_2O_2 is not a radical, but by definition a ROS and may be responsible for many of the O_2^- reactions.

Myeloperoxidase (MPO) is concluded to play an important role in the microbicidal activity of phagocytes. MPO is a haeme containing enzyme similar to that found in haemoglobin. Unlike haemoglobin however MPO is green not red, and is the substance that gives the greenish colour to pus (69).MPO is released from cytoplasmatic granules of neutrophiles and monocytes by a degranulation process. It reacts with H_2O_2 formed by the respiratory burst to form a complex that can oxidise a large variety of substances. (80) The initial product of the MPO-H_2O_2-chloride system is as mentioned the strong non-radical oxidant HOCl.
The MPO induced bactericidal mode of action starts with the activation of neutrophils. The primary function of the neutrophils is the phagocytosis and destruction of microorganisms. The bacterium is ingested into the phagosome. When MPO and H_2O_2 are released into the phagosome the microbicidal effect is often rapid, with bacterial dying within milliseconds. (80, 81). The targets, of MPO system, on the bacteria include the adenosine triphosphate (ATP) generating systems, and the origin of the replication site of DNA synthesis, which appears to be the most sensitive area (82, 83). If MPO and H_2O_2 are released outside the cell they react with extracellular chloride. This reaction can induce tissue damage and contribute to the pathogenesis of disease (80)

The most recognized of the oxy-radicals is ***superoxide (O_2^-)***. The first step in the metabolism of the oxygen molecule is addition of an electron forming O_2^-. O_2^- is a radical, but it is not very reactive, nor is it a powerful oxidant. However, because O_2^- is formed in large amounts it has important impact.
In phagocytic cells the major source of O_2^- is production via NADPH oxidase. Several other enzymes contribute to the O_2^- production, for example xanthine

oxidase, which is present in the cytosol of many tissues, and in circulating blood (84). Xanthine oxidase can be involved in the production of O_2^- in ischemia/reperfusion and sepsis (85). Cytochrome P450 enzymes can produce O_2^- during special conditions (67). Interestingly nitric oxide synthases (NOS) can produce O_2^- when L-arginine, the substrate for NO production, is insufficient (86).

Hydrogen peroxide (H_2O_2) is more stable than O_2^- and can diffuse across membranes. Several enzymes can form H_2O_2, some of these via a O_2^- intermediate and others without the formation of O_2^-. As H_2O_2 is constantly generated, has a relatively long biological half-life, and is highly biomembrane permeable it must under normal conditions immediately be neutralized at the site of production to prevent diffusion through the cell or to the extra cellular space. Examples of H_2O_2 neutralizing anti-oxidant enzymes are catalase and glutathione peroxidase (87).
If accumulated H_2O_2 will diffuse from its site of production and be a part of the generation of other powerful members of ROS. If MPO is present, for example in the neutrophil phagosome, MPO will catalyze the formation of HOCl from chloride and H_2O_2. Alternatively H_2O_2 will react with free iron (Fe^{2+}) to form the powerful hydroxyl radical (°OH) in the following reaction:

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + {}^\circ OH$$

In biological fluids the concentration of free iron is limited. The free iron needed can then be formed by reducing the ferric iron Fe^{3+} by O_2^- as follows:

$$O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2$$

Putting these two together, the sum of the reactions will be:

$$H_2O_2 + O_2^- \rightarrow O_2 + OH^- + {}^\circ OH$$

This reaction is called the O_2^- driven Fenton reaction or the Haber-Weiss reaction (88) (89). Iron is required for the reaction, as H_2O_2 and O_2^- do not interact directly in an appreciable rate (90). In summary H_2O_2 will react either with MPO and chloride to form HOCl or with O2- to form °OH.

Kinetics of °OH and HOCl:. °OH is an extremely reactive radical, which will react with essentially the first molecule it meets. It may cause DNA modification and strand breaks, enzyme inactivation, lipid peroxidation and can result in generation of secondary radicals (91). For °OH to be effective in the struggle against microorganisms it needs to be formed in the immediate vicinity of the target on the bacterial surface, because otherwise it will react before it ever gets to the target (90). However, most of the H_2O_2 will not react directly with O_2^- forming °OH, but be consumed by MPO to form HOCl. HOCl is a non-radical

oxidant with a wide range of ways to react. It is the most bactericidal oxidant known to be produced by the neutrophil and is more selective in its reactions than °OH (92). If HOCl reacts with O_2^- the result will be production of °OH (91).

The nitro-radical system

The term ***reactive nitrogen intermediates (RNI)*** is used to describe another class of oxidants. Principally RNI are oxidation intermediates of the nitrogenous products of nitric oxide synthase (NOS), being mainly nitric oxide (NO), nitrogen dioxide (NO_2^-), S-nitrosothiols (RSNO) and peroxynitrite ($ONOO^-$) (93, 94).
Oxidation of arginin by NOS creates the gas NO. Three distinct isoforms of NOS have been characterized. Two are constitutively expressed, calcium dependent isoforms: neuronal NOS (nNOS or NOS-1) and endothelial NOS (eNOS or NOS-3) primarily involved in signal transduction. The third NOS is an inducible, calcium independent isoform: inducible NOS (iNOS or NOS-2) and is involved in host defence thus of particular interest in this thesis (95-97) .

iNOS was first identified in macrophages, in which its formation was shown to be induced by cytokines and other substances commonly associated to infections. However it is now known that a variety of cells, including macrophages, astrocytes, hepatocytes, and monocytes, can express iNOS in response to exposure to cytokines, endotoxines and/or oxidative stress (98). The iNOS gene has been localized to the region of chromosome 17q0-12. Expression of the enzyme requires a combination of signals. IFN-γ, lipopolysacharide (LPS), interleukin-1 (IL-1) and cyclic adenosine mononucleotide phosphate (cAMP) are examples of substances that may induce iNOS expression (99, 100). iNOS gene expression is also regulated by the transcript factors NF-κB, the Janus kinase (JAK), signal transducer and activator of transcription (STAT) among others. Known inhibitors of iNOS include dexamethasone and transforming growth factor-β (TGF-β) and others(101). NO itself is an inhibitor of iNOS expression.
The traditional view of NOS is that the two constitutive forms produce NO in low concentrations during physiological conditions, and that iNOS during pathophysiolocial events, sometimes over a long period of time, produces NO in very high “cytotoxic” concentrations (102). However, this is a much to simplified picture of iNOS. There are several reports of iNOS expression during non-stimulated conditions in for example the duodenum, jejunum and airways. They are then involved in physiological regulatory processes, and thus not associated to host defence or tissue restitution (42, 103-105).

Nitric oxide (NO) was brought to the attention of biomedical-scientists in 1985 when it was found that E-coli LPS induced the production of inorganic nitrate

and nitrite by LPS-sensitive mouse macrophages (106). In 1987 it was found that the endothelium-derived relaxing factor (EDRF) was in fact NO (107, 108). Some years later it was demonstrated that NO and citrulline are the degradation products of L-arginine, and that NO has its action in a large variety of physiological processes such as neurotransmission, immune function, secretion, haemostasis, vascular tone, cardiac contractility and intestinal peristalsis (96, 109) . NO also was ascribed important roles in pathologic conditions such as septic shock, atherosclerosis, hypertension, ischemia/reperfusion injury, and carcinogenesis (109). NO can be formed via enzymatic reactions as described, but large amounts of NO is also produced non-enzymatically. High concentration of nitrite from the saliva is reduced when it meets the extremely low pH in the stomach. This results in the production of NO and a variety of other nitrogen species (110). The non-enzymatic NO production is probably important for intragastric clearance of digested microorganisms (111). NO reacts rapidly with O_2^- to produce the extremely active radical ***peroxynitrite (ONOO$^-$)***. It may act as an oxidant, or isomerise to nitrate, or protonate and dissociate to give nitrogen dioxide (NO_2) and the hydroxyl radical (OH°) (98). $ONOO^-$ has effects on mitochondrial respiration and can potentially oxidise most vital functions of the mitochondria. It causes oxidation and cross linking of proteins, inhibition of mitochondrial complexes, nitration of tyrosine residues, oxidation of non-protein thiols, oxidation of membrane lipids, and disruption of the cell/organelle membranes (98). As an end product of the nitration of tyrosine residues the more stable nitrotyrosine is formed (described in more detail below). $ONOO^-$ is a primary trigger of multiple forms of DNA damage such as base modifications, single strand breakage, and apoptotic double strand breakage. The DNA damage, in turn, can lead to acute cellular injury via acute cell necrosis or delayed apoptotic cell death (112). Persistent DNA damage, during chronic inflammation, may contribute to increased risk of cancer development.

Nitration of tyrosine results in ***nitrotyrosine***. The nitrate is mainly donated by $ONOO^-$. However an important aspect about nitrotyrosine formation is that it is not solely generated by $ONOO^-$. Nitrite (NO_2^-) can be oxidized to nitrogen dioxide (NO_2) or to nitryl chloride (NO_2Cl). NO_2 and NO_2Cl can then nitrogenate tyrosine to form nitrotyrosine. As shown in Figure 3 both iNOS and MPO activity may be involved in the formation of nitrotyrosine (113, 114). Nitrotyrosine is a biologically active molecule, however it is much more stable than the very reactive $ONOO^-$. Consequently nitrotyrosine can be used as a marker of nitroradical and $ONOO^-$ activity (115). Nitrotyrosine appears as tyrosine in both free and protein-bound forms.

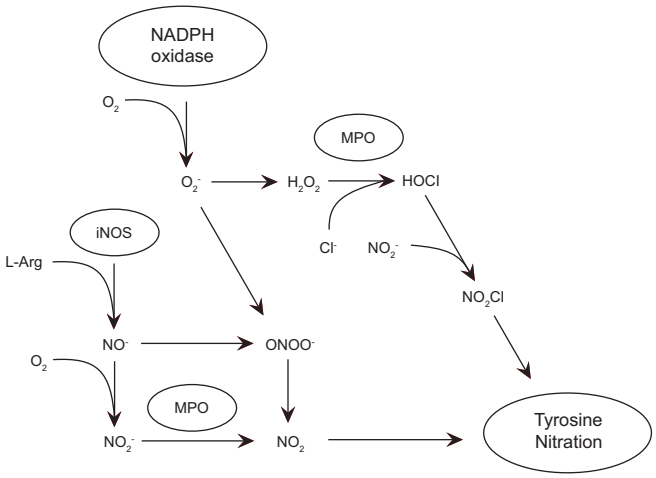

Figure 3: *Nitrotyrosine can be used as a marker of nitroradical and $ONOO^-$ activity. Nitrotyrosine formation is not solely generated by $ONOO^-$. Both iNOS and MPO activity may be involved in the formation of nitrotyrosine.*

THE RENIN-ANGIOTENSIN SYSTEM (RAS)

As mentioned *H. pylori* infection is obligately followed by a profound inflammatory process of the infected mucosa. The nitro- and oxy-radical systems are as described above important in the host defence trying to eliminate the bacteria. During recent years it has become obvious that the renin-angiotensin system (RAS) apart from its effect on body fluid homeostasis is also involved in several pathological conditions e.g. inflammation, wound healing and carcinogenesis (116, 117). The classical endocrine part of RAS with effects on hemodynamic regulation is well described. Less is known about the tissue-based and immuno-modulatory character of this system demonstrated in a number of organs e.g. brain, kidney, adrenals, pancreas, liver and colon. It was considered of interest to investigate if the RAS could be associated with *H. pylori* infection, a brief overview of RAS is therefore given below.

Angiotensin II (Ang II) is the RAS primary biologically active peptide, although other angiotensins exists and may exert biological actions. Ang II is an octapeptide produced by the cleavage of the decapeptide Angiotensin I (Ang I) by Angiotensin I-converting enzyme (ACE). Ang I is produced by cleavage of Angiotensinogen by renin (Fig 4). Ang II works principally through two separate receptors designated Ang II type 1 receptor (AT1R) and Ang II type 2 receptor (AT2R). Well-described actions of Ang II such as vasoconstriction, aldosterone release and cellular proliferation are mediated by the AT1R. In some species (e.g. rodents) there are two types of AT1 receptors; AT1a and AT1b, with slightly different effects. The AT2R is known to be active mainly in fetal tissue

but has been shown to influence adult tissues as well. The highest concentrations of AT2 receptors are found in fetal mesenchymal tissue, adrenal medulla, uterus and ovarian follicles. Several studies indicate that activation of the AT2R has opposing effects to those mediated by the AT1R, thus modulating the responses to stimulation with Ang II (118).

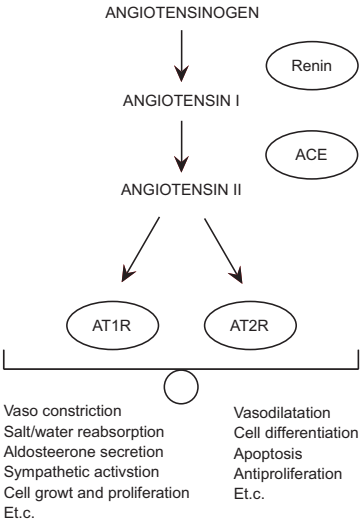

Figure 4: *The classical view of RAS. The proteolytic enzyme renin cleaves angiotensinogen into angiotensin I, wich is transformed into angiotensin II by ACE. Angiotensin II works principally trough two separate receptors AT1R and AT2R.*

Angiotensin II receptors and inflammation

Data are accumulating showing that the Ang II is to be recognised as a modulator of inflammatory reactions. Local increase of vascular permeability is the most important event in the initial phase of inflammation. Ang II increases vascular permeability directly or via the release of cox-metabolits such as leukotriene C4, prostaglandin E2 (PGE2) (116, 119, 120).
Furthermore, Ang II via the AT1R is involved in inducing synthesis of pro-inflammatory mediators e.g. Il-6, Il-12, that participate in signalling pathways via activation of the transcript factor NF-κB and various protein kinases (116). A number of other vital molecules in inflammatory processes are induced by the AT1 receptor, examples being IL1-β and tumour necrosis factor- alpha (TNF-α) (121). Interestingly several of these factors are also active in various phases of cancer development.
The AT2R is also involved in regulation of inflammation. In experimentally induced glomerulonefritis both AT1R and AT2R have been shown participate in the regulation of inflammation via the NF-κB pathway. It has been suggested

that the AT2R activation attenuates inflammation to balance the AT1R activity, but AT2R has also been reported to have some pro-inflammatory qualities. Regulation of the antagonistic characteristics of AT1R and AT2R have been suggested to be a central process in the management of inflammation and wound recovery, and an imbalance in the expression of these receptors may lead to disease (121-123).

Angiotensin II receptors and cancer

Data are accumulating indicating a connection between the RAS and cancer development (117, 121). Over-expression of AT1 is reported in association with cancer of the breast, pancreas, kidney, skin, larynx, adrenal gland and lung (121). AT2 overexpression has been described in at least one paper on colorectal cancer (121). There seems to be a connection between hypoxia/oxidative stress in the growing tumour and expression of different AT receptors. Poor blood supply leads to hypoxia and necrosis, which leads to increased AT1b expression, whereas re-oxygenation leads to oxidative stress and increased AT1a expression in experimental carcinogenesis (121). Expression of both AT1a and AT1b will lead to inflammation- associated angiogenesis, immune suppression and metastases.

Angiotensin II receptors and gastric pathology

Until recently the role of RAS in gastric physiology and disease had been sparsely explored. Some studies have reported actions of Ang II on mucosal blood flow, acid secretion and smooth-muscle contraction. Others have implicated the influence of RAS in gastric ulcer disease (124). However, several recent reports have pointed out connections between the RAS and gastric cancer. An association between polymorphism in the ACE gene and the incidence of *H. pylori* related gastric cancer was recently reported (125, 126). Another study shows that ACE is expressed locally in gastric cancer and that the ACE gene polymorphism influences metastatic behaviour (127).

Oxy-radicals, nitro-radicals and angiotensin II receptors

Ang II has a strong relationship to oxy-radical formation. For example the $p47^{phox}$ subunit, one of the important subunits of NADPH oxidase is phosphorylated by Ang II. Stimulation of Ang II increases the NADPH oxidase activity and results in an increased production of O_2^- . It follows that Ang II is regarded as a pro-oxidant (128). Activation of AT2 receptors in an animal model increase luminal NO output in un-inflamed jejunum. This response was not due to an increase in the expression of iNOS in the mucosa indicating that AT2R stimulation may facilitate the L-arginine/NO pathway on the enzymatic level (105).

OVERALL OBJECTIVES

The general aim of the present thesis was to elucidate how *H. pylori* avoid being eliminated by the host's defence systems. The microbe-associated interferences with the reactive oxygen and nitrogen radical species produced by the mucosa in response to infection was considered to be of particular interest. As described, *H. pylori* infection of the stomach obligately results in mucosal inflammation with a marked host immune response including activation of several pro-inflammatory and bactericidal systems. Despite this profound host response the bacterium avoids elimination and survives in the mucosa. This results in a concomitant chronic inflammation of the gastric mucosa. One theory is that *H. pylori* possesses an ability to inhibit the host defence mechanisms. Previous results from our laboratory indicate that *H. pylori* restricts gastric NO production by pathogen-derived competitive iNOS inhibitors (129, 130). It was considered of great interest to further investigate the possibility of *H. pylori* related functional inhibition of not only the nitro- radical formation, but also interactions with the oxy-radical formation.
Furthermore, because the angiotensin II system has been ascribed immunomodulatory actions, it was also considered of interest to explore if the pathogen influences the presence and location of this regulatory system in the gastric mucosa.

An important methodological consideration

H. pylori usually colonises during childhood and persists as a lifelong chronic infection. The associated morbibities develop over decades, if ever, and a study over time of their mechanisms of action is thus principally impossible in man. The investigational strategy of the present thesis project, therefore, was a translational approach based on an animal model with short lifespan and mimicking the human pathological response to *H. pylori*. The dynamics of the infection versus host defences could then be studied over a reasonable time and novel results could be confirmed/discarded in the human setting as represented by cohorts of infected as well as uninfected people.

The choice of experimental model

Soon after the acceptance of *H. pylori* as a major human pathogen it was found necessary to develop an animal model to elucidate the pathogenic mechanisms of the bacteria, and to improve the treatment of associated disease. Several animal models have been presented, e.g. the pig and cat, but rodent models were best suited for quantitative studies and have been widely used (131-133).

Probably the most used animals are mice. One advantage of using mice is the possibility of using different transgenic mice with alterations in various pathways including immune response and tumour suppressors. Specific gene targeting together with *H. pylori* infection in mice may be followed by adenocarcinoma (134-136) . Carcinoma has also been demonstrated in wild type mice following the introduction of chemical carcinogens, but not as a result of *H. pylori* infection alone (137, 138). When taken together it can be concluded that mice are not the perfect animal model for long term studies of *H. pylori*-associated carcinogenesis. In 1998 both Watanabe et al and Honda et al presented studies where they claimed that the *Mongolian gerbil* was shown to develop gastric adenocarcinoma following infection with *H. pylori* alone without the use of chemical agents (139, 140). The Mongolian gerbil, also known as the desert rat, is actually not a rat. The subfamily Gerbillinae belong to the family Muridae along with mice and rats, and their relatives. The Mongolian gerbil differs in many respects from both rats and mice. For example, since the gerbils get most of their water from their food they do not need to drink. In 1954, Dr. Victor Schwentker brought nine Mongolian gerbils to the USA for scientific work. Since then the gerbil has been widely used both as a laboratory animal and as a pet. Gerbils range in length from 8 to 12 cm, plus a long tail, and have a weight up to 200 grams. The lifespan in the wild is 3-4 years. Because of almost ideal characteristics, including carcinogenesis, the *H. pylori* infected Mongolian gerbil was chosen as model for *H. pylori* related pathology in the present thesis project.

AIMS

The specific aims for this thesis were:

- to validate the suitability of Mongolian gerbils as an experimental model for studies of *H. pylori* induced gastric mucosal pathology,

- to elucidate a possible *H. pylori* strain dependency on the expression of the oxy- and nitro-radical forming enzymes in Mongolian gerbils,

- to investigate whether *H. pylori* infection in Mongolian gerbils results in inhibition of either or both of the nitro- and oxy-radical formation *in situ*,

- to confirm the existence of *H. pylori* related interferences with the nitro- and oxy-radical systems in humans, and

- to investigate the presence, location and expression of angiotensin II receptors in the stomach of non-infected and *H. pylori* infected Mongolian gerbils.

REVIEW OF RESULTS AND COMMENTS

Is the Mongolian gerbil a suitable experimental model for *H. pylori* induced gastric pathology?

A total of 70 seven week old pathogen free Mongolian gerbils were included, and divided into three groups (I). Two of the groups were inoculated with *H. pylori*, one group with the strain TN2GF4 and the other with the strain SS1 suspended in Brucella broth. The third group was sham inoculated with Brucella broth only. The *H. pylori* strains used for inoculation were chosen from the expectation of TN2GF4 being a more virulent strain than SS1. The former strain was originally isolated from a patient with gastric ulcer, and was reported to be motile and urease, catalase, and oxidase positive, as well as vacA and cagA positive (139). Of particular interest was that the TN2GF4 strain had been reported to induce adenocarcinoma in Mongolian gerbils (139). The Sydney strain of *H. pylori* (SS1) was introduced in 1997 by Lee et al for experimental studies primarily on mice (141). The original human isolate came from a 42-year-old Greek-born woman living in Australia. Her medical history included abdominal pain and peptic ulcer disease. Since 1997 the SS1 strain has been widely used in experimental studies allowing interlaboratory comparison of data. When the SS1 strain was introduced for animal studies it was described as a cagA and vacA positive genotype (7). However it has recently been suggested that SS1 probably is functionally negative for both cagA and vacA, (142, 143) .

Groups of animals from each treatment arm were sacrificed 3, 6, 12 and 18 months after inoculation. At the time of sacrifice the stomach was removed, and one half of the stomach was used for culture, and a part of the other half for histopathological examination. *H. pylori* infection was confirmed by analysing the growth of bacteria and was detected in all animals up to 12 months. At 18 months the *H. pylori* growth persisted in the SS1 group, whereas in the TN2GF4 group an overgrowth of other bacteria, interpreted as lactobacilli occurred. Lactobacilli are known to be able to colonize stomachs of *H. pylori* infected Mongolian gerbils (144). In the controls *H. pylori* was not detected in any of the animals at any time during the study.

Histological diagnoses were performed in a blinded fashion by a team of three independent highly experienced gastrointestinal histopathologists. The updated Sydney System was used to score inflammation (122). The scored variables were: 1. grade of chronicity and activity of inflammation, 2. presence of intestinal metaplasia, 3. lymphoid follicles and aggregates, and 4. grade of *H. pylori* colonization. Ulceration was defined as a necrosis reaching the submucosal layer. To define a neoplasia the criteria by the WHO classification were used (145). As shown in Table 1 both the infected groups exhibited a

strong active inflammation, but with an earlier onset in the TN2GF4 group. Gastric ulcers were found only in animals infected with the TN2GF4 strain. Intestinal metaplasia was observed in both infected groups. Glands buried in the submucosal layer were found in both the infected groups, but in a higher percentage in the animals infected with the more virulent TN2GF4 strain. The nuclei of the buried glands showed regenerative abnormalities, but no neoplastic changes. Invasive adenocarcinoma or other neoplastic changes fulfilling the WHO criteria were not found in any of the gerbils at any time of the study (145).

***Table 1**:Histopathological findings in each group at different times after inoculation (I)*

	3 months			6 months			12 months			18 months		
	Control (n=5)	SS1 (n=4)	TN2GF4 (n=3)	Control (n=5)	SS1 (n=5)	TN2GF4 (n=5)	Control (n=5)	SS1 (n=5)	TN2GF4 (n=5)	Control (n=3)	SS1 (n=7)	TN2GF4 (n=10)
Body weight# (g)	102 (±6)	87 (±3)	96 (±11)	90 (±x)	83 (±x)	69* (±x)	111 (±3)	90 (±4)	71** (±3)	107 (±x)	106 (±x)	80** (±x)
H. pylori positive cultures	0/5	4/4	3/3	0/5	5/5	5/5	0/5	5/5	5/5	0/3	7/7	0/7
Inflammation score##												
Grade of activity	0.0	1.0	2.0	0.0	1.0	2.0	0.0	3.0	2.0	0.0	2.0	2.0
Grade of chronicity	0.0	1.5	2.0	0.0	2.0	2.0	0.0	2.0	2.0	0.0	2.0	2.0
Intestinal metaplasia	0	1/4	0	0	2/5	1/5	0	0	2/5	0	4/7	3/10
Gastric ulcers	0	0	1/3	0	0	3/5	0	0	5/5	0	0	4/10
Buried glands	0	0	0	0	0	3/5	0	1/4	4/5	0	1/7	8/10

*# body weights expressed as means ± SEM. , **p<0.01, *p<0.05*
##Numbers are median values

Comments: In the previous reports of adenocarcinoma following long-term infection with *H. pylori* by Watanabe et al and Honda et al, invasive neoplasia was diagnosed by the presence of atypical glands that invaded the proper muscular layer (139, 140). In the present study with an observation period extended up to 18 months, we also found glands in association to the muscularis propria. However, these glands did not show any atypical structural changes speaking towards malign transformation. The histopathological picture rather appeared as the healing state following gastric ulcerations. There is an obvious risk misinterpreting the finding of buried glands and ecstatic mucinous cysts in the submucosal layer and the muscularis propria for being adenocarcinoma. Our results thus do not speak in favour of that the Mongolian gerbils develop adenocarcinoma. Furthermore it has not been reported that experimentally *H. pylori* infected Mongolian gerbils diagnosed with adenocarcinoma have

developed local or distant lymph node metastasis, or that any Mongolian gerbils have died of gastric cancer(146).
In summary infection of Mongolian gerbils with two different *H. pylori* strains resulted in development of histopathological changes such as gastritis, gastric ulcers, intestinal metaplasia and glands buried in the submucosal layer. The onset of inflammation was earlier, and the number of animals developing pathological changes was higher, in the animals infected with *H. pylori* strain TN2GF4, compared to the ones infected with the SS1 strain. No animals developed neoplastic changes fulfilling the criteria of the WHO classification of tumours in the gastrointestinal tract.
In conclusion, our results indicate that Mongolian gerbils infected with *H. pylori* has not yet been confirmed being a cancer model but are suitable for studies of acute and chronic mucosal inflammation.

Is expression of the oxy- and nitro-radical forming enzymes in Mongolian gerbils dependent on the infecting *H. pylori* strain?

In paper II gastric tissues were analysed with regard to expression of iNOS and MPO. These two enzymes were considered as adequate representatives of the nitro- and oxy-radical forming pathways, respectively. Tissue specimens were obtained from the animals described in paper I, thus 5 animals from each group were sacrificed 3,6,12 or 18 months after inoculation. As a result of early deaths for other reasons, some of the groups contained fewer than five animals. At the time of sacrifice the stomachs were opened and full thickness wall specimens were immediately collected and snap frozen. Western blot analyses were performed using commercially available antibodies with confirmed selectivity. To optimize the comparison between results from different time points, and to be able to make a legible graphic presentation, the results were calculated as a percentage of the mean data from the uninfected control group obtained during each Western blot procedure. This can only be done if there is a certainty that the expressions of iNOS and MPO in the uninfected controls are stable over time. Western blot analyses of iNOS and MPO expressions in control animals of different time points were carried out separately and showed no variation between the groups

Both iNOS and MPO expression were markedly upregulated in the corpus and antrum areas of the *H. pylori* infected animals as compared to non-infected controls. However, the time course differed between the two tested strains. The TN2GF4 infected animals exhibited a rapid and very marked increase in iNOS expression that then rapidly declined, whereas SSI-infected animals had a slower onset of the increase in iNOS expression both in the corpus and antrum regions (Fig 5).

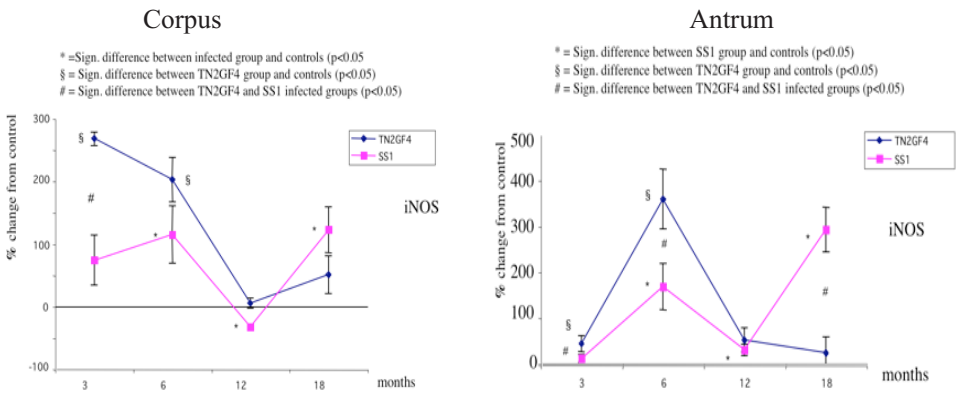

Figure 5. *Results from Western blot showing expression of iNOS in gerbils infected with the H. pylori strains TN2GF4 and SS1. The uninfected group at each time was used as a baseline. Data are presented as the percentage change in each infected group from the mean value of the uninfected controls at each time, and are plotted as group means ± SEM.*

In both the infected groups MPO expression did initially increase to very high levels compared to controls. The TNF2G4 infected group showed a significantly more pronounced MPO expression up to 12 months compared to the SSI group (Fig 6). Interestingly, at 18 months the MPO expression in the TN2GF4 infected animals did not differ from controls, but the SS1 infected animals were still at significantly higher level in both the corpus and antrum areas (Fig 6).

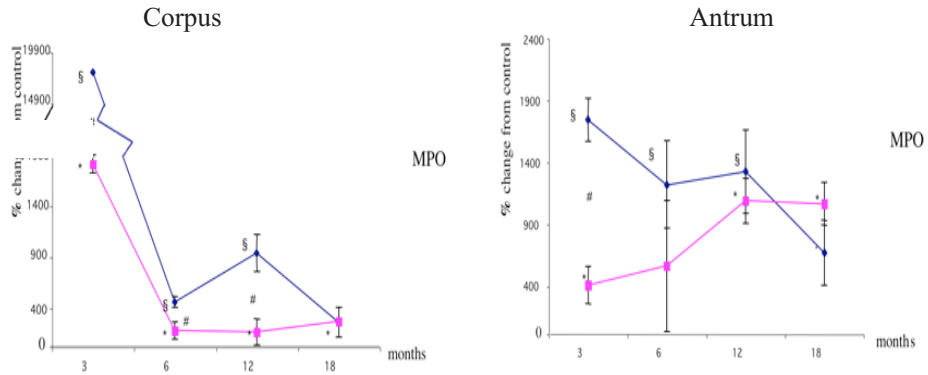

Figure 6: *Results from Western blot showing expression of MPO in gerbils infected with the H. pylori strains TN2GF4 and SS1. The uninfected group at each time was used as a baseline. Data are presented as the percentage change in each infected group from the mean value of the uninfected controls at each time, and are plotted as group means ± SEM. (Significant differences are indicated in figure, and are explained in Figure 5)*

Comments: The inoculation of Mongolian gerbils with either of the two different *H. pylori* strains SS1 and TN2GF4 resulted in a markedly up-regulation of enzymes involved in both the nitro- and oxy-radical formation. Several groups have shown previously that *H. pylori* infection leads to increased expression of nitro- and oxyradical producing enzymes (129, 147). The use of two different strains in this study made it possible to elucidate a strain dependency in the ability to induce enzyme expression. The finding that infection with *H. pylori* strain TN2GF4 results in a prompt upregulation of iNOS and MPO expression is in concordance with the finding of an earlier onset of inflammation in this group compared to the SS1-infected group (I). There seems to be a direct relationship between the inflammatory response and the expression of radical producing enzymes in the early stages of infection. After 18 months of infection the histological picture of inflammatory activity is almost equal in both infected groups (I). However, at this time point these results indicate a stronger up-regulation of enzyme expression in the animals infected with the less virulent *H. pylori* strain. It should be noted, though, that the decreased enzyme expression observed in the TN2GF4-group occurred simultaneously with that the *H. pylori* infection had disappeared.
In conclusion: the lapse of time of *H. pylori* induced up-regulation of nitro- and oxy-radical forming enzymes in Mongolian gerbils is dependent on the infecting strain.

Does *H. pylori* infection in Mongolian gerbils result in inhibition of either or both of the nitro- and oxy-radical forming pathways?

Two different strategies were used. In paper II quantification of nitrotyrosine was used as a marker for $ONOO^-$ production, and in paper III the actual juxtamucosal levels of NO and H_2O_2 were assessed *in vivo* using microelektrodes.

Nitrotyrosine measurements: Nitrotyrosine formation in the corpus region was elevated in the TN2GF4 group at 3 and 6 months but returned to levels not significantly from control at 12 and 18 months (Fig 7). In the SS1 infected group nitrotyrosine formation was not different from control at 3 and 6 months but was then reduced to levels significantly below control (Fig 7). Nitrotyrosine formation in the antral region was significantly elevated in both the TN2GF4 and SS1 infected groups at 3 and 6 months. However, as nitrotyrosine levels decreased to or below controls in the SS1 group it remained slightly above controls in the TN2GF4 group (Fig 7)

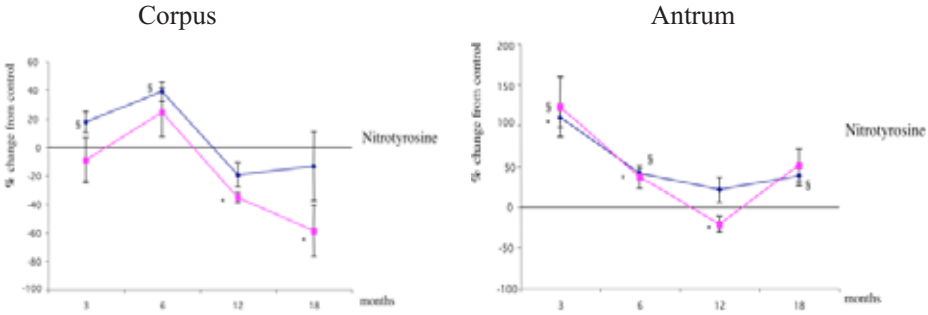

Figure 7: *Results from Western blot showing levels of nitrotyrosine in gerbils infected with the H. pylori strains TN2GF4 and SS1. The uninfected group at each time was used as a baseline. Data are presented as the percentage change in each infected group from the mean value of the uninfected controls at each time, and are plotted as group means ± SEM. (Significant differences are indicated in figure, and are explained in Figure 5. Lines represent TN2GF4 and SS1 groups respectively, as explained in Figure 5)*

Comment 1: The levels of nitrotyrosine were not increased in association to the up-regulated iNOS and MPO expression. These results indicate that there is an inhibitory action somewhere in the radical forming pathways.

To evaluate the $ONOO^-$ formation it would have been desirable to measure $ONOO^-$ levels directly in the gastric mucosa. $ONOO^-$ is however an extremely unstable radical and it is impossible to make a reliable quantification of $ONOO^-$ *in vivo*. Nitrotyrosine is a rather stable end product of nitration of tyrosine and can be used as a marker for $ONOO^-$ (115, 148). However, there are several reasons to be careful in the interpretation of the Western blot analysis of nitrotyrosine. Nitrotyrosine is not formed solely from the reaction between $ONOO^-$ and tyrosine, but there has been recognized involvement of nitrite and peroxidase enzymes as well, suggesting nitrotyrosine not to be considered a specific marker of $ONOO^-$ formation, but as a collective indicator for the involvement of reactive nitrogen species (113). Furthermore there is a risk for underestimation of the actual levels of nitrotyrosine due to temperature related reduction of nitrotyrosine during sample preparation (149). Such an underestimation seems unlikely as test series of Western blot analyses, involving samples from all three groups, using different preparation temperatures showed consistent results (unpublished data) (Fig 8).

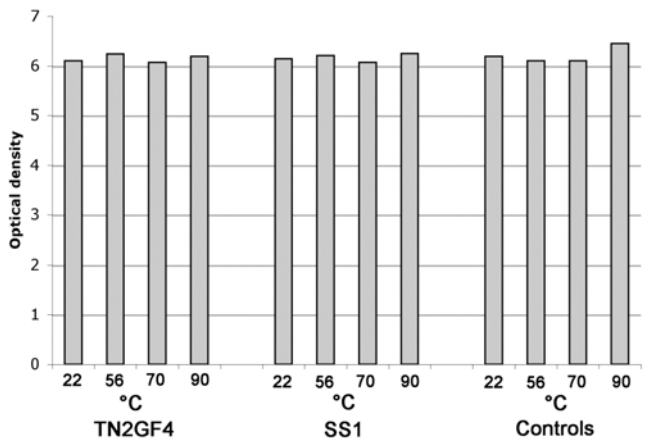

Figure 8: *Diagram showing results from Western blot analysis of nitrotyrosine using different temperatures during preparation. Western blot was performed on animals from all three groups at four different temperatures (22, 56, 70 and 90 °C). The data presented are from one Western blot gel with 12 different samples, each bar represents one sample. No temperature related differences were found.*

Radicals *in-situ:* The reduced or inhibited nitrotyrosine levels could be due to either the inhibition of NO production, or of oxy-radical formation or of both. To elucidate this issue direct *in vivo* recordings of NO and H_2O_2 in *H. pylori* infected Mongolian gerbils were performed. *In vivo* measurement of NO and H_2O_2 has the great advantage of being a direct measurement of unstable radicals in a living animal. It is however important to remember that anaesthesia and surgery may affect the results. The Apollo 4000 NO/Free Radical Analyzer was used, detecting nitric oxide and hydrogen peroxide in real time using electrochemical micro sensors. The technique has previously been described for use *in vivo* (150). The NO/Free Radical Analyzer was calibrated using solutions with known concentrations of NO and H_2O_2. Twenty-seven Mongolian gerbils in three groups were inoculated with the *H. pylori* strains TN2GF4 or SS1, or sham inoculated with Brucella broth only, similarly as in paper I. Six months after inoculation the animals were anasthetized and following laparotomy and gastrotomy the probes were introduced close to the mucosa of the distal stomach. In the animals infected with TN2GF4, the juxtamucosal level of NO was significantly lower compared to the controls (Fig 9). NO levels in the SS1 infected animals did not differ significantly from the controls or the TN2GF4 infected gerbils. The level of H_2O_2 was significantly higher in the SS1 infected animals compared to the controls. However there was no significant difference between the TN2GF4 infected animals and the controls (Fig 9)

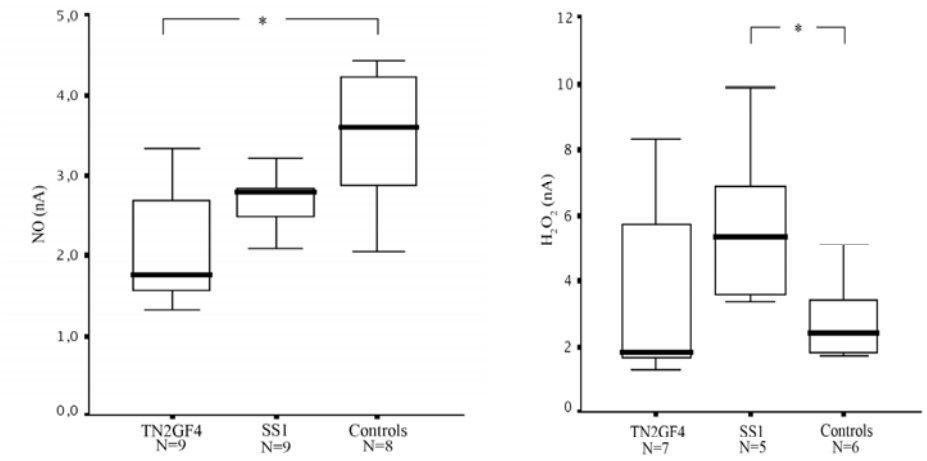

Figure 9. *Box-plots showing in vivo juxtamucosal levels of nitric oxide (left) and hydrogen peroxide (right) in gerbils infected with H. pylori strains TN2GF4 or SS1, and in controls. NO and H_2O_2 levels are presented in nanoampere (nA); (*=p<0.05)*

Comment 2: The results from paper III confirms that *H. pylori* infection is associated with reduced levels of intraluminal NO. The reduction in NO seems to be stronger in the animals infected with the more virulent *H. pylori* strain TN2GF4. Furthermore, the study showed significant increase of H_2O_2 in the animals infected with the less virulent *H. pylori* strain SS1, but not in the ones infected with the more virulent TN2GF4, compared to controls. These results together with the results from paper II showing low levels of nitrothyrosine in the *H. pylori* infected animals suggest that *H. pylori* is able to inhibit formation of both NO and oxy-radicals, but that a strain dependent effect on oxy-radicals may be present.
In conclusion *H. pylori* infection in Mongolian gerbils results in a strain-dependent inhibition of both the nitro- and oxy-radical formation.

Does *H. pylori* interfere with the nitro- and oxy-radical systems in humans?

Biopsies from the antral wall of 21 individuals, 13 *H. pylori* negative (mean age 39 yrs, f/m= 6/7) and 8 *H. pylori* positive (mean age 53 yrs, f/m = 3/5), were collected and snap frozen. Four individuals in the negative and one in the positive group had gastro-esophageal reflux. Two individuals in the positive group had developed duodenal ulcer. Western blot analyses of iNOS, MPO and nitrothyrosine was performed in a similar way as in Mongolian gerbils (II). The analyse was enlarged to also enclose the NADPH-oxidase subunit p47-phox.

As shown in Figure 10 Western blot analysis demonstrated an upregulation of iNOS, MPO and NADPH oxidase in biopsies from the antral region of subjects infected with *H. pylori*, compared to non-infected controls. However there was no significant change in nitrotyrosine formation in the antral region of the infected subjects compared to non-infected controls.

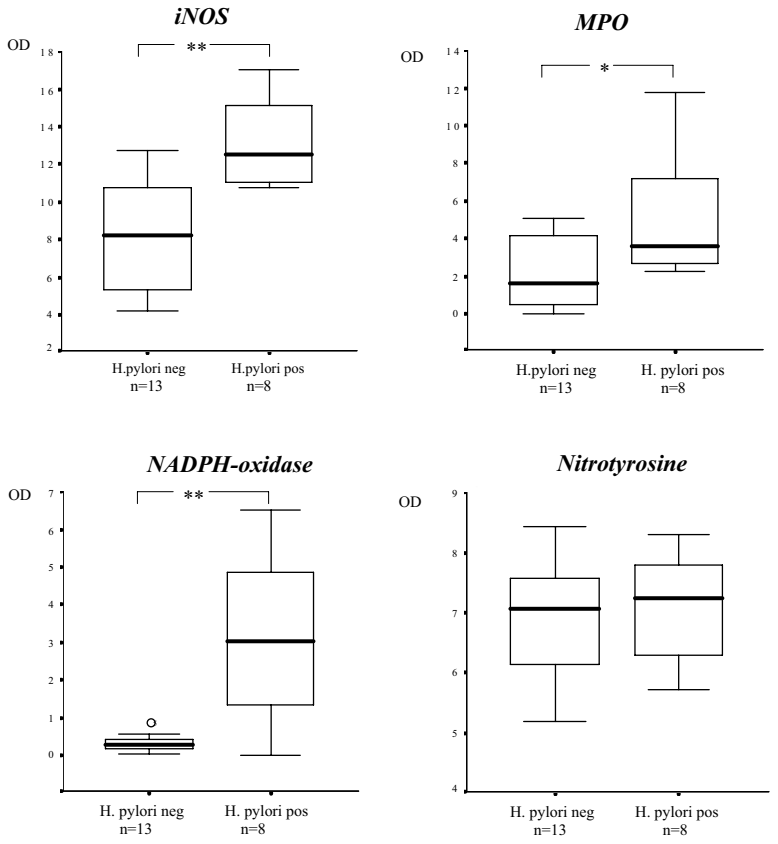

Figure 10: *Box plots showing results from Western blot analysis of antral biopsies of H. pylori negative (n=13) and positive (n=8) groups. Analysed proteins are iNOS, MPO, NADPH-oxidase (subunit p47-phox) and nitrotyrosine containing proteins. OD =optical density. ** = p<0.01, *=p<0.05*

Comments: The results in paper IV confirms that *H. pylori* infection in humans leads to an increased expression of enzymes involved in the formation of nitro- and oxy-radicals. Furthermore it shows that this up-regulation does not result in any significant increase, nor decrease in the nitrotyrosine production compared

to non-infected subjects. These findings are in concordance with our previous results from *H. pylori* infected Mongolian gerbils (II).
In conclusion the results support the existence of a *H. pylori* related interference with the nitro- and oxy-radical forming systems in humans.

Is there a difference in presence, location and expression of angiotensin II receptors in the stomach of non-infected compared to *H. pylori* infected Mongolian gerbils?

Tissues were obtained from the animals described in paper I. Animals sacrificed 6 and 12 months after inoculation were used to analyze the angiotensin II receptors in the gastric antral wall. Using quantitative reverse transcription polymerase chain reaction (qRT-PCR), mRNA of AT1R and AT2R were detected in antral samples from all gerbils. No significant differences were found between the groups with regard to gene transcripts.
Both AT1R and AT2R proteins were identified by Western blot technique in both infected and uninfected antral samples. 12 months after inoculation, the AT1R protein expression was significantly higher in the SS1 infected animals compared to the controls (Fig 11).

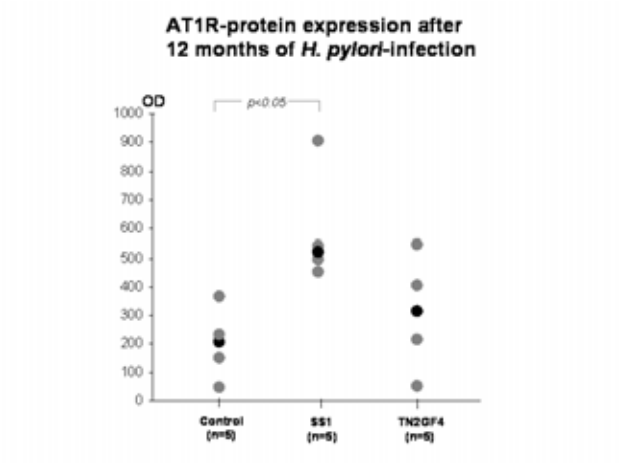

Figure 11. *Plots showing AT1R protein expression 12 months after inoculation in gerbils infected with H. pylori strains TN2GF4, SS1 and in controls. Protein levels are presented as optical density (OD) and the black dots shows the median values.*
The AT1R-protein expression was significantly higher in the SS1-infected animals compared with the controls (Mann-Whitney U-test with Dunnet correction for multiple comparisons).

Immunohistochemical preparation of mucosal sections exhibited staining for the AT1R and the AT2R protein was observed in all specimens studied. The AT1R-antibody induced in general a more distinct immunohistochemical staining than did the AT2R antibody. Both the AT1R and the AT2R were found in endothelial cells of vessels located in the submucosa and muscularis propria, in smooth-muscle cells in the muscular layers of the mucosa and muscularis propria, in some mesenchymal cells, and in the basal part of surface epithelial cells. AT1R was also found in vascular smooth-muscle cells of vessels located in the submucosa, muscularis propria and in the serosa and in cells with a typical appearance of endocrine cells. Aggregates of lymphocytes showing immunoreactivity for both the AT1R and the AT2R protein were found only in the mucosa of the infected gerbils, and not in the uninfected controls. No obvious differences in staining patterns or intensity could be seen between animals infected with the TN2GF4 strain and the SS1 strain, or between animals sacrificed at 6 and 12 months after inoculation.

Comments: Angiotensin II receptors are present in the gastric wall of the Mongolian gerbil. Immunohistochemistry was used to localize the receptors in the different cell-types described. Western blot technique was able to demonstrate an increase of AT1R-protein expression after infection with *H. pylori* strain SS1, but this technique can not conclude in which cell type the increase is most prominent.
In conclusion, this study shows that angiotensin II receptors AT1R and AT2R are present in the antral wall of the Mongolian gerbil. The results suggest a *H. pylori* strain dependent influence on the AT1R expression.

CONCLUSIONS

The principal conclusions drawn from the studies underlying this thesis are as follows:

- the results in paper I indicate that Mongolian gerbils infected with *H. pylori* has not yet been confirmed being a cancer model, but is suitable for studies of acute and chronic mucosal inflammation,

- the results in paper II indicate that the laps of time for up-regulation of nitro- and oxy-radical forming enzymes induced by *H. pylori* in Mongolian gerbils is dependent on the infecting strain,

- the data of paper II and III indicate that *H. pylori* infection in Mongolian gerbils results in inhibition of both the nitro- and oxy-radical formation,

- the results in paper IV indicate the existence of an *H. pylori* related interference with the nitro- and oxy-radical system in humans, and

- the data of paper V indicate that angiotensin II receptors AT1R and AT2R are present in the antral wall of the Mongolian gerbil. The results suggest a *H. pylori* strain dependent influence on the AT1R expression.

GENERAL DISCUSSION

Another important methodological consideration

The above-reviewed research concludes the existence of *H. pylori* derived inhibition of the nitro- and oxy-radical formation in the infected gastric mucosa. Before interpreting this finding into a pathophysiological context some methodological considerations have to be made.
The up-regulated expression of the radical-forming enzymes iNOS, MPO and NADPH oxidase appears very clear in both Mongolian gerbils and humans and generally confirm observations reported by other authors. However the present conclusion regarding inhibition of radical formation is partly controversial. Presence of nitrotyrosine is commonly used as a surrogate variable for radical (*ie* peroxynitrite) activity. In contrast to the present results (II) other groups have reported increased nitrotyrosine production related to *H. pylori* infection (115, 148, 151, 152). These contrasting results are probably due to methodological differences in the quantification of nitrotyrosine levels. Immunohistochemistry or various modifications of immunoblot are often used to analyse nitrotyrosine (115, 148, 152, 153). Because immunohistochemistry is a weak method for quantitative assessments we used a western blot protocol and prospective comparison to un-infected time-controls. This study-design proved to be very robust, although the loss of some individuals over time indicates that it would have been preferable to initially include a larger group of animals in paper II. The results in paper II showed consistently small or no changes in nitrotyrosine contents between the groups. This observation taken together with the upregulated expression of radical-forming enzymes in infected and inflamed mucosae indicate strongly that radical formation was inhibited downstreams the enzyme expression. An identical picture was found in *H. pylori* infected human gastric mucosal specimens. However, it was recently reported that nitrotyrosine assessed by antibody-dependent techniques can be underestimated due to technical reasons (heating of the samples). Such an underestimation could be an important error in the present thesis because conclusions are partly dependent on the observed low nitrotyrosine expression. As reported in Review of Results temperature dependency has been tested not to be the case in our protocol. In addition, using the same analysis it has been found that human esophageal mucosal specimens with confirmed esophagitis due to gastoesophageal acid reflux exhibit high nitrotyrosine levels when compared to controls without reflux esophagitis (un-published results). Reflux-associated esophagitis is induced chemically and thus lack microbe-associated inhibition of radical formation. Also this finding speaks against that the analysis is markedly influenced by heating. The assessment of nitrotyrosine as performed in our hands is thus believed to be valid and indicates that radical activity is low in the *H. pylori*

infected mucosa in both the Mongolian gerbil and man. Furthermore, this conclusion is strongly supported by direct juxtamucosal recordings of NO and H_2O_2 as reported in paper III.

H. pylori, an outstanding survivor

The present thesis project thus shows that gastric *H. pylori* infection in both Mongolian gerbils and humans causes a marked up-regulation of the expression of iNOS, MPO and NADPH oxidase (II, IV) as a part of the host defence mechanism trying to eliminate the microbe (77, 90, 92, 154-156). However, *H. pylori* is apparantly an outstanding survivor in the gastric mucosa withstanding not only the hostile intragastric acidic and peptic activity but also the induced inflammation. One fundamental observation of the present thesis project was the existence of *H. pylori* derived inhibition of the nitro- and oxy-radical formation very probably being an important factor that enables the bacterium to survive and establish a chronic infection. Several target points for the infection to reduce radical synthesis can be suggested and the most obvious ones are depicted in Figure 12.

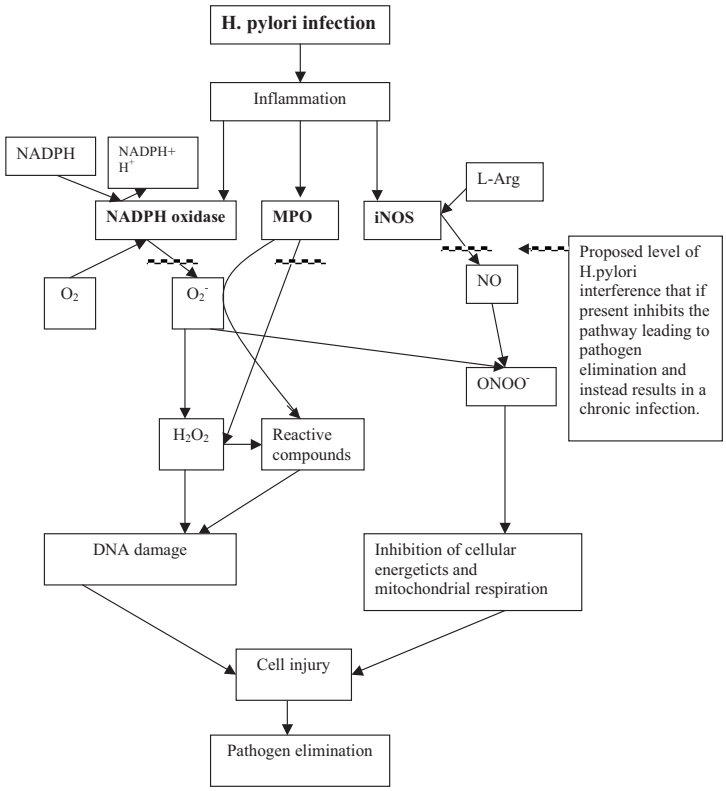

Figure 12: *Simplified overview of infection induced inflammation that causes increased expression of several enzymes in the oxy- nitroradical system. Under normal conditions this results in increased formation of superoxide (O_2^{-}), nitric oxide (NO), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$) which contribute to cellular injury that eliminates the pathogen. A proposed level of interference with this host defence is indicated that, if present, results in a chronic infection. It follows that an inhibition of the radical formation enables the H. pylori to avoid being eliminated and instead cause a chronic infection. It should be noted that strain differences may exist with differential patterns of radical formation and, consequently, different pathological effects.*

The inhibition of the radical production

It is well known that NO exerts direct toxic effects on *H. pylori*. *In vitro* studies have shown that *H. pylori* can be killed by NO released from activated macrophages, and from chemical sources of NO and $ONOO^-$ (157-159). However, when located in-situ at the gastric mucosa *H. pylori* may use several

mechanisms to reduce NO attacks. *H. pylori* produce arginase, which converts L-arginine to urea and L-ornitidine. Because L-arginine is also used by iNOS to produce NO, arginase can compete with iNOS for available substrate and thereby reduce NO synthesis. (157). Another principle by the microbe is to produce L-arginine analogues that blocks iNOS. (130) It follows that at low L-arginine concentrations *H. pylori* inhibits NO production, whereas high concentrations of L-arginine is not associated with such inhibition (130). Recently published *in vitro* studies confirm that L-arginine availability regulates the iNOS-dependent host defence against *H. pylori* (160). A finding of outmost interest is that *H. pylori* produced arginase not only inhibits iNOS, and thereby the NO-dependent apoptosis of macrophages, but arginase also have the ability to direct induce apoptosis (161). In this way *H. pylori* is able to induce apoptosis in macrophages by a process that occurs independently of iNOS expression but is mediated by arginase.
A possible iNOS inhibitor is *H. pylori* produced asymmetrical dimethyl arginine (ADMA), a methylated form of arginine that blocks iNOS by competitive inhibition. ADMA levels of antral mucosal specimens from humans have been shown to be markedly higher in *H. pylori* positive subjects (129). Furthermore, agmatine, a decarboxylated form of arginine has been reported to have NOS inhibitory qualities (162). Agmatine has been found in the gastric juice of *H. pylori* infected individuals, and has been suggested as a *H. pylori* virulence factor (163). Taken together, numerous data in the literature support the conclusion of this thesis that NO production *in vivo* is inhibited by *H. pylori* despite a pronounced iNOS expression in the infected mucosa.
What about oxy-radials? A few years ago little was known about the *H. pylori* enzymatic systems combating oxy-radical formation(164). Recent studies have shown that *H. pylori* have a number of enzymatic systems that respond to oxidative stress (165). *H. pylori* produced SOD dissipates O2- as previously described (166). Catalase produced by *H. pylori* is encoded by the gene katA, and removes H_2O_2 (167). *H. pylori* also produces a number of other antioxidant proteins. For example, KatA-associated protein assists Catalase to translocate into the site of action in the periplasm(167). The enzymes alkyl hydroperoxidereductase and peroxynitrite reductase are involved in detoxification of $ONOO^-$ (168, 169). Thiol-peroxidase is described as being a scavanger of H_2O_2. (170) As a second line of defence against oxidative stress it is also shown that *H. pylori* is equipped with protein and DNA repair systems to repair the damage caused by oxyradicals (171-173). Thus, the literature shows that *H. pylori* possess protection against oxidative attacks and support the present results suggesting that *H. pylori* exerts inhibition of the enzymatic activity of not only the nitroradical- but also the oxy-radical formation.

Interestingly, large amounts of NO are also formed in the gastric juice by the acidification of swallowed salivary nitrite (174-178). Such non-enzymatic NO formation is probably important for the intragastric clearance of ingested living microorganisms and the regulation of gastroduodenal functions like inhibition of

antral gastrin release in man (41, 42). Furthermore, NO formed from salivary nitrite increases the blood flow in the gastric mucosa and the thickness of the mucus layer in an anesthetised animal model (179). The importance of non-enzymatically formed NO in the gastric juice in the protection against *H. pylori* infection is not fully elucidated. As described in Background urease is important for *H. pylori* survival as it protects the bacteria from the acidic conditions through the formation of ammonia. A maintained high pH in the immediate vicinity of the microbe also reduces the risk of non-enzymatic NO-formation and probably contributes to the persistence of the infection.

The RAS a novel immuno-modulating link?

H. pylori has evolved complex mechanisms to interfere with several levels of the immunological signalling all the way from antigen presentation to antibody production (63, 180). T-cell response to *H. pylori* infection is a central part of the immunologic reaction to the infection (181). However the pro-inflammatory response is counteracted by the presence of *H. pylori* specific regulatory T-cells that actively suppress the immunological actions (64, 182). The presence of regulatory T-cells may thus be an important part of bacterial persistence (183) (184). The *H. pylori* virulence factor VacA has been shown to inhibit the immune response by interfering with antigen processing in B-cells and subsequent presentation of the resulting peptides to T-cells (185). Furthermore recent studies have suggested that the inability to clear the infection may be related to insufficient activation of dendritic cells (DC). It is suggested that in chronic *H. pylori* infection DC´s become inhibited by the prolonged antigen exposure leading to suboptimal Th1 development (66).
A novel link to immuno-modulation by *H. pylori* by way of the renin angiotensin system is presented in this thesis. Angiotensin II activation of AT1R has been reported to induce powerful pro-inflammatory cytokines, chemokines and adhesion molecules (121). AT1 is known to cause vasoconstriction, but it has also been shown to increase vascular permeability enabling extravasation and tissue-infiltration of inflammatory cells (119, 120). Ang II is via the AT1R involved in several steps of the inflammatory process e.g. recruitment of inflammatory cells into the tissue and direct activation of infiltrating immunocompetent cells. Ang II is also involved in transcriptional regulation via activation of NF-κB (116). Furthermore, AT1R antagonists have been found to have anti-inflammatory effects in experimentally induced gastritis (186). When taking this in consideration it is not surprising that AT1R expression is increased in the SS1 infected gerbils compared to controls (V). More confusing is the finding that AT1R expression is not significantly increased in the gerbils infected with *H. pylori* strain TN2GF4. In paper V this finding is discussed from the hypothesis that certain *H. pylori*-strains may produce a compound with ability to inhibit AT1R expression. As AT1R is an initiator and supporter of inflammation it could be beneficial for the bacteria to partially inhibit AT1R

expression to keep the inflammation at a level that is optimal for bacterial survival.

The chronic infection

In summary, *H. pylori* possess several mechanisms that reduce host defences and allow its persistence in the gastric mucosa. This results in a longstanding chronic inflammation unless the bacterium has been eradicated by use of antibiotics. It is reasonable to propose that the chronic inflammation in fact is beneficial for the bacterium. By allowing a mild inflammation with increased blood flow and vascular permeability the microbes can obtain nutrients, while at the same time limiting the efficacy of the immune response (180).
However, in some of the infected subjects the longstanding infection and chronic inflammation eventually results in stomach cancer. At start of this research project the intention was to establish a cancer model using Mongolian gerbils (I). However, no adenocarcinoma was found in the present investigation. This is in contrast to Watanabe et al who reported adenocarcinoma in 37% of Mongolian gerbils after 62 weeks of infection with *H. pylori* strain TN2GF4 (139). Later Honda et al reported that 2 of 5 animals developed adenocarcinoma 18 months after inoculation with the *H. pylori* strain ATCC-43504, and Hirayama et al reported adenocarcinoma in 1 of 56 gerbils 96 weeks after *H. pylori* inoculation (140, 187). By then the Mongolian gerbil was accepted and recommended as the animal model of choice for studying *H. pylori* related gastric adenocarcinoma (188). It is important to note that there are no common criteria for diagnosing adenocarcinoma in these animals (189). In addition there exist wellknown differences between Western and Japanese histopathologists regarding their respective diagnostic classification of gastrointestinal epithelial neoplastic lesions in humans. In the year 2000 pathologists from different countries gathered to develop a common worldwide terminology for gastrointestinal epithelial neoplasia called the Vienna classification of gastrointestinal epithelial neoplasia(190). The Vienna classification is based on the WHO classification of tumours in the gastrointestinal tract (145, 190). Two of the pathologists involved in paper I are co-authors of the Vienna classification, one representing the Western classification system, and the other representing the Japanese system (190). The morphological findings in paper I were generally regarded as benign and the conclusion was that adenocarcinoma had not been shown to develop in Mongolian gerbils infected with *H. pylori*.
The choice of *H. pylori* strain TN2GF4 for paper I was made with the apprehension of that the strain was reported being carcinogenic. Our conclusion is that neither we, nor Watanabe found any real neoplastic changes (I). One must, therefore, question if the strain is really carcinogenic as it was collected from a patient with ulcer disease and not cancer. An interesting publication from 2005 describes the development of gastric adenocarcinoma in gerbils following infection with *H. pylori* strain 7.13 (191, 192). The gastric adenocarcinomas

were characterized by marked cellular pleomorpism, cellular atypia, and euchromatic nuclei that exhibited bizarre morphologic features. In their study 17% of the infected animals showed adenocarcinoma already after 4 weeks of infection. At 16 weeks after inoculation adenocarcinoma was found in 59% of infected animals. However, it has so far not been described by Franco et al, nor by anyone else, that *H. pylori* infection in gerbils causes lymphatic invasion or invasion into blood vessels, or any local or distant lymph node metastases. It follows that the proposal of the *H. pylori* infected Mongolian gerbil being a cancer model still awaits confirmation.

Radicals and carcinogenesis

Nitro- and oxy-radicals are often considered to be important pro-carcinogenic factors in *H. pylori* infected subjects (115, 155, 193-195). However, the role of radicals in the carcinogenic process has shown to be extremely complex and both tumour progressive and regressive actions are described. The physiological role of inflammation and the associated radical formation is often forgotten in this debate. Basically the reactive radicals are formed to defend body homeostasis from noxious influences on the tissue level. This means that radicals are used not only to eliminate intruding microbes but also to eliminate tissue that has been traumatised, chemically poisoned or by other means diseased. A next action in the inflammatory process is restitution to resume the tissue's structure and function. In this complex process the radicals are executive as cytotoxic agents but also important as signalling molecules in the cell and tissue dynamics (67, 79, 91, 196).
The complexity is well illustrated by iNOS and NO. The latter is commonly associated with the development of malignant disease but involved mechanisms are obscure and involve several bioregulatory systems (196). In *H. pylori* associated gastric cancer, for example, the combination of increased iNOS and cyclooxygenase-2 (COX-2) expression is related to tumour progression and poor prognosis (197). Depending on the local concentration of NO both tumour promoting and deterring actions have been described. Local formation of NO alone is an excellent signalling molecule involved in various physiological regulation, e.g. vasodilatation, but when formed at higher rate, and particularly when exposed to oxy-radicals resulting in peroxynitrite formation, it exerts toxic actions on invading microorganisms and simultaneously also damage cells of the host. If this situation is extended over years there is an increased risk for radical induced DNA-damages and possibly cancer development (109, 198, 199). Furthermore it has recently been reported that the formation of nitrites and nitrates from NO may result in subsequent formation of nitrosamines, which are highly carcinogenic substances that may play a key role in gastric carcinogenesis (200). On the other hand, NO derived from macrophages has cytotoxic effects also on atypic cells and is used by the body to eliminate them (196, 201-203). It is apparent that the role of NO, with or without oxy-radicals, in carcinogenesis is multifaceted.

H. pylori and carcinogenesis: Are radicals involved?

It is beyond doubt that chronic *H. pylori* induced gastritis in humans is strongly related to development of adenocarcinoma. What about the radical formation and *H. pylori* carcinogenesis? During the course of this project two obstacles were encountered that prevented fair conclusions concerning this subject: First, despite a long observation period the model chosen, *H. pylori* infected Mongolian gerbils, did not develop adenocarcinoma (I). Secondly, chronic gastritis was confirmed but radical formation was not increased, rather the opposite (II, III). By referring to Correa's cascade (see Background) it is plausible to assume that the Mongolian gerbil-model reflects a condition that foregoes the epithelial cell transformation to malignant state. This is supported by the translational study (IV) showing a similar picture in human inflamed mucosa from mainly asymptomatic individuals. Assuming that this is the case and including the present finding of inhibited radical formation the paradigm should be; radical-induced cellular injuries are not a main reason to malignant transformation in the *H. pylori* infected stomach. Alternatively, a more sophisticated explanation may be that a strain dependent imbalance between the nitro- and oxy-radical formation is of importance for the risk of future cellular transformation. Such a situation is actually indicated in paper III where the *H. pylori* strain SS1 was associated with an increased H_2O_2 production, whereas TN2GF4 infected animals showed no increase compared to controls. As both strains inhibited NO-formation, the results suggest that the former animals would over time be subjected to more pronounced oxy-radical load on the gastric mucosa that may be of importance for potential risk of malignant transformation. Future studies are needed to elucidate this possibility then utilising confirmed cancer-associated *H. pylori* strains.

Perspectives

Even though that the *H. pylori* infected Mongolian gerbil cannot be regarded as an optimal cancer model, at least not with the *H. pylori* strains tested in the present project, the model is very suitable for studies of additional factors that together with the chronic gastritis, initiates malignant transformation. Such additional factors can be ingested carcinogens, but also various *H. pylori* strain-related virulence factors. In future studies utilising this model attention should also be put on direct interaction between *H. pylori* and the epithelial cells as well as indirect signalling via the inflammatory cascade reactions resulting in promotion of, for example, hyper-proliferation and a changed cell turn-over.

To make a complex situation even more confused we have in this thesis pointed out a possible relationship between *H. pylori*, angiotensin receptors, radical

formation and carcinogenesis. This is highly interesting, because it was recently reported that a polymorphism in the angiotensinogen gene is related to an increased risk for *H. pylori* related gastric cancer (204, 205). This confirms that the angiotensin II system is important for the carcinogenic process following *H. pylori* infection. Studies on established gastric cancers in humans indicate that ACE as well as AT1R and AT2R are expressed locally in gastric cancer (127, 206). The AT1R seems to be involved in spreading of the cancer to lymph nodes and an ACE gene polymorphism is related to metastatic behaviour. How the finding in this thesis of a possible *H. pylori* strain dependent AT1R inhibition is related to the carcinogenesis needs to be further studied. Such research may utilise existing pharmacological tools like ACE–inhibitors and AT1-receptor antagonists and offers translational research possibilities as these drugs are commonly prescribed antihypertensives in humans.

This thesis project has demonstrated that *H. pylori* infection of the gastric mucosa results in an activation of the host-defence system including up-regulation of enzymes involved in nitro-and oxy-radical production. The project has elucidated how *H. pylori,* by inhibiting the host defence system at several sites, survives in the gastric mucosa. This may be a more generalised microbial behaviour as also other microbes have developed various mechanisms to escape the effect of radical formation. For example, E-coli, Burkholderia cepacia and some yeast for example have developed ways to avoid the upregulation of NO and oxy-radical production following infection (207-209) Based on data from the present thesis the impact of oxy- and nitro-radicals in the development of *H. pylori* related adenocarcinoma should perhaps be questioned. However the relationship between nitro- and oxy-radicals and carcinogenesis is complex as each radical-type has dual effects in the carcinogenic process. Future studies of interest would be to compare the development of *H. pylori* related adenocarcinoma with carcinogenesis related to a chemically induced inflammation without involvement of infecting agents, for example esophageal adenocarcinomas following reflux esophagitis and Barrets esophagus. Furthermore the gained knowledge may be useful in exploring the pathogenesis of other neoplasm's associated with infectious agents or chronic inflammation, such as those of liver, lung, prostate and cervix.

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REFERENCES

1.Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet. 1984;1(8390):1311-5.
2.Megraud F. Epidemiology of Helicobacter pylori infection. Gastroenterol Clin North Am. 1993;22(1):73-88.
3.Rowland M, Daly L, Vaughan M, et al. Age-specific incidence of Helicobacter pylori. Gastroenterology. 2006;130(1):65-72; quiz 211.
4.Rowland M, Kumar D, Daly L, et al. Low rates of Helicobacter pylori reinfection in children. Gastroenterology. 1999;117(2):336-41.
5.Graham DY, Malaty HM, Evans DG, et al. Epidemiology of Helicobacter pylori in an asymptomatic population in the United States. Effect of age, race, and socioeconomic status. Gastroenterology. 1991;100(6):1495-501.
6.Fiedorek SC, Malaty HM, Evans DL, et al. Factors influencing the epidemiology of Helicobacter pylori infection in children. Pediatrics. 1991;88(3):578-82.
7.Lee A, Fox JG, Otto G, et al. Transmission of Helicobacter spp. A challenge to the dogma of faecal-oral spread. Epidemiol Infect. 1991;107(1):99-109.
8.Thomas JE, Gibson GR, Darboe MK, et al. Isolation of Helicobacter pylori from human faeces. Lancet. 1992;340(8829):1194-5.
9.Goodwin CS, Armstrong JA. Microbiological aspects of Helicobacter pylori (Campylobacter pylori). Eur J Clin Microbiol Infect Dis. 1990;9(1):1-13.
10.Clyne M, Dolan B, Reeves EP. Bacterial factors that mediate colonization of the stomach and virulence of Helicobacter pylori. FEMS Microbiol Lett. 2007;268(2):135-43.
11.Weeks DL, Eskandari S, Scott DR, et al. A H+-gated urea channel: the link between Helicobacter pylori urease and gastric colonization. Science. 2000;287(5452):482-5.
12.Harris PR, Mobley HL, Perez-Perez GI, et al. Helicobacter pylori urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production. Gastroenterology. 1996;111(2):419-25.
13.Blaser MJ, Perez-Perez GI, Kleanthous H, et al. Infection with Helicobacter pylori strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. Cancer Res. 1995;55(10):2111-5.
14.Backert S, Ziska E, Brinkmann V, et al. Translocation of the Helicobacter pylori CagA protein in gastric epithelial cells by a type IV secretion apparatus. Cell Microbiol. 2000;2(2):155-64.
15.Segal ED, Cha J, Lo J, et al. Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by Helicobacter pylori. Proc Natl Acad Sci U S A. 1999;96(25):14559-64.
16.Crabtree JE, Covacci A, Farmery SM, et al. Helicobacter pylori induced interleukin-8 expression in gastric epithelial cells is associated with CagA positive phenotype. J Clin Pathol. 1995;48(1):41-5.
17.Viala J, Chaput C, Boneca IG, et al. Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. Nat Immunol. 2004;5(11):1166-74.
18.Tummuru MK, Sharma SA, Blaser MJ. Helicobacter pylori picB, a homologue of the Bordetella pertussis toxin secretion protein, is required for induction of IL-8 in gastric epithelial cells. Mol Microbiol. 1995;18(5):867-76.

19.Kuipers EJ. Review article: exploring the link between Helicobacter pylori and gastric cancer. Aliment Pharmacol Ther. 1999;13 Suppl 1:3-11.
20.Cover TL. The vacuolating cytotoxin of Helicobacter pylori. Mol Microbiol. 1996;20(2):241-6.
21.Boquet P, Ricci V, Galmiche A, et al. Gastric cell apoptosis and *H. pylori*: has the main function of VacA finally been identified? Trends Microbiol. 2003;11(9):410-3.
22.Tummala S, Keates S, Kelly CP. Update on the immunologic basis of Helicobacter pylori gastritis. Curr Opin Gastroenterol. 2004;20(6):592-7.
23.Gerhard M, Lehn N, Neumayer N, et al. Clinical relevance of the Helicobacter pylori gene for blood-group antigen-binding adhesin. Proc Natl Acad Sci U S A. 1999;96(22):12778-83.
24.Marshall BJ, Armstrong JA, McGechie DB, et al. Attempt to fulfil Koch's postulates for pyloric Campylobacter. Med J Aust. 1985;142(8):436-9.
25.Morris A, Nicholson G. Ingestion of Campylobacter pyloridis causes gastritis and raised fasting gastric pH. Am J Gastroenterol. 1987;82(3):192-9.
26.Graham DY, Opekun AR, Osato MS, et al. Challenge model for Helicobacter pylori infection in human volunteers. Gut. 2004;53(9):1235-43.
27.Dixon MF, Genta RM, Yardley JH, et al. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. Am J Surg Pathol. 1996;20(10):1161-81.
28.Kumar C, Robbins. Basic Pathology; 1997.
29.Sipponen P, Kosunen TU, Valle J, et al. Helicobacter pylori infection and chronic gastritis in gastric cancer. J Clin Pathol. 1992;45(4):319-23.
30.Leung WK, Sung JJ. Review article: intestinal metaplasia and gastric carcinogenesis. Aliment Pharmacol Ther. 2002;16(7):1209-16.
31.Rokkas T, Filipe MI, Sladen GE. Detection of an increased incidence of early gastric cancer in patients with intestinal metaplasia type III who are closely followed up. Gut. 1991;32(10):1110-3.
32.Filipe MI, Munoz N, Matko I, et al. Intestinal metaplasia types and the risk of gastric cancer: a cohort study in Slovenia. Int J Cancer. 1994;57(3):324-9.
33.Genta RM, Rugge M. Review article: pre-neoplastic states of the gastric mucosa--a practical approach for the perplexed clinician. Aliment Pharmacol Ther. 2001;15 Suppl 1:43-50.
34.Parsonnet J. Helicobacter pylori in the stomach--a paradox unmasked. N Engl J Med. 1996;335(4):278-80.
35.Kuipers EJ. Helicobacter pylori and the risk and management of associated diseases: gastritis, ulcer disease, atrophic gastritis and gastric cancer. Aliment Pharmacol Ther. 1997;11 Suppl 1:71-88.
36.Olbe L, Fandriks L, Hamlet A, et al. Mechanisms involved in Helicobacter pylori induced duodenal ulcer disease:an overview. World J Gastroenterol. 2000;6(5):619-23.
37.Olbe L, Hamlet A, Dalenback J, et al. A mechanism by which Helicobacter pylori infection of the antrum contributes to the development of duodenal ulcer. Gastroenterology. 1996;110(5):1386-94.
38.Harris AW, Gummett PA, Misiewicz JJ, et al. Eradication of Helicobacter pylori in patients with duodenal ulcer lowers basal and peak acid outputs to gastrin releasing peptide and pentagastrin. Gut. 1996;38(5):663-7.

39.El-Omar EM, Oien K, El-Nujumi A, et al. Helicobacter pylori infection and chronic gastric acid hyposecretion. Gastroenterology. 1997;113(1):15-24.40.Moss SF, Legon S, Bishop AE, et al. Effect of Helicobacter pylori on gastric somatostatin in duodenal ulcer disease. Lancet. 1992;340(8825):930-2.
41.Holm M, Johansson B, Pettersson A, et al. Acid-induced duodenal mucosal nitric oxide output parallels bicarbonate secretion in the anaesthetized pig. Acta Physiol Scand. 1998;162(4):461-8.
42.Holm M, Powell T, Casselbrant A, et al. Dynamic involvement of the inducible type of nitric oxide synthase in acid-induced duodenal mucosal alkaline secretion in the rat. Dig Dis Sci. 2001;46(8):1765-71.
43.Isenberg JI, Selling JA, Hogan DL, et al. Impaired proximal duodenal mucosal bicarbonate secretion in patients with duodenal ulcer. N Engl J Med. 1987;316(7):374-9.
44.Fandriks L, von Bothmer C, Johansson B, et al. Water extract of Helicobacter pylori inhibits duodenal mucosal alkaline secretion in anesthetized rats. Gastroenterology. 1997;113(5):1570-5.
45.Dixon MF. Patterns of inflammation linked to ulcer disease. Baillieres Best Pract Res Clin Gastroenterol. 2000;14(1):27-40.
46.Hansson LE, Nyren O, Hsing AW, et al. The risk of stomach cancer in patients with gastric or duodenal ulcer disease. N Engl J Med. 1996;335(4):242-9.
47.El-Omar EM, Carrington M, Chow WH, et al. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature. 2000;404(6776):398-402.
48.Hansson LE, Engstrand L, Nyren O, et al. Helicobacter pylori infection: independent risk indicator of gastric adenocarcinoma. Gastroenterology. 1993;105(4):1098-103.
49.Schistosomes, liver flukes and Helicobacter pylori. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon, 7-14 June 1994. IARC Monogr Eval Carcinog Risks Hum. 1994;61:1-241.
50.Brown LM, Devesa SS. Epidemiologic trends in esophageal and gastric cancer in the United States. Surg Oncol Clin N Am. 2002;11(2):235-56.
51.Feldman F, Brandt. Sleisenger and Fordtran`s, Gastrointestinal and liver disease; 2006.
52.Vieth M, Stolte M. Elevated risk for gastric adenocarcinoma can be predicted from histomorphology. World J Gastroenterol. 2006;12(38):6109-14.
53.Sipponen P, Marshall BJ. Gastritis and gastric cancer. Western countries. Gastroenterol Clin North Am. 2000;29(3):579-92, v-vi.
54.Correa P. Helicobacter pylori and gastric cancer: state of the art. Cancer Epidemiol Biomarkers Prev. 1996;5(6):477-81.
55.Meining A, Morgner A, Miehlke S, et al. Atrophy-metaplasia-dysplasia-carcinoma sequence in the stomach: a reality or merely an hypothesis? Best Pract Res Clin Gastroenterol. 2001;15(6):983-98.
56.Kuipers EJ, Perez-Perez GI, Meuwissen SG, et al. Helicobacter pylori and atrophic gastritis: importance of the cagA status. J Natl Cancer Inst. 1995;87(23):1777-80.
57.Peek RM, Jr. The biological impact of Helicobacter pylori colonization. Semin Gastrointest Dis. 2001;12(3):151-66.
58.Joossens JV, Hill MJ, Elliott P, et al. Dietary salt, nitrate and stomach cancer mortality in 24 countries. European Cancer Prevention (ECP) and the INTERSALT Cooperative Research Group. Int J Epidemiol. 1996;25(3):494-504.

59.Spechler SJ. Carcinogenesis at the gastroesophageal junction: free radicals at the frontier. Gastroenterology. 2002;122(5):1518-20.
60.Konturek PC, Konturek SJ, Bielanski W, et al. Role of gastrin in gastric cancerogenesis in Helicobacter pylori infected humans. J Physiol Pharmacol. 1999;50(5):857-73.
61.Wong BC, Zhu GH, Lam SK. Aspirin induced apoptosis in gastric cancer cells. Biomed Pharmacother. 1999;53(7):315-8.
62.Ristimaki A, Honkanen N, Jankala H, et al. Expression of cyclooxygenase-2 in human gastric carcinoma. Cancer Res. 1997;57(7):1276-80.
63.Wilson KT, Crabtree JE. Immunology of Helicobacter pylori: insights into the failure of the immune response and perspectives on vaccine studies. Gastroenterology. 2007;133(1):288-308.
64.Lundgren A, Suri-Payer E, Enarsson K, et al. Helicobacter pylori-specific CD4+ CD25high regulatory T cells suppress memory T-cell responses to *H. pylori* in infected individuals. Infect Immun. 2003;71(4):1755-62.
65.El-Omar EM. The importance of interleukin 1beta in Helicobacter pylori associated disease. Gut. 2001;48(6):743-7.
66.Mitchell P, Germain C, Fiori PL, et al. Chronic exposure to Helicobacter pylori impairs dendritic cell function and inhibits Th1 development. Infect Immun. 2007;75(2):810-9.
67.Magder S. Reactive oxygen species: toxic molecules or spark of life? Crit Care. 2006;10(1):208.
68.Imlay JA. Pathways of oxidative damage. Annu Rev Microbiol. 2003;57:395-418.
69.Babior BM. Phagocytes and oxidative stress. Am J Med. 2000;109(1):33-44.
70.Metchnikoff. Untersuchungen uber die intracelluläre verdauung beim wirbellosen thieren. Arb Zoologischen Inst Univ Wien. 1883;5(141).
71.Baldridge C.W GRW. Am J Physiol. 1933;103(235).
72.Rossi Z. Biochemical aspects of phagocytosis in polymorphonuclear leucocytes. Experientia. 1964;20:21-3.
73.Babior BM, Kipnes RS, Curnutte JT. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. J Clin Invest. 1973;52(3):741-4.
74.Cross AR, Segal AW. The NADPH oxidase of professional phagocytes--prototype of the NOX electron transport chain systems. Biochim Biophys Acta. 2004;1657(1):1-22.
75.Quinn MT, Gauss KA. Structure and regulation of the neutrophil respiratory burst oxidase: comparison with nonphagocyte oxidases. J Leukoc Biol. 2004;76(4):760-81.
76.Bokoch GM, Knaus UG. NADPH oxidases: not just for leukocytes anymore! Trends Biochem Sci. 2003;28(9):502-8.
77.Babior BM. NADPH oxidase: an update. Blood. 1999;93(5):1464-76.
78.Roussyn I, Briviba K, Masumoto H, et al. Selenium-containing compounds protect DNA from single-strand breaks caused by peroxynitrite. Arch Biochem Biophys. 1996;330(1):216-8.
79.Halliwell B. Oxidative stress and cancer: have we moved forward? Biochem J. 2007;401(1):1-11.
80.Klebanoff SJ. Myeloperoxidase. Proc Assoc Am Physicians. 1999;111(5):383-9.

81.Albrich JM, Hurst JK. Oxidative inactivation of Escherichia coli by hypochlorous acid. Rates and differentiation of respiratory from other reaction sites. FEBS Lett. 1982;144(1):157-61.
82.McKenna SM, Davies KJ. The inhibition of bacterial growth by hypochlorous acid. Possible role in the bactericidal activity of phagocytes. Biochem J. 1988;254(3):685-92.
83.Barrette WC, Jr., Hannum DM, Wheeler WD, et al. General mechanism for the bacterial toxicity of hypochlorous acid: abolition of ATP production. Biochemistry. 1989;28(23):9172-8.
84.White CR, Darley-Usmar V, Berrington WR, et al. Circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits. Proc Natl Acad Sci U S A. 1996;93(16):8745-9.
85.Mueller CF, Laude K, McNally JS, et al. ATVB in focus: redox mechanisms in blood vessels. Arterioscler Thromb Vasc Biol. 2005;25(2):274-8.
86.Szabo C, Ohshima H. DNA damage induced by peroxynitrite: subsequent biological effects. Nitric Oxide. 1997;1(5):373-85.
87.Boveris A, Cadenas E. Mitochondrial production of hydrogen peroxide regulation by nitric oxide and the role of ubisemiquinone. IUBMB Life. 2000;50(4-5):245-50.
88.Fenton. Oxidation of tartaric acid in the presence of iron. J Chem Soc. 1894;65:899-910.
89.Haber W. The catalytic decomposition of hydrogen peroxide by iron salts. Proc R Soc Lond. 1934;147:332-51.
90.Klebanoff SJ. Myeloperoxidase: friend and foe. J Leukoc Biol. 2005;77(5):598-625.
91.Hampton MB, Kettle AJ, Winterbourn CC. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood. 1998;92(9):3007-17.
92.Klebanoff SJ. Myeloperoxidase: contribution to the microbicidal activity of intact leukocytes. Science. 1970;169(950):1095-7.
93.Fang FC. Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. J Clin Invest. 1997;99(12):2818-25.
94.Nathan C, Shiloh MU. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proc Natl Acad Sci U S A. 2000;97(16):8841-8.
95.Marletta MA, Yoon PS, Iyengar R, et al. Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. Biochemistry. 1988;27(24):8706-11.
96.Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev. 1991;43(2):109-42.
97.Nathan C. Nitric oxide as a secretory product of mammalian cells. Faseb J. 1992;6(12):3051-64.
98.Brown GC. Nitric oxide and mitochondrial respiration. Biochim Biophys Acta. 1999;1411(2-3):351-69.
99.Xie QW, Whisnant R, Nathan C. Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide. J Exp Med. 1993;177(6):1779-84.
100.Koide M, Kawahara Y, Nakayama I, et al. Cyclic AMP-elevating agents induce an inducible type of nitric oxide synthase in cultured vascular smooth

muscle cells. Synergism with the induction elicited by inflammatory cytokines. J Biol Chem. 1993;268(33):24959-66.
101.Vodovotz Y, Bogdan C, Paik J, et al. Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor beta. J Exp Med. 1993;178(2):605-13.
102.Palmer RM, Bridge L, Foxwell NA, et al. The role of nitric oxide in endothelial cell damage and its inhibition by glucocorticoids. Br J Pharmacol. 1992;105(1):11-2.
103.Guo FH, De Raeve HR, Rice TW, et al. Continuous nitric oxide synthesis by inducible nitric oxide synthase in normal human airway epithelium in vivo. Proc Natl Acad Sci U S A. 1995;92(17):7809-13.
104.Snygg J, Casselbrant A, Pettersson A, et al. Tonometric assessment of jejunal mucosal nitric oxide formation in anaesthetized pigs. Acta Physiol Scand. 2000;169(1):39-45.
105.Ewert S, Laesser M, Johansson B, et al. The angiotensin II receptor type 2 agonist CGP 42112A stimulates NO production in the porcine jejunal mucosa. BMC Pharmacol. 2003;3:2.
106.Stuehr DJ, Marletta MA. Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide. Proc Natl Acad Sci U S A. 1985;82(22):7738-42.
107.Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature. 1987;327(6122):524-6.
108.Ignarro LJ, Buga GM, Wood KS, et al. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sci U S A. 1987;84(24):9265-9.
109.Bentz BG, Simmons RL, Haines GK, 3rd, et al. The yin and yang of nitric oxide: reflections on the physiology and pathophysiology of NO. Head Neck. 2000;22(1):71-83.
110.Weitzberg E, Lundberg JO. Nonenzymatic nitric oxide production in humans. Nitric Oxide. 1998;2(1):1-7.
111.Bjorne H, Weitzberg E, Lundberg JO. Intragastric generation of antimicrobial nitrogen oxides from saliva--physiological and therapeutic considerations. Free Radic Biol Med. 2006;41(9):1404-12.
112.Lin KT, Xue JY, Nomen M, et al. Peroxynitrite-induced apoptosis in HL-60 cells. J Biol Chem. 1995;270(28):16487-90.
113.Eiserich JP, Hristova M, Cross CE, et al. Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. Nature. 1998;391(6665):393-7.
114.Mohiuddin I, Chai H, Lin PH, et al. Nitrotyrosine and chlorotyrosine: clinical significance and biological functions in the vascular system. J Surg Res. 2006;133(2):143-9.
115.Mannick EE, Bravo LE, Zarama G, et al. Inducible nitric oxide synthase, nitrotyrosine, and apoptosis in Helicobacter pylori gastritis: effect of antibiotics and antioxidants. Cancer Res. 1996;56(14):3238-43.
116.Suzuki Y, Ruiz-Ortega M, Lorenzo O, et al. Inflammation and angiotensin II. Int J Biochem Cell Biol. 2003;35(6):881-900.
117.Deshayes F, Nahmias C. Angiotensin receptors: a new role in cancer? Trends Endocrinol Metab. 2005;16(7):293-9.

118.de Gasparo M, Catt KJ, Inagami T, et al. International union of pharmacology. XXIII. The angiotensin II receptors. Pharmacol Rev. 2000;52(3):415-72.
119.Newton CR, Curran B, Victorino GP. Angiotensin II type 1 receptor activation increases microvascular hydraulic permeability. Surgery. 2004;136(5):1054-60.
120.Newton CR, Curran B, Victorino GP. Angiotensin II type 2 receptor effect on microvascular hydraulic permeability. J Surg Res. 2004;120(1):83-8.
121.Smith GR, Missailidis S. Cancer, inflammation and the AT1 and AT2 receptors. J Inflamm (Lond). 2004;1(1):3.
122.Esteban V, Lorenzo O, Ruperez M, et al. Angiotensin II, via AT1 and AT2 receptors and NF-kappaB pathway, regulates the inflammatory response in unilateral ureteral obstruction. J Am Soc Nephrol. 2004;15(6):1514-29.
123.Okada H, Inoue T, Kikuta T, et al. A possible anti-inflammatory role of angiotensin II type 2 receptor in immune-mediated glomerulonephritis during type 1 receptor blockade. Am J Pathol. 2006;169(5):1577-89.
124.Seno K, Zhu JH, Barrett JD, et al. Cigarette smoke increases gastric ulcer size in part by an angiotensin II-mediated mechanism in rats. Dig Dis Sci. 1997;42(1):74-8.
125.Goto Y, Ando T, Nishio K, et al. The ACE gene polymorphism is associated with the incidence of gastric cancer among *H. pylori* seropositive subjects with atrophic gastritis. Asian Pac J Cancer Prev. 2005;6(4):464-7.
126.Ebert MP, Lendeckel U, Westphal S, et al. The angiotensin I-converting enzyme gene insertion/deletion polymorphism is linked to early gastric cancer. Cancer Epidemiol Biomarkers Prev. 2005;14(12):2987-9.
127.Rocken C, Lendeckel U, Dierkes J, et al. The number of lymph node metastases in gastric cancer correlates with the angiotensin I-converting enzyme gene insertion/deletion polymorphism. Clin Cancer Res. 2005;11(7):2526-30.
128.Bataller R, Schwabe RF, Choi YH, et al. NADPH oxidase signal transduces angiotensin II in hepatic stellate cells and is critical in hepatic fibrosis. J Clin Invest. 2003;112(9):1383-94.
129.von Bothmer C, Edebo A, Lonroth H, et al. Helicobacter pylori infection inhibits antral mucosal nitric oxide production in humans. Scand J Gastroenterol. 2002;37(4):404-8.
130.von Bothmer C, Bolin I, Pettersson A, et al. Stimulated murine macrophages as a bioassay for *H. pylori*-related inhibition of nitric oxide production. Scand J Gastroenterol. 2003;38(4):380-6.
131.O'Rourke JL, Lee A. Animal models of Helicobacter pylori infection and disease. Microbes Infect. 2003;5(8):741-8.
132.Eaton KA. Animal models of Helicobacter gastritis. Curr Top Microbiol Immunol. 1999;241:123-54.
133.Lee A. Animal models for host-pathogen interaction studies. Br Med Bull. 1998;54(1):163-73.
134.Fox JG, Rogers AB, Ihrig M, et al. Helicobacter pylori-associated gastric cancer in INS-GAS mice is gender specific. Cancer Res. 2003;63(5):942-50.
135.Fox JG, Wang TC, Rogers AB, et al. Host and microbial constituents influence Helicobacter pylori-induced cancer in a murine model of hypergastrinemia. Gastroenterology. 2003;124(7):1879-90.

136.Wang TC, Dangler CA, Chen D, et al. Synergistic interaction between hypergastrinemia and Helicobacter infection in a mouse model of gastric cancer. Gastroenterology. 2000;118(1):36-47.
137.Tatematsu M, Ogawa K, Hoshiya T, et al. Induction of adenocarcinomas in the glandular stomach of BALB/c mice treated with N-methyl-N-nitrosourea. Jpn J Cancer Res. 1992;83(9):915-8.
138.Han SU, Kim YB, Joo HJ, et al. Helicobacter pylori infection promotes gastric carcinogenesis in a mice model. J Gastroenterol Hepatol. 2002;17(3):253-61.
139.Watanabe T, Tada M, Nagai H, et al. Helicobacter pylori infection induces gastric cancer in mongolian gerbils. Gastroenterology. 1998;115(3):642-8.
140.Honda S, Fujioka T, Tokieda M, et al. Development of Helicobacter pylori-induced gastric carcinoma in Mongolian gerbils. Cancer Res. 1998;58(19):4255-9.
141.Lee A, O'Rourke J, De Ungria MC, et al. A standardized mouse model of Helicobacter pylori infection: introducing the Sydney strain. Gastroenterology. 1997;112(4):1386-97.
142.Crabtree JE, Ferrero RL, Kusters JG. The mouse colonizing Helicobacter pylori strain SS1 may lack a functional cag pathogenicity island. Helicobacter. 2002;7(2):139-40; author reply 40-1.
143.Kawazoe T, Sakagami T, Nakajima K, et al. Role of bacterial strain diversity of Helicobacter pylori in gastric carcinogenesis induced by N-methyl-N-nitrosourea in Mongolian gerbils. Helicobacter. 2007;12(3):213-23.
144.Sun YQ, Monstein HJ, Nilsson LE, et al. Profiling and identification of eubacteria in the stomach of Mongolian gerbils with and without Helicobacter pylori infection. Helicobacter. 2003;8(2):149-57.
145.Hamilton SR AL, editors. . WHO classification. Tumours of the gastrointestinal tract. Pathology and genetics. Lyon: IARC Press; 2000. 2000.
146.Chen D, Stenstrom B, Zhao CM, et al. Does Helicobacter pylori infection per se cause gastric cancer or duodenal ulcer? Inadequate evidence in Mongolian gerbils and inbred mice. FEMS Immunol Med Microbiol. 2007;50(2):184-9.
147.Iguchi M, Shiotani A, Nishioka S. Helicobacter pylori infection reduces intraluminal nitric oxide. Scand J Gastroenterol. 2000;35(7):694-8.
148.Goto T, Haruma K, Kitadai Y, et al. Enhanced expression of inducible nitric oxide synthase and nitrotyrosine in gastric mucosa of gastric cancer patients. Clin Cancer Res. 1999;5(6):1411-5.
149.Soderling AS, Hultman L, Delbro D, et al. Reduction of the nitro group during sample preparation may cause underestimation of the nitration level in 3-nitrotyrosine immunoblotting. J Chromatogr B Analyt Technol Biomed Life Sci. 2007;851(1-2):277-86.
150.Iijima K, Henry E, Moriya A, et al. Dietary nitrate generates potentially mutagenic concentrations of nitric oxide at the gastroesophageal junction. Gastroenterology. 2002;122(5):1248-57.
151.Sakaguchi AA, Miura S, Takeuchi T, et al. Increased expression of inducible nitric oxide synthase and peroxynitrite in Helicobacter pylori gastric ulcer. Free Radic Biol Med. 1999;27(7-8):781-9.
152.Sun YQ, Petersson F, Monstein HJ, et al. Long-term morpho-functional development of Helicobacter pylori-induced gastritis in Mongolian gerbils. Scand J Gastroenterol. 2005;40(10):1157-67.
153.Pignatelli B, Li CQ, Boffetta P, et al. Nitrated and oxidized plasma proteins in smokers and lung cancer patients. Cancer Res. 2001;61(2):778-84.

154.Geiszt M, Leto TL. The Nox family of NAD(P)H oxidases: host defense and beyond. J Biol Chem. 2004;279(50):51715-8.
155.Keenan JI, Peterson RA, 2nd, Hampton MB. NADPH oxidase involvement in the pathology of Helicobacter pylori infection. Free Radic Biol Med. 2005;38(9):1188-96.
156.Wilson KT, Ramanujam KS, Mobley HL, et al. Helicobacter pylori stimulates inducible nitric oxide synthase expression and activity in a murine macrophage cell line. Gastroenterology. 1996;111(6):1524-33.
157.Gobert AP, McGee DJ, Akhtar M, et al. Helicobacter pylori arginase inhibits nitric oxide production by eukaryotic cells: a strategy for bacterial survival. Proc Natl Acad Sci U S A. 2001;98(24):13844-9.
158.Dykhuizen RS, Fraser A, McKenzie H, et al. Helicobacter pylori is killed by nitrite under acidic conditions. Gut. 1998;42(3):334-7.
159.Kuwahara H, Miyamoto Y, Akaike T, et al. Helicobacter pylori urease suppresses bactericidal activity of peroxynitrite via carbon dioxide production. Infect Immun. 2000;68(8):4378-83.
160.Chaturvedi R, Asim M, Lewis ND, et al. L-Arginine Availability Regulates Inducible Nitric Oxide Synthase-dependent Host Defense Against Helicobacter pylori. Infect Immun. 2007.
161.Gobert AP, Cheng Y, Wang JY, et al. Helicobacter pylori induces macrophage apoptosis by activation of arginase II. J Immunol. 2002;168(9):4692-700.
162.Abe K, Abe Y, Saito H. Agmatine suppresses nitric oxide production in microglia. Brain Res. 2000;872(1-2):141-8.
163.Molderings GJ, Burian M, Homann J, et al. Potential relevance of agmatine as a virulence factor of Helicobacter pylori. Dig Dis Sci. 1999;44(12):2397-404.
164.Hazell SL. Evasion of the toxic effects of oxygen. In Helicobacter pylori physiology and genetics. washington DC; 2001.
165.Wang G, Alamuri P, Maier RJ. The diverse antioxidant systems of Helicobacter pylori. Mol Microbiol. 2006;61(4):847-60.
166.Seyler RW, Jr., Olson JW, Maier RJ. Superoxide dismutase-deficient mutants of Helicobacter pylori are hypersensitive to oxidative stress and defective in host colonization. Infect Immun. 2001;69(6):4034-40.
167.Harris AG, Hazell SL. Localisation of Helicobacter pylori catalase in both the periplasm and cytoplasm, and its dependence on the twin-arginine target protein, KapA, for activity. FEMS Microbiol Lett. 2003;229(2):283-9.
168.Olczak AA, Seyler RW, Jr., Olson JW, et al. Association of Helicobacter pylori antioxidant activities with host colonization proficiency. Infect Immun. 2003;71(1):580-3.
169.Bryk R, Griffin P, Nathan C. Peroxynitrite reductase activity of bacterial peroxiredoxins. Nature. 2000;407(6801):211-5.
170.Wang G, Olczak AA, Walton JP, et al. Contribution of the Helicobacter pylori thiol peroxidase bacterioferritin comigratory protein to oxidative stress resistance and host colonization. Infect Immun. 2005;73(1):378-84.
171.Wang G, Alamuri P, Humayun MZ, et al. The Helicobacter pylori MutS protein confers protection from oxidative DNA damage. Mol Microbiol. 2005;58(1):166-76.

172.O'Rourke EJ, Chevalier C, Pinto AV, et al. Pathogen DNA as target for host-generated oxidative stress: role for repair of bacterial DNA damage in Helicobacter pylori colonization. Proc Natl Acad Sci U S A. 2003;100(5):2789-94.
173.Alamuri P, Maier RJ. Methionine sulphoxide reductase is an important antioxidant enzyme in the gastric pathogen Helicobacter pylori. Mol Microbiol. 2004;53(5):1397-406.
174.McKnight GM, Smith LM, Drummond RS, et al. Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans. Gut. 1997;40(2):211-4.
175.Fandriks L, von Bothmer C, Aneman A, et al. Intragastric nitric oxide/nitrite in Helicobacter pylori-infected subjects. Scand J Gastroenterol. 2001;36(4):347-50.
176.Benjamin N, O'Driscoll F, Dougall H, et al. Stomach NO synthesis. Nature. 1994;368(6471):502.
177.Aneman A, Snygg J, Fandriks L, et al. Continuous measurement of gastric nitric oxide production. Am J Physiol. 1996;271(6 Pt 1):G1039-42.
178.Lundberg JO, Weitzberg E, Cole JA, et al. Nitrate, bacteria and human health. Nat Rev Microbiol. 2004;2(7):593-602.
179.Bjorne HH, Petersson J, Phillipson M, et al. Nitrite in saliva increases gastric mucosal blood flow and mucus thickness. J Clin Invest. 2004;113(1):106-14.
180.Baldari CT, Lanzavecchia A, Telford JL. Immune subversion by Helicobacter pylori. Trends Immunol. 2005;26(4):199-207.
181.Eaton KA, Mefford M, Thevenot T. The role of T cell subsets and cytokines in the pathogenesis of Helicobacter pylori gastritis in mice. J Immunol. 2001;166(12):7456-61.
182.Lundgren A, Stromberg E, Sjoling A, et al. Mucosal FOXP3-expressing CD4+ CD25high regulatory T cells in Helicobacter pylori-infected patients. Infect Immun. 2005;73(1):523-31.
183.Svennerholm AM, Quiding-Jarbrink M. Priming and expression of immune responses in the gastric mucosa. Microbes Infect. 2003;5(8):731-9.
184.Rad R, Brenner L, Bauer S, et al. CD25+/Foxp3+ T cells regulate gastric inflammation and Helicobacter pylori colonization in vivo. Gastroenterology. 2006;131(2):525-37.
185.Molinari M, Salio M, Galli C, et al. Selective inhibition of Ii-dependent antigen presentation by Helicobacter pylori toxin VacA. J Exp Med. 1998;187(1):135-40.
186.Bregonzio C, Armando I, Ando H, et al. Anti-inflammatory effects of angiotensin II AT1 receptor antagonism prevent stress-induced gastric injury. Am J Physiol Gastrointest Liver Physiol. 2003;285(2):G414-23.
187.Hirayama F, Takagi S, Iwao E, et al. Development of poorly differentiated adenocarcinoma and carcinoid due to long-term Helicobacter pylori colonization in Mongolian gerbils. J Gastroenterol. 1999;34(4):450-4.
188.Pritchard P. How useful are the rodent animal models of gastric adenocarcinoma? Aliment Pharmacol Ther. 2004;19:841-59.
189.Sugiyama T, Hige S, Asaka M. Development of an *H. pylori*-infected animal model and gastric cancer: recent progress and issues. J Gastroenterol. 2002;37 Suppl 13:6-9.
190.Schlemper RJ, Riddell RH, Kato Y, et al. The Vienna classification of gastrointestinal epithelial neoplasia. Gut. 2000;47(2):251-5.

191.Franco AT, Israel DA, Washington MK, et al. Activation of beta-catenin by carcinogenic Helicobacter pylori. Proc Natl Acad Sci U S A. 2005;102(30):10646-51.
192.Peek RM, Jr., Crabtree JE. Helicobacter infection and gastric neoplasia. J Pathol. 2006;208(2):233-48.
193.Li CQ, Pignatelli B, Ohshima H. Increased oxidative and nitrative stress in human stomach associated with cagA+ Helicobacter pylori infection and inflammation. Dig Dis Sci. 2001;46(4):836-44.
194.Son HJ, Rhee JC, Park DI, et al. Inducible nitric oxide synthase expression in gastroduodenal diseases infected with Helicobacter pylori. Helicobacter. 2001;6(1):37-43.
195.Xia HH, Wong BC. Nitric oxide in Helicobacter pylori-induced apoptosis and its significance in gastric carcinogenesis. J Gastroenterol Hepatol. 2003;18(11):1227-30.
196.Lechner M, Lirk P, Rieder J. Inducible nitric oxide synthase (iNOS) in tumor biology: the two sides of the same coin. Semin Cancer Biol. 2005;15(4):277-89.
197.Chen CN, Hsieh FJ, Cheng YM, et al. Expression of inducible nitric oxide synthase and cyclooxygenase-2 in angiogenesis and clinical outcome of human gastric cancer. J Surg Oncol. 2006;94(3):226-33.
198.Salgo MG, Stone K, Squadrito GL, et al. Peroxynitrite causes DNA nicks in plasmid pBR322. Biochem Biophys Res Commun. 1995;210(3):1025-30.
199.Beckman JS. The double-edged role of nitric oxide in brain function and superoxide-mediated injury. J Dev Physiol. 1991;15(1):53-9.
200.Lechner M, Rieder J, Tilg H. Helicobacter pylori infection, iNOS, and gastric cancer: the impact of another possible link. J Surg Oncol. 2007;95(3):271-2.
201.Kwak JY, Han MK, Choi KS, et al. Cytokines secreted by lymphokine-activated killer cells induce endogenous nitric oxide synthesis and apoptosis in DLD-1 colon cancer cells. Cell Immunol. 2000;203(2):84-94.
202.Gansauge S, Nussler AK, Beger HG, et al. Nitric oxide-induced apoptosis in human pancreatic carcinoma cell lines is associated with a G1-arrest and an increase of the cyclin-dependent kinase inhibitor p21WAF1/CIP1. Cell Growth Differ. 1998;9(8):611-7.
203.Mortensen K, Skouv J, Hougaard DM, et al. Endogenous endothelial cell nitric-oxide synthase modulates apoptosis in cultured breast cancer cells and is transcriptionally regulated by p53. J Biol Chem. 1999;274(53):37679-84.
204.Sugimoto M, Furuta T, Shirai N, et al. Influences of chymase and angiotensin I-converting enzyme gene polymorphisms on gastric cancer risks in Japan. Cancer Epidemiol Biomarkers Prev. 2006;15(10):1929-34.
205.Sugimoto M, Furuta T, Shirai N, et al. Role of angiotensinogen gene polymorphism on Helicobacter pylori infection-related gastric cancer risk in Japanese. Carcinogenesis. 2007.
206.Rocken C, Rohl FW, Diebler E, et al. The angiotensin II/angiotensin II receptor system correlates with nodal spread in intestinal type gastric cancer. Cancer Epidemiol Biomarkers Prev. 2007;16(6):1206-12.
207.Nunoshiba T, deRojas-Walker T, Wishnok JS, et al. Activation by nitric oxide of an oxidative-stress response that defends Escherichia coli against activated macrophages. Proc Natl Acad Sci U S A. 1993;90(21):9993-7.

208.Smart WC, Coffman JA, Cooper TG. Combinatorial regulation of the Saccharomyces cerevisiae CAR1 (arginase) promoter in response to multiple environmental signals. Mol Cell Biol. 1996;16(10):5876-87.
209.Saini LS, Galsworthy SB, John MA, et al. Intracellular survival of Burkholderia cepacia complex isolates in the presence of macrophage cell activation. Microbiology. 1999;145 (Pt 12):3465-75.

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