Carbonate Ions and Gastric Cancer

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Carbonate ions and gastric cancer

Abstract

Nearly one million new cases of gastric cancer are diagnosed annually throughout the world. Even though the incidence has fallen dramatically in recent decades, this disease is still the second leading cause of cancer death in a global perspective. The geographic distribution of gastric cancer varies markedly, with the highest rates in Asian countries such as Japan, Korea, and China. This variation is presumably associated with modifiable risk factors, primarily H. pylori infection and diet, which have dominated the debate on this topic for more than a decade. The incidence of spontaneous gastric cancer is extremely low in rats, which has led to testing of different experimental models in attempts to generate gastric tumors in these animals. In the first study underlying this thesis, rats were subjected to gastric resection to generate duodenogastric reflux (DGR) and subsequent development of gastric adenocarcinomas. The effects of various food supplements on the incidence of cancer were studied using a total of 256 male Wistar rats. Surprisingly, in the first set of experiments in the second study, ingestion of food supplemented with calcium carbonate more than tripled the incidence of carcinomas (61%) compared to controls (17%). In a second set of experiments, calcium ions were switched to sodium ions, which revealed that carbonate ions caused the remarkable increase in cancer in the rats given an altered diet (54%, compared to 12% for controls). Both experimental and clinical studies have shown that DGR is associated with the development of gastric cancer. It has also been found that pancreaticoduodenal juice is responsible for the neoplastic formation, and that such fluid is especially rich in carbonate ions. The final study examined non-transformed mucosa in a rat model of gastric cancer to determine expression of COX-2 and ODC as markers of tumor promotion and to measure production of Ki67 as an indication of cell proliferation. This was done to assess the effect of carbonate ions on gastric tumorigenesis. The results indicated that the gastric resection per se increased COX-2 expression and significantly augmented cell proliferation. Dietary supplementation of carbonate ions did not further enhance the levels of COX-2. However, in the resected animals, carbonate-supplemented food led to elevated expression of ODC and a further increase in cell proliferation in the non-transformed mucosa. In conclusion, an environment entailing persistent chronic inflammation and increased levels of COX-2, induced by either duodenogastric reflux or a factor such as H. pylori infection, increases the risk of malignant transformation. Moreover, extra carbonate intake raises the levels of ODC in the gastric mucosa in a COX-2-dependent manner, which magnifies the proliferative drive and results in an even higher risk of gastric carcinoma.

Key words: **Experimental gastric cancer, Wistar rats, gastrojejunostomy, duodenogastric reflux, inflammation, carbonate ions, COX-2, ODC, proliferation**

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At universities in Sweden, a doctoral thesis is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers that have already been published or are manuscripts in various stages of preparation (*in manuscript, submitted, or in press*).

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To Ylva
and the boys
Johan
André

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List of Papers

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

I. **Roy A Ehrnström**, Clas G. Lindström, Stefan Arvidsson, Nils H. Sternby, Elise Nilsson and Ylva M Ehrnström. An experimental study of gastric stump carcinoma in Wistar rats. *APMIS*, 103; 878–884, 1995

II. **Roy A Ehrnström**, Béla Veress, Stefan Arvidsson, Nils H Sternby, Tommy Andersson & Clas G. Lindström. Dietary supplementation of carbonate promotes spontaneous tumorigenesis in a rat gastric stump model. *Scandinavian Journal of Gastroenterology*, 2006; 41:12–20

III. **Roy A Ehrnström**, Otto Ljungberg, Monica E Haglund, Lars Magnus Bjursten, Clas G Lindström and Tommy Andersson. Dietary supplementation with carbonate increases expression of ornithine decarboxylase and cell proliferation in gastric mucosa in a rat model of gastric cancer. S*ubmitted.*

Abbreviations

AICR	American Institute for Cancer Research
BI	Billroth I resection
BII	Billroth II resection
BabA	blood group antigen-binding adhesin
babA	gene encoding BabA in *H. pylori*
BMDC	bone-marrow-derived stem cell
CagA	cytotoxin-associated protein
cagA	gene encoding CagA in *H. pylori*
CIS	carcinoma *in situ*
COX-2	cyclooxygenase-2
CT	computed tomography
DGOR	duodenogastroesophageal reflux
DGR	duodenogastric reflux
EGC	early gastric cancer
EMR	endoscopic mucosal resection
ESD	endoscopic submucosal dissection
GORD	gastroesophageal reflux disease
H&E	hematoxylin and eosin
IHC	immunohistochemical
IL	interleukin
Ki67	immunohistochemical marker for proliferative activity
MDCT	multidetector CT
MNNG	*N*-methyl-*N´*-nitro-*N*-nitrosoguanidine
MNU	*N*-methyl-*N´*-nitrosourea
MRI	magnetic resonance imaging
MSC	mesenchymal stem cells
ODC	ornithine decarboxylase
PET	positron emission tomography
PET-CT	combined PET and CT scans
TNF	tumor necrosis factor
TNM	tumor-node-metastasis
VacA	vacuolating toxin
vacA	gene encoding VacA in *H. pylori*
WCRF	World Cancer Research Fund
WHO	World Health Organization

Abstract

Nearly one million new cases of gastric cancer are diagnosed annually throughout the world. Even though the incidence has fallen dramatically in recent decades, this disease is still the second leading cause of cancer death in a global perspective. The geographic distribution of gastric cancer varies markedly, with the highest rates in Asian countries such as Japan, Korea, and China. This variation is presumably associated with modifiable risk factors, primarily *H. pylori* infection and diet, which have dominated the debate on this topic for more than a decade.

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The final study examined non-transformed mucosa in a rat model of gastric cancer to determine expression of COX-2 and ODC as markers of tumor promotion and to measure production of Ki67 as an indication of cell proliferation. This was done to assess the effect of carbonate ions on gastric tumorigenesis. The results indicated that the gastric resection per se increased COX-2 expression and significantly augmented cell proliferation. Dietary supplementation of carbonate ions did not further enhance the levels of COX-2. However, in the resected animals, carbonate-supplemented food led to elevated expression of ODC and a further increase in cell proliferation in the non-transformed mucosa.

In conclusion, an environment entailing persistent chronic inflammation and increased levels of COX-2, induced by either duodenogastric reflux or a factor such as *H. pylori* infection, increases the risk of malignant transformation. Moreover, extra carbonate intake raises the levels of ODC in the gastric mucosa in a COX-2-dependent manner, which magnifies the proliferative drive and results in an even higher risk of gastric carcinoma.

Clinical introduction (Humans)

Demographics

From a global perspective, gastric cancer is one of the most common forms of malignant disease. However, the overall incidence has declined in recent decades, and it is now the fourth most widespread malignancy after lung, breast, and colorectal cancers. Nevertheless, in developing countries and various other parts of the world, the incidence of gastric cancer is still high, and the disease continues to be either the principal form of cancer or second only to lung cancer. According to Global Cancer Statistics 2002, it is estimated that annually there are 934 000 new cases and the mortality rate is 700 000, which makes gastric cancer the second most common cause of cancer death in the world.[1] In Sweden, the pattern of incidence is somewhat different, with prostate cancer being most common, followed by breast, colorectal, skin, and lung cancers, and stomach cancer is 10th on the list.[2]

There is tremendous geographical variation in the distribution of stomach cancer. More specifically, this disease is predominant in Eastern Asia (e.g., Japan, China, and Korea) and some South American countries (e.g., Chile and Peru), together with parts of Europe, such as Portugal, Estonia, Belarus, and the Russian Federation (Fig. 1). By comparison, the incidence of stomach cancer is low in the United States, Northern Africa, India, Indonesia, and most countries in Western Europe, including Sweden (Fig. 1).[1, 3, 4]

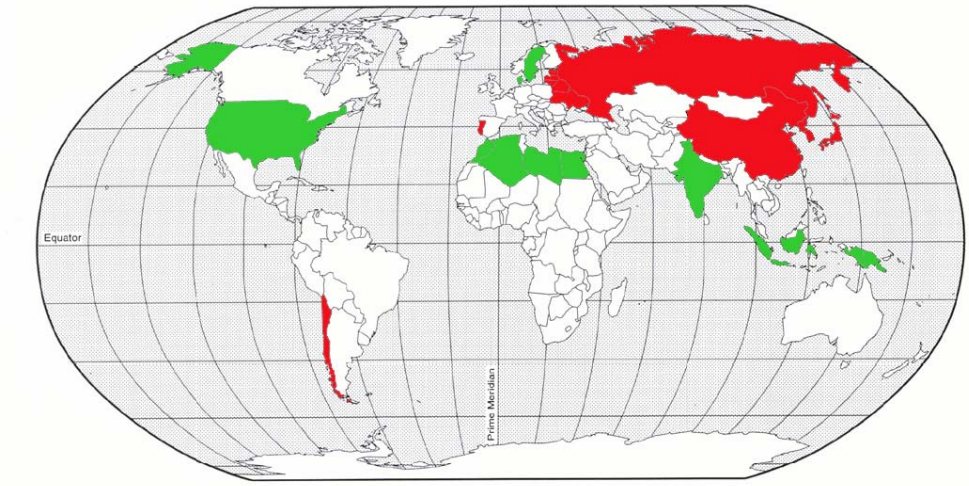


Figure 1. Map showing the countries with the lowest (green) and highest (red) incidence of gastric cancer.

Men are affected by stomach cancer almost twice as often as women.[3] Furthermore, it is well recognized that the sex ratio (male/female) is directly related to the histological type of gastric adenocarcinoma, with a high ratio for the intestinal type (see p. 16) and a low ratio for the diffuse type (see p. 16), regardless of the differences between countries. In fact, the diffuse type of stomach cancer is dominant in Caucasians and the intestinal type is most common among the Japanese and in colored populations.[5] In addition, most cases of gastric cancer are diagnosed between the ages of 50 and 70 in Western countries.[4] Age is even related to the histological type of the cancer: the intestinal type tends to be more common among older patients, whereas the diffuse type is seen in younger patients.[6] Furthermore, the intestinal type predominates in Asia, but the diffuse type is most frequent in the United States and Europe.[5-7] Notably, an increase in stomach cancer has been observed in the youngest age group in China, which is especially interesting because it may represent the introduction of new environmental factors.[7]

Individuals living in high risk areas, such as Korea, show a striking decrease in risk when they emigrate to low incidence regions like the United States.[8] This is illustrated more precisely by research results demonstrating that second and third generation Korean immigrants acquire risk levels similar to those of the host country.[9]

Although the incidence of gastric cancer has declined over the past decades, this is not true regarding gastric cardia cancer, the frequency of which has instead increased.[10] Traditionally, this type of cancer has been very difficult to differentiate from lower esophageal adenocarcinomas, and thus it has been troublesome to characterize its pathology. However, considering epidemiology, it seems that gastric cardia cancer is quite different from cancers in the corpus and antral regions of the stomach.[10]

Symptoms

In most cases, the clinical presentation of manifest, widely spread stomach cancer is well recognized. However, symptoms of the initial phases of the disease can be quite subtle, and patients with early gastric cancer are usually asymptomatic or have unspecific symptoms such as dyspepsia.[11] Indigestion or dyspepsia can comprise heartburn, mild nausea, or loss of appetite, and occasionally also slight abdominal pain or the feeling of being full after eating only a small meal. Moreover, patients can complain of excessive gas (flatus).[12] Clearly, a symptom such as dyspepsia is of limited value as a predictor of early cancer, since dyspeptic symptoms occur in up to 40% of the population.[11]

When the cancer moves into more advanced stages, there may be additional symptoms, such as difficulty swallowing, vomiting without or with blood (hematemesis), or passing blood in the stools that become dark (melena), heavy abdominal pain, and breath odor. With the spread of a tumor, the symptoms become graver and can include weight loss, anemia, jaundice, ascites, and a general decline in health (Fig. 2).[13]

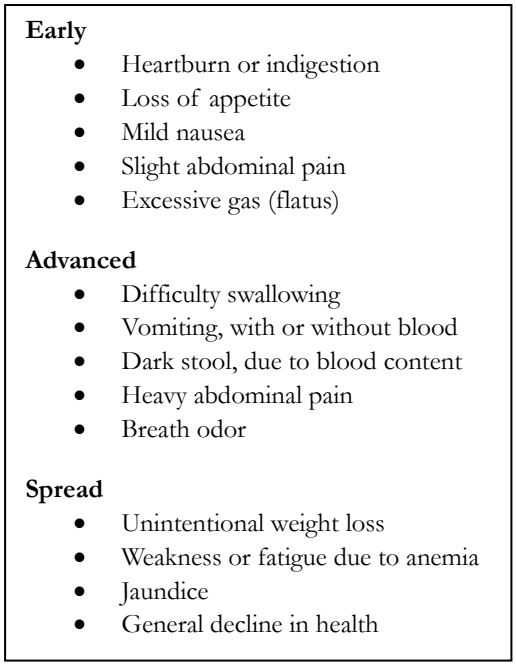


Figure 2. Symptoms during different phases of gastric cancer

Diagnosis and histopathology

Diagnosis of gastric cancer requires an upper endoscopic assessment and a biopsy for histopathological examination.[14, 15]

Anatomically, the stomach is located just under the diaphragm (Fig. 3), and it is divided into five major regions: the cardia, fundus, body or corpus, antrum, and pylorus (Fig. 4). The wall itself has five layers: the inner mucosa containing glands, followed by the thin muscularis mucosa, the submucosa, a thick level of muscle (muscularis externa or m. propria), and outermost the serosa (Fig. 5). Malignant tumors can arise in any part of the stomach, although most occur in the distal third and particularly the lesser curvature. The majority of these malignancies are carcinomas that probably develop from glandular cells (90–95%), followed by non-epithelial tumors such as lymphomas that arise from B or T cells (3%), and leiomyosarcomas that begin in muscle cells in the stomach wall (2%). A small fraction of stomach tumors are referred to as carcinoid tumors, which are well-differentiated endocrine neoplasms.[16]

In humans, it has long been known that the sequential changes in the gastric mucosa that result in invasive neoplasia involve chronic gastritis, atrophy, and intestinal metaplasia (incomplete or complete) and dysplasia, which ultimately lead to malignant transformation.[17] Throughout the gastrointestinal tract, dysplasia of the glandular epithelium is categorized as either low grade or high grade, the latter referring to carcinoma *in situ*.

Over the years, several different systems have been used to classify gastric cancer. The classification developed by Lauren in the middle of the 1960s is still used today, and the WHO recently published a somewhat modified version of that scheme. According to Lauren's classification, the intestinal and the diffuse type of the disease are subdivided into categories based on morphology. Compared to the diffuse variant, the intestinal type of adenocarcinoma has a better prognosis, and it is made up of tubular or papillary structures with enough cell cohesion to create glandular formations that can in some cases have solid components. The neoplastic cells are usually quite distinct and have large and irregular nuclei. Adenocarcinomas are most often differentiated as being of high, medium, or low grade, and the grade is considered a prognostic factor (Fig. 6). In diffuse gastric cancer, which is often called the undifferentiated type, cell cohesion is significantly reduced or lost due to the lowered levels of E-cadherin,[18] and thus there is little or no gland formation. The cells are small and organized in tiny clusters that infiltrate and thicken the stomach wall without forming a mass.[9] A particular subgroup of this type is the well-known signet-ring cell carcinoma, which is easily recognized because of its characteristic cells with a clear cytoplasm and a nucleus pushed towards the plasma membrane to create the picture of a signet ring. The macroscopic behaviors of the intestinal and the diffuse

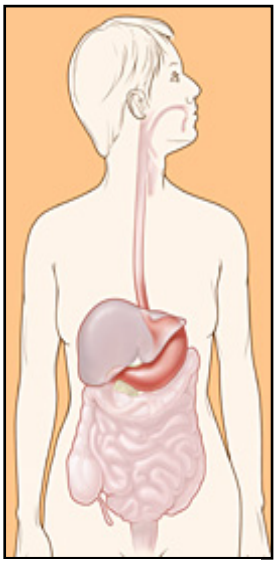


Figure 3. Location of the stomach

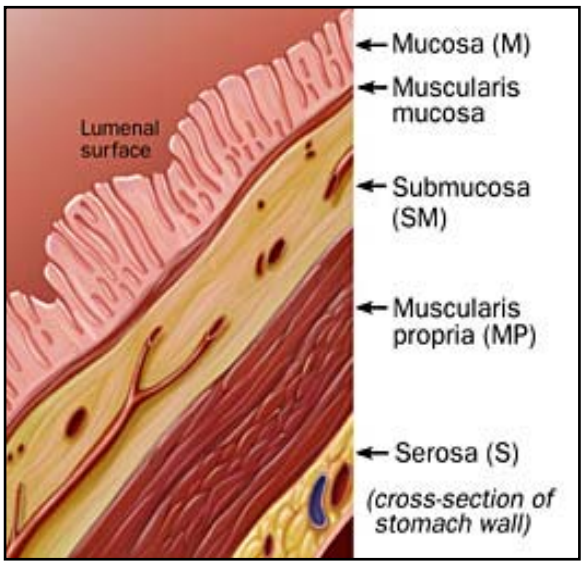


Figure 5. Cross-section showing the layers of the stomach wall.

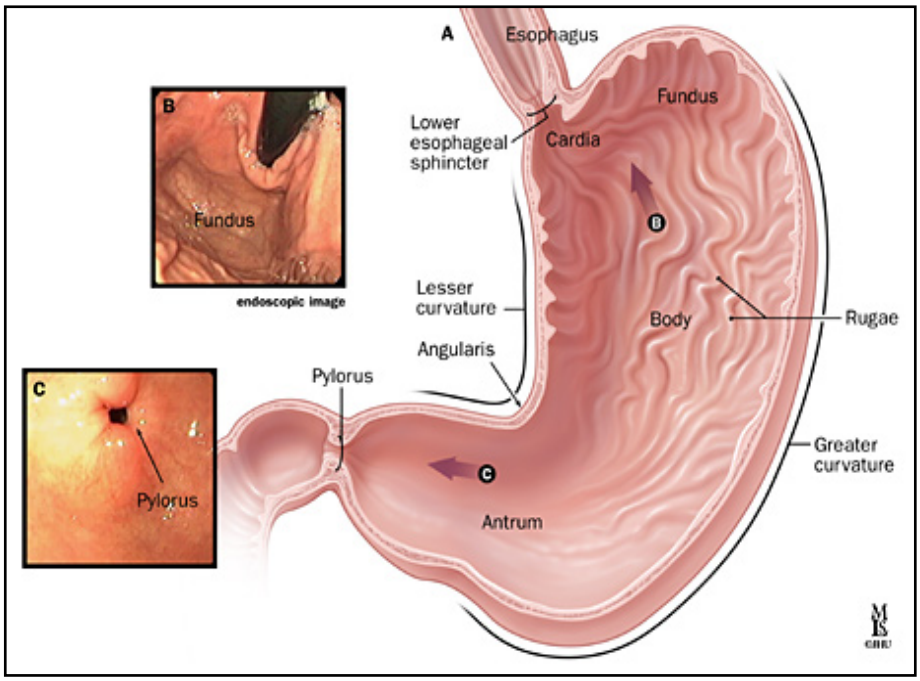


Figure 4. Normal internal anatomy (A) of the stomach and endoscopic views (B and C) of the stomach.

type of gastric cancer reflect the microscopic growth patterns: in the former the macroscopic margins match the microscopic spread, whereas the latter type extends under the mucosa and beyond its macroscopic borders.[19]

Lauren's classification has become exceptionally widespread because, in addition to histological differences, the two main forms of gastric cancer are also dissimilar with regard to epidemiology, etiology, and pathogenesis. The intestinal type dominates in high-risk areas, is more often found in the distal part of the stomach, and is frequently preceded by a protracted precancerous phase.[9] In contrast, the diffuse type prevails in young patients and women and is often a hereditary disease. By comparison, the WHO classification of gastric carcinomas includes different subtypes, such as adenocarcinoma (intestinal and diffuse, as discussed above; Fig. 6). However, there are also a number of less common and rare forms of cancer, such as papillary adenocarcinoma, tubular adenocarcinoma, mucinous adenocarcinoma, signet-ring cell carcinoma, adenosquamous carcinoma, squamous cell carcinoma, small cell carcinoma, and undifferentiated carcinoma.[16]

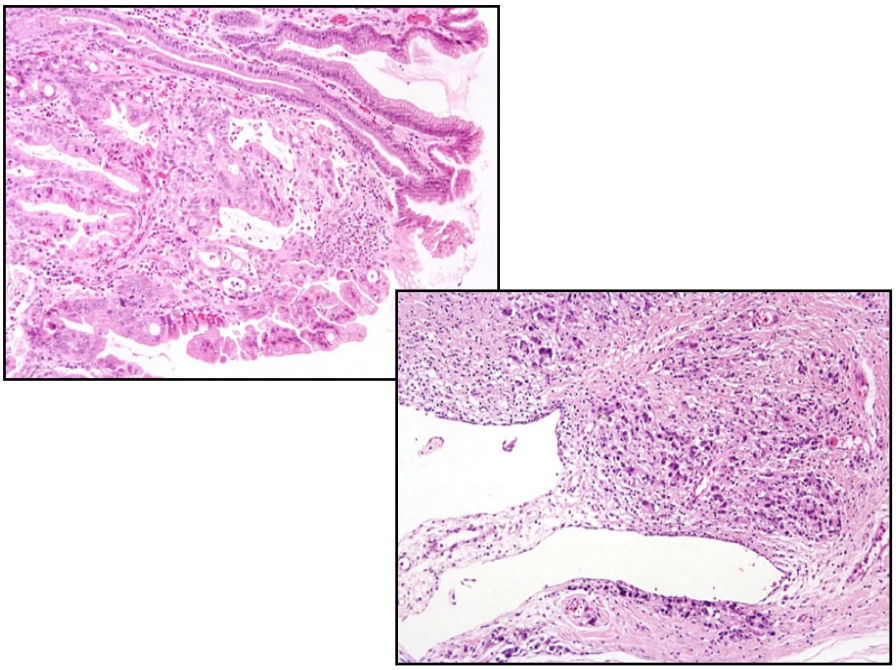


Figure 6. Intestinal (left) and diffuse (right) type of gastric cancer according to Lauren's classification.

In recent years, confidence in the Lauren classification system has diminished due to the suboptimal correlation between the phenotypes and the clinical behaviors of the tumors. Tay et al.[20] categorized gastric cancers into three groups that they called tumorigenic, reactive, and gastric-like types based on their unique molecular signatures, which were detected by applying a combination of comparative genomic hybridization and global gene expression profiling analyses. These investigators considering patients in all three of the designated groups and found that those with the gastric-like type of the disease had considerably longer survival, regardless of other variables such as sex, age at diagnosis, clinical stage, tumor site, or Lauren classification.[20] Furthermore, molecular signatures correlated well with the clinical stage in each subtype, especially for tumor stages I and IV (early and late). In the future, molecular signatures may prove to be much more useful than morphology for staging and assessing the prognosis of different kinds of gastric cancer.

An interesting theory is that gastric cancer might develop from stem cells. In short, the idea that terminally differentiated cells proliferate endlessly in response to extensive genetic damage is becoming increasingly obsolete. Therefore, it is now assumed that stem cells are the targets of transformation, and an incredible amount of attention is being focused on tissue-derived stem cells, mesenchymal stem cells (MSCs), and bone-marrow-derived stem cells (BMDCs)[21-23] due to their capacity for long-term self-renewal and relatively marked resistance to apoptosis.

Many studies in recent years have explored the role of angiogenesis in tumor progression. Folkman and his research group addressed that subject and concluded that microscopic cancers are present in most of the ageing population.[24] For instance, those investigators claim that 98% of people who die of trauma at the age of 50 to 70 harbor microscopic cancers in the thyroid gland, but that only a small fraction of those cell changes (0.1%) would have developed into detectable cancer if the individuals had lived longer. What capacity must the cells exhibit in order to be able to grow and proliferate? According to Folkman, it is the "angiogenic switch" that is needed, in other words the ability of a tumor to progress from a non-angiogenic to an angiogenic phenotype by recruiting and sustaining its own blood supply. Oxygen diffusion in a mammalian cell is limited to a distance of 100–200 μm. Accordingly, blood vessels that are farther away cannot help microscopic tumors to expand in mass, and hence such cancers will remain "dormant."[24] The mechanism involved in making the angiogenic switch has not yet been fully elucidated.

Clinical staging

It is very important to establish whether a cancer has spread beyond its primary site before considering what kinds of treatment are feasible.[14] Is there any malignant extension to adjacent structures? Has the cancer spread to distant sites or to lymph nodes? The answers to these questions determine the choice of therapy. The typical dissemination of gastric cancer comprises regional lymph nodes, the liver, the lungs, and/or the peritoneal cavity.

The staging methods vary somewhat between countries. Routine staging usually includes endoscopic inspection, computed tomography (CT), multidetector CT scanning (MDCT, a form of spiral CT), or magnetic resonance imaging (MRI) to map the appearance of the intragastric tumor and metastasis to organs in the thorax or abdomen, or the regional lymph nodes.[25, 26] Complementary examinations often comprise endoscopic ultrasound to assess the stomach wall and lymph nodes, along with transabdominal ultrasound to investigate the liver and possible ascites.[27] In some cases laparoscopy is needed, for example, if ascites is suspected.[14] Furthermore, there is now more widespread use of the new PET-CT (positron emission tomography-computed tomography) imaging technique, which combines functional and anatomical information to enable more accurate visualization of form and performance.[28]

The two main reasons for classifying malignant tumors are to identify stage-independent prognostic factors and to generate numerical models for prediction of the outcome.[29] There are several systems for staging gastric cancers, but the most widely used is no doubt tumor-node-metastasis (TNM) classification (Table 1 and Fig. 7), which is based on the size of the primary tumor, the extent of spread to regional lymph nodes, and the presence or absence of detectable distant metastasis.[30] When the cancer is staged, it is given a number to designate one of these three characteristics (Table 2). For example, T1 denotes disease that is often called "early gastric cancer" (EGC), in which the malignancy is limited to the mucosa or submucosa and does not invade the muscularis propria. The methods currently available for clinical staging are not perfect, and hence new techniques and tests are constantly being developed. In many cases, more advanced disease is found during surgery than was detected by diagnostic procedures, which indicates the need for further improvement of diagnostic tools.

Classification	Tumor Extent
T0	No Tumor
Tis	Mucosa
T1	Lamina propria, submucosa
T2a	Muscularis propria
T2b	Subserosa
T3	Penetrates serosa
T4	Adjacent structures
N0	No metastasis
N1	1-6 nodes
N2	7-15 nodes
N3	> 15 nodes
M0	No metastasis
M1	Distant metastasis

Table 1. TNM classification of gastric cancer

Stage	TNM
0	Tis N0 M0
IA	T1 N0 M0
IB	T1 N1 M0; T2a/b N0 M0
II	T1 N2 M0; T2a/b N1 M0; T3 N0 M0
IIIA	T2a/b N2 M0; T3 N1 M0; T4 N0 M0
IIIB	T3 N2 M0
IV	T4 N1,N2,N3 M0; T1,T2,T3 N3 M0; Any T Any N M1

Table 2. Stages of gastric cancer according to TNM classification

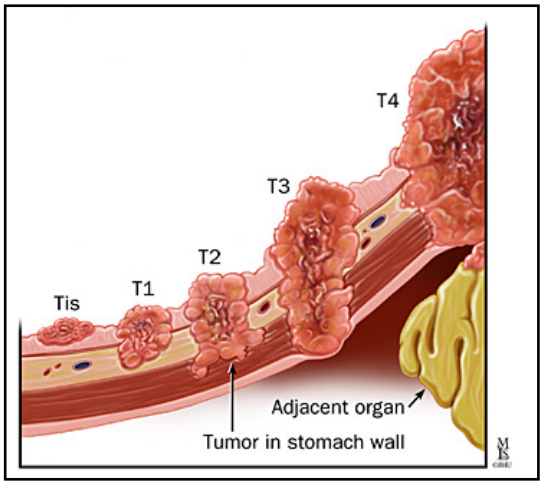


Figure 7. TNM staging of gastric cancer, showing depth of invasion.

Treatment and prognosis

Complete surgical resection is still the principal treatment for gastric cancer. Notwithstanding, the five-year survival rate is poor, in the range 10–20%.[4] For many years, the standard method to treat all stomach cancer included total gastrectomy with splenic and celiac lymph node resection, splenectomy, and pancreatectomy. The reconstruction involves anastomosis of a Roux-en-Y limb of jejunum to the esophagus to reduce reflux of bile into the esophagus (Fig. 8). In proximal cancers (i.e., those affecting the proximal two-thirds of the stomach), total gastrectomy or esophagogastrectomy continues to be the favored option,[14] despite the high morbidity linked to this type of operation. For distal gastric tumors, the current method of choice is subtotal gastrectomy, which can be performed with or without a Roux-en-Y limb (Figs. 9 and 10).[14, 31]

As mentioned above, stomach cancer is a very common type of tumor in Japan, and thus various centers in that country have vast experience in detecting and treating EGC.[12] The rate of EGC is approximately 10% in most of the world, whereas in Japan over 50% of stomach cancer patients fall into this category. This level of detection is a major triumph for Japanese endoscopists, especially because the five-year survival rate is greater than 90% in EGC patients.[11] But why is the rate of findings so high in Japan? A plausible explanation is that endoscopists in Western countries look for a visible mass or an ulcer, whereas their Japanese counterparts to a greater extent use gastric distension, anti-foaming agents, and chromoscopy, which can identify small gastric lesions that would otherwise be easily overlooked.[11]

Globally, the most commonly used treatment for EGC is gastrectomy (distal, proximal, or total).[12] Laparoscopy-assisted gastrectomy is a safe, useful, and minimally invasive approach that can be applied in the majority of cases, but it is not yet well accepted by surgeons.[32] In Japan, there is a trend towards a shift from more aggressive methods to more conservative techniques.[11] One such strategy is endoscopic mucosal resection (EMR; Fig. 12), which permits en bloc removal of the cancer by use of a snare loop. However, that was initially often associated with incomplete removal of the tumor, mainly due to slipping of the snare loop during the resection, as well as the limited diameter of the loop itself.[5] To rectify this problem, a new method called endoscopic submucosal dissection (ESD) was developed in Japan. Another well-accepted technique is to use an insulated tip (IT) knife equipped with a ceramic bulb, which allows the endoscopist to excise a wider area in the submucosa and more accurately define tumor borders.[5, 11]

Lymph node metastases are a major prognostic factor, and node dissections are performed as part of the complete surgical resection, but the optimal extent of node removal is still a subject of debate. The lymph nodes around the stomach are classified into 16 stations, which are in turn assigned to three different clusters or

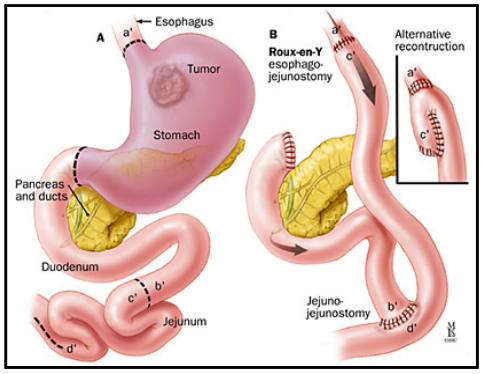


Figure 8. Total gastrectomy (A) and Roux-en-Y esophagojejunostomy (B).

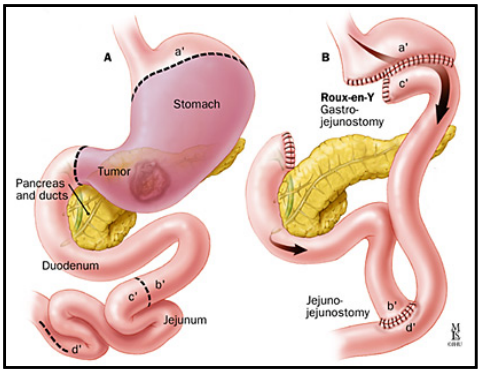


Figure 9. Subtotal gastrectomy (A) with Roux-en-Y gastrojejunostomy (B).

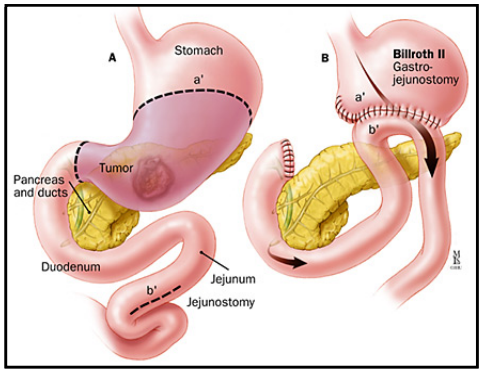


Figure 10. Subtotal gastrectomy (A) with Billroth II anastomosis (B).

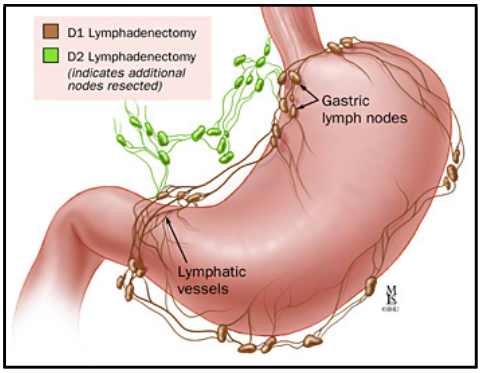


Figure 11. Surgical lymphadenoectomy. D1 and D2 indicate the extent of lymph node removal.

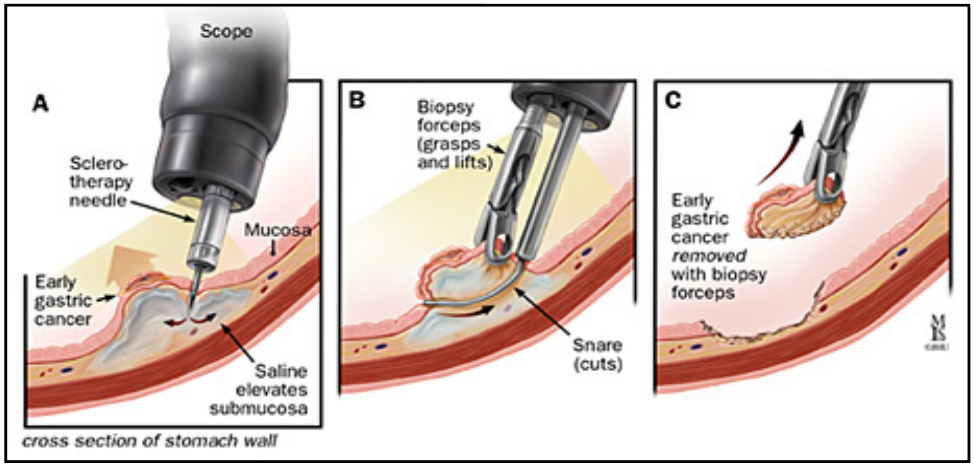


Figure 12. Endoscopic mucosal resection of EGC. Injection and snare technique.

groups designated D1, D2, and D3.[33] Among Japanese surgeons,[14] it is general practice to perform lymphadenectomy (Fig. 11) according to the D2 protocol, which calls for excision of the first two clusters of lymph nodes that drain the area affected by the tumor. However, in Western countries, most surgical centers use D1 protocols, which confine the lymphadenectomy to the first cluster of lymph nodes. Unfortunately, despite evidence supporting the use of D2 resection, it is a demanding procedure and thus it will probably take time to evolve as a routine method in the West as well. Indeed, because of the limited experience of the D2 strategy, Western countries report much higher surgical mortality and notably inferior overall survival rates compared to Japan.[14]

It is extremely important to remember that the majority of patients in countries other than Japan are diagnosed in tumor stages with little chance of curable surgery.[14] Even patients with tumor-free margins, constituting close to 80% of all cases of gastric cancer, will in most cases experience recurrence and die from their disease.[31] Therefore, it is considered imperative that adjuvant therapies be developed to improve the outcome, and numerous trials are now in progress. Only a handful of studies have focused on adjuvant chemotherapy, and they have indicated that such treatment offers only weakly significant improvement or no apparent improvement at all in the results of complete resection.[33] Trials employing chemoradiotherapy have shown an increase in the survival rate from 27 to 36 months, but those investigations have been criticized for their lack of surgical consistency.[33] Nonetheless, there is extensive use of palliative therapy, such as surgery or endoscopic procedures and radiation.

Risk factors

The cause of stomach cancer is multifactorial. The disparity in geographic distribution, the migratory effects of the disease, and the recently observed shift in anatomical location of such cancer suggest that environmental or lifestyle factors make the chief contributions to the etiology.[4, 31] In the last two decades, the debate on this subject has centered on two major aspects—*Helicobacter pylori* infection and dietary factors—both of which can be modified.

Diet

In 1997, a global account of diet and cancer prevention was published jointly by the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR) in a report entitled *Food, Nutrition and the Prevention of Cancer: a Global Perspective*. Briefly, this report is a comprehensive review of scientific studies concerning diet, nutrition, and cancers. The panel and contributors estimated that 30–40% of all cancers are linked to dietary choices, and they proclaimed that there is quite convincing evidence that high consumption of vegetables and fresh fruit protects against stomach cancer.[34] They also concluded that the use of refrigerators has a substantial protective effect in that it enables year-round consumption of vegetables and fresh fruit, which makes it possible to avoid salt as a preservative. Moreover, these experts proposed that the risk of gastric cancer can be reduced by a diet that includes foods containing carotenoids, allium compounds, whole-grain cereals, and green tea. In contrast, it was assumed that the risk is not lowered by substances such as fiber, selenium, and garlic, and also that coffee, black tea, nitrates, sugar, vitamin E, and retinol are not related to gastric cancer. Alcohol was not believed to increase the risk for gastric cancer in general, except for gastric cardia cancer. In 2001, the WCRF and AICR laid plans to publish a second report in 2006. However, due to the enormous amount of data accumulated, they have been forced to postpone publication until autumn 2007.

The hypothesis indicating that extended and excessive use of salt is linked to the etiology of gastric cancer was first presented in 1965. Prolonged and extreme salt intake may cause irritation and in the long run atrophic gastritis, and along with that an increased propensity for cancer.[34] Salt intake has been assessed in numerous ways, for example by analyzing consumption of salted foods, use of table salt, salt added during cooking, and, more accurately, by assessing urinary sodium excretion.[10] However, there are some problems associated with evaluating studies of this specific exposure. For instance, it is difficult to precisely measure salt intake, since dietary studies may not accurately observe salt added during cooking or at the table.[10] The pathogenesis related to salt is considered to occur through irritation of the mucosa and eventually the development of atrophic gastritis. Moreover, it is assumed that the

same mode of action is connected with *H. pylori* infection, and, lack of adjustment for infection could easily confound the effects of salt.[10]

The role of dietary nitrite and *N*-nitrosamines in the pathogenesis of gastric cancer has continued to be a matter of discussion for more than three decades. Nitrate (NO_3-) is a widespread contaminant of ground and surface waters worldwide, and it represents the most oxidized chemical form of nitrogen found in natural systems.[35] Nitrate is converted to nitrite (NO_2-) in the intestinal tract or during the processing of food (e.g., grilling). Nitrate and nitrite are not carcinogenic in themselves,[34] but they become so through a process called nitrosation, which results in the formation of *N*-nitroso compounds. Nitrosation is inhibited by vitamin C present in the gastric juice.[10] Despite enormous efforts to establish a link between stomach cancer and nitrosamines, thus far epidemiological studies have not provided any conclusive evidence of such a connection.[10, 34, 36]

Randomized trials have suggested that it is the antioxidant capacity of fruit and vegetables, which can be provided by vitamin C, that is responsible for the capacity of these food products to decrease the risk of gastric cancer.[10] Interestingly, infections with *H. pylori* are associated with reduced bioavailability and plasma concentrations of vitamin C, and Woodward and colleagues[37] have speculated that that effect may constitute a causative factor in gastric cancer. In support of that hypothesis, those investigators observed that eradication of *H. pylori* improved secretion of vitamin C into the stomach.

H. pylori infection

Ever since *H. pylori* was identified in 1983, intensive research and many studies have established a clear correlation between this 3-μ-long, gram-negative bacterium and gastric carcinoma.[1, 4, 10, 38-40] In addition to causing stomach cancer, *H. pylori* is also involved in the pathogenesis of peptic ulcer disease and MALT (mucosa-associated lymphoid tissue) lymphoma.[41] This microbe has been developing survival mechanisms for a very long time, and it is capable of existing in the highly acidic environment of the human stomach.[40] A unique feature of *H. pylori* is that it excretes the enzyme urease, which hydrolyzes urea into ammonia and bicarbonate in order to create the appropriate microenvironment for the bacteria, which in turn causes gastric inflammation in the host. It is assumed that persistent chronic inflammation induced by *H. pylori* infection leads to gastric atrophy and eventually to intestinal metaplasia, and in some subjects ultimately to development of adenocarcinoma, all according to Correa's model of cacinogenesis.[42]

H. pylori infects about half the world's population. In developed countries, the rates of infection are in the range 30–40%, whereas the rates in developing and high-

prevalence countries are as high as 80–90%.[43] Notably, there are still disagreements about why the bulk of people infected with *H. pylori* remain asymptomatic, and only a small fraction of them develop distal gastric adenocarcinoma.[4, 38]

There is evidence that bacterial virulence factors and pro-inflammatory factors in the host determine who will develop gastric cancer.[39] Clearly enhanced risks are associated with strains of *H. pylori* that carry the cytotoxin-associated gene A (*cagA*). That gene encodes the 120–140-kDa protein CagA. Bacterial strains that express CagA have been found to cause more severe inflammation, a higher degree of atrophy, and increased incidence of peptic ulcer and gastric adenocarcinoma of intestinal type.[44] In Japan, all *H. pylori* strains are *cagA* positive, whereas only about 60% of the strains in Western countries carry that gene.

The difference in virulence between *H. pylori* strains also depends on the presence or absence of vacuolating toxin (VacA) and the blood group antigen-binding adhesin (BabA).[45] The latter protein is encoded by the *babA* gene, and it promotes bacterial colonization by supporting tighter adhesion of the microbes to the gastric epithelial cells via the Lewis antigen that is expressed on the surface of those host cells.[45] The VacA protein is encoded by the *vacA* gene, and it is known to be toxic to epithelial cells *in vitro*. VacA exerts its effect by inducing large intracellular vacuoles and apoptosis, which it achieves by inserting itself into the cell membrane and forming a channel though which organic anions and bicarbonate can be released.[44] It has also been reported that VacA disrupts the cytoskeletal architecture, and that it damages cell-cycle-related genes and thus breaks the balance between cell proliferation and cell death.[40] Furthermore, genetic polymorphisms with high levels of the pro-inflammatory cytokine IL-1β have been observed to enhance the susceptibility to neoplastic transformation.[38, 39] One of the cited investigations comprised an evaluation of the results of many independent studies, and the findings indicated an up to 20-fold increase in the relative risk of developing adenocarcinoma in patients infected with *H. pylori* strains of the triple-positive virulent genotype.[38]

The observation that several countries in Asia, among them India, Thailand, and Indonesia, have a high rate of *Helicobacter pylori* infections but a considerably low incidence of gastric cancer is termed the "Asian paradox."[38] Similarly, the expression "African enigma" refers to the fact that the northern and western parts of Africa differ markedly with regard to incidences of gastric cancer (which are low) and the rates of *H. pylori* infection (which are high).[38, 43] Although there is probably some underreporting from these countries, it seems plausible that the mentioned disparity can be explained by the presence of different bacterial virulence factors and genetic host polymorphisms. There may be other explanations for this paradox as well (see "Calcium carbonate in food processing" on page 46).

Genetic predisposition

A positive family history is a significant risk factor, and case-control studies have indicated a two- to threefold increase in risk among first-degree relatives.[46] Moreover, the results of an epidemiological investigation have suggested that 1–3% of gastric carcinomas are associated with a hereditary gastric cancer predisposition syndrome.[47]

In 1999, the International Gastric Cancer Linkage Consortium[47] defined hereditary diffuse gastric cancer (HDGC) by either of two clinical criteria: (1) any family with two or more documented cases of diffuse gastric cancer in first- or second-degree relatives, and one of the cases under the age of 50; or (2) three documented diffuse gastric cancers in first- or second-degree relatives of any age. Based on these criteria, it has been estimated that the incidence of germline mutations in HDGC is 25–40% in this group of patients. The first germline mutation identified as a molecular genetic cause of HDGC was the *CDH1* gene, which was described in 1998. Since then, at least forty distinct *CDH1* germline mutations have been reported for this disease. The *CDH1* gene encodes E-cadherin, which is a protein that is necessary for cell cohesion and plays an important role in tumor invasion and metastasis, and expression of E-cadherin is typically reduced or lost in the diffuse type of gastric cancer. Investigations in recent years have examined and extended the known functions of E-cadherin in carcinogenesis. One study has found evidence that this protein is involved in modulating intracellular signaling, and in that way it can promote tumor growth.[48] Even somatic mutations in E-cadherin frequently occur in sporadic gastric cancer of the diffuse type, as well as in sporadic lobular breast carcinomas.[47] In fact, reduced or lost expression of E-cadherin is now a well-known phenomenon in many human carcinomas, such as those arising in breast, colon, stomach, head and neck, lung, skin, thyroid, and ovary.[48]

Lately, research has indicated that functional polymorphism in the interleukin-1 and tumor necrosis factor alpha genes are associated with increased risk of gastric cancer. Carriers with genotypes leading to elevated production of the potent proinflammatory cytokine IL-1β exhibit severe suppression of acid secretion, which rapidly leads to gastric atrophy and hypochlorhydria, and along with that a rise in the risk of developing stomach cancer.[46]

Hereditary nonpolyposis colorectal cancer syndrome (HNPCC) is an inherited autosomal dominant tumor predisposition condition that is characterized by the familial occurrence of colorectal carcinoma. The molecular genetic cause of this syndrome is germline mutations in DNA mismatch repair genes. Patients with HNPCC often shows microsatellite instability (MSI-H) leading to frequent replication errors that usually are corrected by an intact DNA mismatch repair system. This syndrome is also associated with carcinoma outside of the colon, such as in ureter,

duodenum, and stomach. Most gastric carcinomas linked to HNPCC are of the intestinal type.[47]

Germline mutation of the p53 tumor suppressor gene is responsible for a rare form of inherited cancer known as Li-Fraumeni syndrome. The tumor protein p53 is encoded by the gene designated *TP53*, which occurs on chromosome 17 in humans and is very important for the ability of multicellular organisms to suppress cancer.[47] If DNA is damaged, the p53 protein arrests the cell cycle at the G1/S regulation point and initiates apoptosis (programmed cell death). The types of tumors seen most often in Li-Fraumeni syndrome include osteosarcoma, brain tumors, and soft-tissue sarcomas, followed by gastrointestinal tumors (7%). Approximately half of the tumors are gastric carcinomas.[47]

Another rare condition known to be associated with an increased risk of stomach cancer is Peutz-Jeghers syndrome, which is an inherited autosomal disease characterized by hamartomatous polyps in the gastrointestinal tract and mucocutaneous pigmentation of the lips, buccal mucosa, and digits. This syndrome is caused by mutations in the tumor suppressor gene STK11 that lead to disrupted regulation of cell division and formation of non-cancerous polyps and cancerous tumors.

Miscellaneous factors

Several medical conditions have been proposed to increase the risk of gastric cancer. It has been shown that adenomatous polyps elevate the incidence of such malignancy by 20%,[31] and conditions like pernicious anemia, intestinal metaplasia, achlorhydria, Menetrier's disease, previous stomach surgery for benign diseases, and Epstein-Barr virus infections have all been linked to stomach cancer. Moreover, having blood type A has long been considered to be a risk factor for diffuse-type gastric cancer.[49]

The issue of a possible relationship between tobacco and gastric cancer has received considerable attention over the years, but, despite that, the issue still remains unclear. A few case-controlled studies have addressed this question, but they have not detected any definite association between these two factors, although some have demonstrated what appears to be a dose-dependent relationship.[50] Furthermore, it has been suggested that smoking is mainly related to the distal type of stomach cancer.[51]

Epidemiological investigations have shown excess risks of stomach cancer in connection with occupational exposure to ionizing radiation, nitrogen oxides, dust, and *N*-nitroso compounds.[52, 53] Carpenters, steelworkers, and tin miners are among those with the highest risk of gastric cancer, and increased risks are also seen in people working in chemical industries, coal mines, oil refineries, and rubber manufacturing plants.[53]

Corpulence is positively correlated with cancer in general, but results in the literature are inconsistent with regard to stomach cancer in particular. However, most studies have constituted a relationship between obesity and increased risk of gastric cardia cancer, gastroesophageal reflux, and Barrett's esophagus.[4, 10]

Age and gender are also risk factors for gastric cancer (see "Demographics" on p. 13), and the risk rises with age. At the time of diagnosis, most people are between 50 and 70 years of age and rarely younger than 40.[1, 3]

Experimental background (Animals)

Why animal experiments?

Etiology is the study of the causes of disease, and pathogenesis concerns the way in which a disease evolves, induces tissue damage, and spreads within an organism. Thus it is imperative to understand both of these processes in order to be able to develop approaches to either stop or limit a specific disease. The etiology and pathogenesis of most disorders are very complex and include dynamic interactions between molecular and cellular systems that determine the progression of the condition in question. Animal research is a highly effective method of studying the intricate interplay that occurs between molecules, cells, and organs in this context.

The use of animals in experiments is a subject that should not be taken lightly. There are unquestionably clear ethical arguments that must be given consideration. Is the anticipated outcome of my study of importance for humans? Is there any other way of finding answers to my questions? Has such an investigation been done before? Do I need this number of animals? How will my experiments affect the lives and welfare of the animals I have chosen?

In May 2005, the Nuffield Council on Bioethics published a consensus report entitled "The Ethics of Research Involving Animals."[54] This report is nearly 400 pages long, and it covers a broad range of ethical issues in the field of animal research. There are some statements in the report that I consider to be of utmost value in the debate concerning the use of animals in experimental research.

"While we trust that more progress in the moral debate can be made, we are aware that, for the near future, further moral argument alone cannot provide a universal answer as to whether or not research on animals is justified. But practical advances in scientific methods can reduce areas of conflict. For this reason, the importance of the Three Rs (Refinement, Reduction and Replacement), and especially of the need to find replacements, cannot be overstated" (page 22).[54]

"We conclude that because of evolutionary continuities in the form of behavioral, anatomical, physiological, neurological, biochemical and pharmacological similarities between animals and humans there are sufficient grounds for the scientific hypothesis that, in specific cases, animals can be useful models to study particular aspects of biological processes in humans, and to examine the effects of therapeutic and other interventions" (page 23).[54]

When a decision is finally made to use animals in a research project, there are several things that must be taken into account. For example, it is very important to consider whether the model offers physiological similarities to humans with respect to the particular application being studied. In tumor investigations such those included in

the present research, it is critical that the animal tumor resembles the specific human disease, both histopathologically and at a molecular level.

When scrutinizing the "materials and methods" sections of papers describing animal studies, it is astonishing how little space, if any at all, that the authors allot to describing the postoperative mortality. Nonetheless, Langhans *et al.*[55] have reported a survival rate of 44.8% in their series of Billroth I and II resections performed on rats. More precisely, only 209 out of a total of 466 rats survived the operations, and thus the mortality rate was in fact 55.2%. Also, a rather recent investigation conducted by Viste *et al.*[56] was declared to have led to a mortality rate of 34%. So why is the postoperative mortality so remarkably high?

Of course the surgical technique and the skill of the surgeon have a decisive impact on the final results. However, I personally believe that one of the most essential and critical stages in any animal experiment involving surgical procedures is the postoperative phase or, more correctly, the postoperative intensive care that is given. I have seen and experienced the following situation several times. You spend hours performing a complicated microsurgical procedure, and afterwards you put your patient (rat) in its cage and leave for home. The next morning you arrive only to find that the patient (rat) is dead. When you have gone through this embarrassing series of events a few times, you usually stop leaving the patient (rat) unattended. Clearly, you must realize that the experimental animal needs the same type of consideration and attention that people get in an intensive care ward. This means that before you can consider leaving your animal patient alone, you must make sure that it receives adequate pain control, and that it is sufficiently rehydrated, awake and able to find and drink water on its own.

The present investigations (Animals)

Aims

The aim of the research described in this thesis was to examine factors that are involved in promoting gastric cancer. It is known that the cause of this particular form of cancer is multifactorial, and there is now evidence indicating that environmental and lifestyle elements are of particular importance. Notably, recent medical advances in this area have to some extent focused on diet and food supplements.

More precisely, the aims of the present studies were as follows:

- To establish a laboratory animal model to investigate the incidence of gastric carcinoma in rats given various dietary supplements without simultaneous administration of carcinogenic substances (Paper I).
- To ascertain whether acetic acid can increase the incidence of adenocarcinoma (Paper I).
- To evaluate how dietary supplementation of calcium in the form of calcium carbonate affects the incidence of gastric cancer (Paper II).
- To determine whether it is the calcium or the carbonate ion that is responsible for the markedly increased occurrence of gastric adenocarcinomas (Paper II).
- To study the effect of dietary supplementation of carbonate ions on expression of the two cancer-related proteins COX-2 and ornithine decarboxylase (ODC), and to investigate the correlation between the expression of those proteins and cell proliferation (measured as Ki67 staining) in the non-transformed mucosa in a rat model of gastric cancer (Paper III).

Results and discussion

An experimental study of gastric stump carcinoma in Wistar rats (Paper I)

In a gigantic investigation performed in 1930, Bullock and Curtis[57] undertook post-mortem examinations of 33 000 laboratory rats and discovered one solitary case of spontaneous adenocarcinoma with no detectable metastases. This finding demonstrates the extremely low incidence of spontaneous gastric carcinomas in these animals. However, the scarceness of tumors also suggests that this particular species is almost perfect for use in experimental studies, because there is virtually no risk of confounding factors such as spontaneously developing tumors.

One way to experimentally induce adenocarcinomas in the stomach is to use carcinogenic substances. In 1915, Yamagiwa and Ichikawa[58] were pioneers in this area when they succeeded in generating squamous cell carcinoma in rabbits by painting coal tar on the ears of the animals. Even today, their experimental specimens are still on display in the museum of the Faculty of Medicine, University of Tokyo. In 1935, other investigators induced hepatomas in rats by feeding the animals o-aminozotoluene. [58] The real use of experimental carcinogenesis in the gastrointestinal tract began in 1952 with the discovery that colon cancers could be provoked by administration of aminobiphenyl.[58] At that time, aminobiphenyl was used as a rubber antioxidant and a dye intermediate, but it is no longer commercially manufactured. In 1967, Sugimura and Fujimura[59] gave rats subcutaneous injections of *N*-methyl-*N´*-nitro-*N*-nitrosoguanidine (MNNG) to obtain fibrosarcomas. Surprisingly enough, these authors also found that adenocarcinomas occurred in the glandular stomach of rats that had ingested MNNG dissolved in their drinking water, and a few of those animals also exhibited metastases to the liver and lymph nodes. This was a very fortunate finding, given that so many procedures had previously been unsuccessful in producing gastric cancers in animals. Since the time of the fundamental studies of Sugimura and Fujimura, MNNG has become one of the most reliable means of inducing carcinogenesis in the glandular stomach of the rat, and an abundance of series of histopathological lesions caused by this agent have been described in the literature. However, a disadvantage of MNNG is its non-specific tumor effect, for example, it has been found to cause leiomyosarcoma in the upper intestinal tract.[59] In my opinion, it is not ideal to use carcinogenic substances in experiments that are conducted to examine the effects of different dietary factors. Carcinogenic substances such as MNNG often have systemic effects, so the whole organism becomes the target. The experimental model I have chosen entails reflux into the stomach, and there is only a local effect. Moreover, the situation in the rat model corresponds to that in humans, where enhanced reflux increases the risk of gastric malignancy.[56, 60, 61]

Another way to experimentally generate carcinomas in the stomach of rats is to perform a gastric resection. That technique was first used by Schlake and Nomura[62] in

1979, when they performed a BI resection that in itself provoked carcinogenic predisposition. In 1981, Langhans *et al.*[55] found a cancer incidence of 30% in rats after BII resection, whereas a complete absence of carcinoma was observed in rats subjected to BII resection with Roux-en-Y, and hence they suggested that surgically induced duodenogastric reflux (DGR) is directly related to tumorigenesis. Mason *et al.*[63] investigated the role of DGR and found that combined reflux led to adenocarcinomas in 58% of the rats they used. In an attempt to determine what fraction of the reflux (i.e., bile or pancreaticoduodenal secretions) was responsible for the malignant transformation, those investigators conducted a new study.[64] The results showed that bile reflux alone did not generate any cancers. However, pancreaticoduodenal reflux secretions alone induced carcinomas in 71% of the treated animals, confirming that it was this component of the reflux, and not bile, that was responsible for the malignant potential.

In the present investigation (Paper I), a gastrojejunostomy resection (Billroth II) was performed on a total of 44 animals. After surgery, the animals were randomized into two groups, which respectively received a normal diet and a special diet containing acetic acid. The results show that that 25% (5/20) of the animals that underwent BII resection and ate a normal diet developed carcinomas, whereas the corresponding rate was only 17% (4/23) in the BII-resected group that received a diet supplemented with acetic acid (Table 3). No tumors were detected in the sham-operated animals or the control group.[65]

Food	Normal	Acetic Acid
Number of animals	20	23
Adenocarcinoma	5	3
High grade dysplasia	0	1

Table 3. The incidence of major histological changes in the different resected groups

Today, it is well known that DGR can provoke gastric cancer, both in humans and in animals used in laboratory experiments.[60] A number of research groups have explored and used this gastrojejunostomy method and induced gastric tumors at incidences ranging from 12% to 60%.[55, 56, 63-68] The frequency differs greatly between the studies, which might be explained by the use of different surgical techniques, resulting in varying degrees of reflux. The length of a trial is also an important variable, since more cancers develop in prolonged experiments. The cited studies certainly entailed *ad hoc* variation, and they also used different histopathological criteria. Specific attention should be given to histological evaluation of adenocystic

proliferation, which might be mistaken for adenocarcinomas. Similarly, it is important to carefully examine glandular structures adjacent to an inflammation, which can easily be misjudged as cytological atypia.

Another important aspect of the histological evaluation concerns dissimilarities between species. In humans, the development of adenocarcinoma is preceded by chronic inflammation, intestinal metaplasia, and atrophic gastritis.[17] In contrast, rats often display inflammation, cystic dilatation, and metaplasia, together with mucosal hyperplasia and derangement.[56] The metaplasia seen in rats is specific to that species and is nothing like the type observed in humans (i.e., intestinal metaplasia). In rats, the metaplastic areas are lighter in color than the surrounding mucosa, and they are clearly visible and frequently seen in the deeper part of the mucosa, always in the vicinity of the gastrojejunal anastomosis (Fig. 13). This raises the question of whether this type of metaplasia might precede tumor evolution and be the rodent form of the human counterpart (i.e., intestinal metaplasia). We have designated this special form of transformation "light-cell metaplasia."

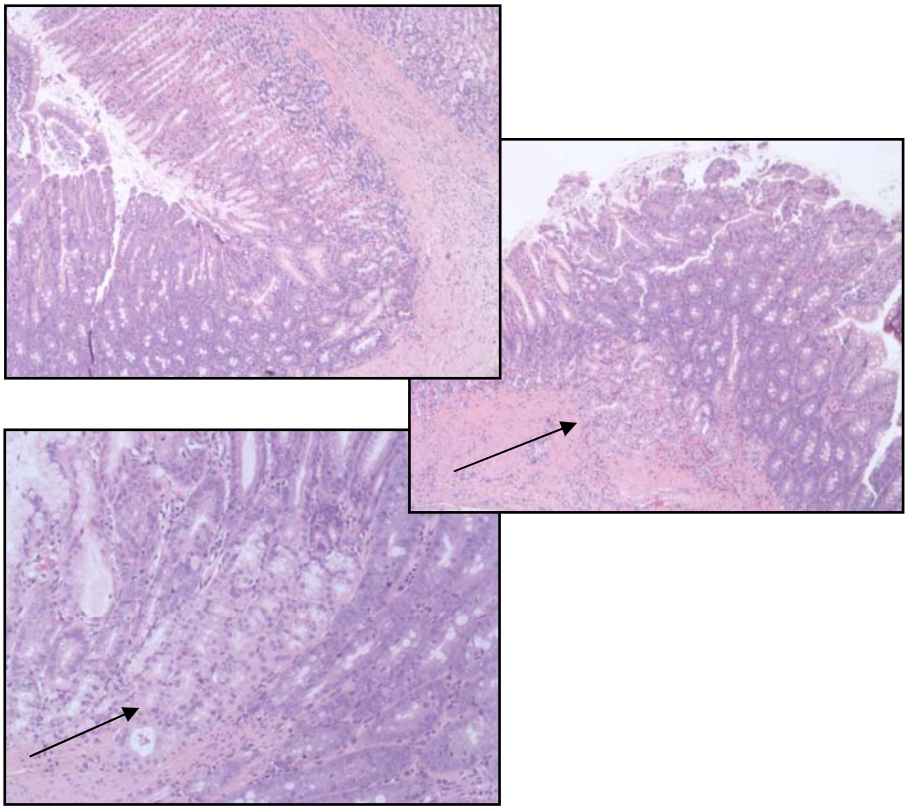


Figure 13. Normal gastrojejunal anastomosis (top) and light-cell metaplasia (middle, arrow), and the latter shown at higher magnification (bottom).

Gastric tumors behave differently in humans and Wistar rats. For instance, in the animals nearly all tumors arise in the pyloric region, whereas in humans they develop in the distal third of the stomach, especially in the small curvature (curvatura minor). In my experimental model involving a gastrojejunostomy, the tumors occur in the raphi area (anastomosis). Furthermore, there are also disparities between different experimental models. In my rat model, tumors that develop in the glandular part of the stomach are of mucinous or non-mucinous (tubular) type, and the degree of differentiation is high or moderate, and there are no metastases. By comparison, the use of MNNG to induce tumors in rats[69] has been found to result in almost the same variability and wide range of different types of tumors as are seen in humans (discussed in the section headed "Diagnosis and histopathology" on page 16), and there is also metastasis.

It should also be kept in mind that rats and humans differ with regard to their anatomy. In rats, the stomach is divided into two parts. The first part is a non-glandular forestomach that is connected to the esophagus and is lined with keratinized squamous epithelium, and its main function is the storage of food. The second (distal) part is called the glandular stomach (Fig. 14), and between the two parts there is a distinct elevated fold known as the limiting ridge.[69] In humans, the stomach is lined exclusively with glandular epithelium, which is described in the section "Diagnosis and histopathology."

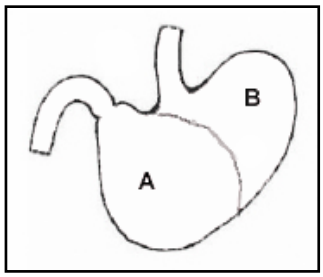


Figure 14. Anatomy of the rat stomach showing the two parts referred to as the glandular stomach (A) and the forestomach (B), which are separated by the limiting ridge.

We used sodium acetate as a possible inducer of cancer. In the stomach, this compound is transformed into acetic acid, which has been used experimentally to cause gastritis and ulcers, and has also been employed in research on colitis.[70-72] Our hypothesis was that, in gastric-resected rats, acetic acid might induce chronic inflammation that would eventually result in a higher rate of carcinoma compared to animals given a normal diet. Unfortunately, we found no such difference between these two groups of resected animals. Examining the amount of inflammation, we noted that the two diet groups differed by only 8%, possibly because we used sodium acetate instead of acetic acid. This was done because the use of acetic acid was not approved by the animal ethics committee. The study design indicated that the

concentration of acetic acid would be 5%, which is comparable to the level found in ordinary pickled cucumbers. The idea was that sodium acetate could be used instead of acetic acid, because it would be transformed into acetic acid when it was mixed with water in the stomach. Interestingly, all the animals that were given a diet containing acetic acid and were found to have adenocarcinomas also showed inflammation.

Dietary supplementation of carbonate promotes spontaneous tumorigenesis in a rat gastric stump model (Paper II)

Calcium is necessary to maintain the structure and function of all living cells.[73] In humans, calcium is absorbed primarily from cheese and other dairy products, as well as from dietary supplements. In the body, the bulk (99%) of the calcium is found in mineralized tissues like bone and teeth. The remaining 1% occurs chiefly in the blood and extracellular fluid. In plasma, the level of ionized calcium is regulated within a very small range through variations in the amounts that are taken up by absorption in the intestines, removed by renal excretion, and affected by skeletal turnover.[73] Data concerning calcium in relation to cancer prevention are contradictory. It has been suggested that dietary supplementation of calcium can reduce the risk of breast cancer[73] and also lower the risk of colorectal cancer.[74] In addition, studies on rats have indicated that diets high in calcium may prevent the cancer-promoting effect of red meat.[75] However, a large randomized, double-blind and placebo-controlled trial did not succeed in establishing any effect of calcium on the incidence of colorectal cancers in women.[76] Similarly, a randomized trial[77] found no evidence that calcium supplementation increases the risk of prostate cancer. In contrast, a case-controlled study conducted in France showed that a diet rich in calcium does result in an increased risk of gastric cancer.[78]

Food	Control	$CaCO_3$
Number of animals	18	18
Adenocarcinoma	3	11
High grade dysplasia	2	1

Table 4. The incidence of major histological changes in the first set of experiments shown in relation to dietary supplements.

The objective of the study reported in Paper II was to use the exact same experimental approach as in the first investigation (Paper I), namely, gastric resection with gastrojejunostomy in rats, to achieve DGR and subsequent development of

malignant transformation, and also to investigate the incidence of gastric adenocarcinoma after consumption of a calcium-supplemented diet. In the first set of experiments, two groups of resected rats were randomized to either a normal diet or a diet supplemented with calcium. The animals were given calcium carbonate simply because that is the most inexpensive form of the calcium. Quite unexpectedly, the results showed that the incidence of adenocarcinoma was more than tripled in the calcium-fed animals as compared to the controls (61% and 17%, respectively; Table 4). None of the unoperated animals showed any cancers. These findings were so astonishing and unanticipated, that we decided to repeat the experiment, this time changing the ions used in order to establish which ion (the calcium anion or the carbonate cation) was responsible for the cancer-promoting effect.

In the second experiment, only a single carcinoma developed in the group fed a diet supplemented with calcium ions (given as $CaHPO_4 \cdot 2H_2O$). Conversely, there was a definite positive relationship between an increased incidence of gastric cancer and a diet supplemented with carbonate, as indicated by a malignancy rate of 54% in the carbonate-fed animals as compared to 12% in the controls (Table 5).

Food	None	$CaHPO_4$	$NaHCO_3$
Number of animals	26	26	24
Adenocarcinoma	3	1	13
High grade dysplasia	2	0	2

Table 5. The incidence of major histological changes in the second set of experiments and with different food additives.

Calcium carbonate is not soluble in pure water. Nevertheless, any calcium salt (e.g., calcium carbonate, citrate, chloride, or phosphate) is soluble in an acidic environment such as exists in the stomach and can therefore be easily absorbed[79]. A great deal of calcium carbonate is in solution even at an intraluminal pH of approximately 6 (as is found in the jejunum).[79] In my investigation, the pH varied between 4 and 6 in the gastric remnant, with a mean of 5.0 for all operated animals, which indicates fairly good solubility in the postoperative remnant.

Even though it is evident that DGR causes malignant transformation in the stomach[55, 60, 63, 65-67] and esophagus,[61, 80] the pathogenesis of this effect is not entirely understood. Different coexistent factors have been suggested as plausible causes of such neoplastic alteration, among them *H. pylori* infection,[81] ulcers,[82] and excessive fat intake.[83] However, very little notice has been given to the hypothesis that carbonate is involved in the pathogenesis, or to the fact that there is an abundance of carbonate in

the pancreaticoduodenal succus that is refluxed to the stomach during the DGR process.

To our knowledge, the investigation described in Paper II is the first to show that carbonate anions can increase the occurrence of stomach adenocarcinoma in rats that have been subjected to gastric resection to ensure DGR and spontaneous carcinogenesis. Furthermore, it is noteworthy that carbonate is the chief ingredient in such reflux, which has also been shown to be related to the development of stomach cancer.

Dietary supplementation with carbonate increases expression of ornithine decarboxylase and cell proliferation in gastric mucosa in a rat model of gastric cancer (Paper III)

The aim of the study reported in Paper III was to further examine the effects of carbonate ions on gastric tumorigenesis. This was achieved by investigating non-transformed gastric mucosa in a rat model of gastric cancer with regard to expression of the tumor promotion markers COX-2 and ornithine decarboxylase (ODC) and the level of cell proliferation (measured as Ki67 staining).

Cyclooxygenase-2 (COX-2) is an enzyme that controls the rate at which prostaglandin (PG) is synthesized. There are two COX isoenzymes designated COX-1 and COX-2. COX-1 is omnipresent and has several physiological functions, whereas COX-2 is an inducible enzyme that is involved in inflammation and the development of cancer (Fig. 15).[84, 85] Studies of both humans and animals have established that inflammation induced by *H. pylori* is associated with increased production of COX-2, and that the level of expression is strongly correlated with the severity of gastric disease and the extent of inflammation. It has also been shown that production of COX-2 in antral mucosa is decreased, but not eliminated, after eradication therapy.[86, 87] Moreover, immunoreactivity of COX-2 has been observed after such treatment, especially in cases of intestinal metaplasia.[88]

There are numerous reports indicating that elevated expression of COX-2 is an essential factor in the development of gastric cancer.[88] The precise role of COX-2 in carcinogenesis is not yet clear, but it is obvious that cells overexpressing this protein acquire invasive phenotypes, and they also induce activation of angiogenesis, exhibit abnormal cell-to-cell interactions, and inhibit apoptosis.[89]

Various clinical studies and observations have suggested that non-selective and selective NSAIDs can inhibit gastric transformation and cell growth.[86] Treatment with COX inhibitors results in decreased production of PGs accompanied by increased apoptosis.[86] The pathogenesis behind this effect is still a matter of controversy.[86] Many investigators suggest that NSAIDs act directly on COX, but several recent

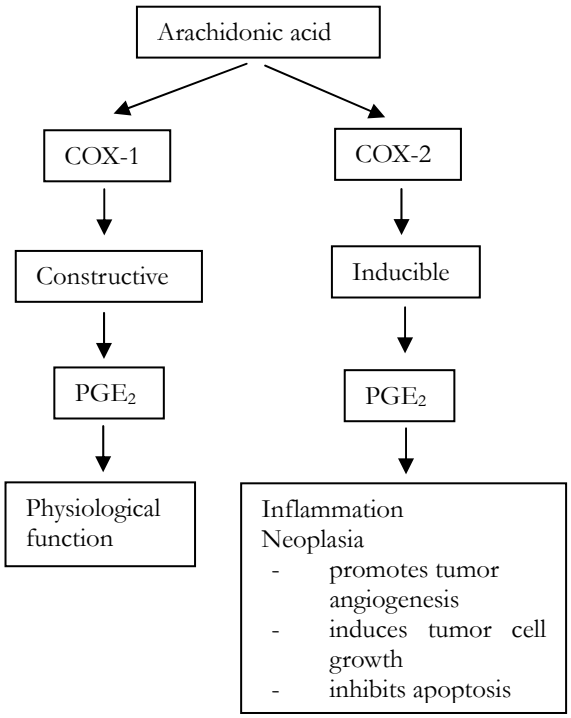


Figure 15. Schematic diagram summarizing the actions of COX-1 and COX-2.

studies have implied that there are also other as of yet unidentified COX-dependent or COX-independent targets.[86] In addition, it is noteworthy that the activity of ODC decreases dramatically in cells treated with NSAIDs, particularly when a selective NSAID drug such as celecoxib is used.[90, 91] Also, a close relationship has been found between increased levels of COX-2 and elevated expression of ODC.[89, 90, 92]

A continuous supply of polyamines (i.e., putrescine, spermidine, and spermine) is required for cell growth and is also necessary for regulation of cell differentiation, apoptosis, and malignant transformation.[93] The first and regulatory enzyme in the biosynthesis of polyamines is ODC.[94] The initial step in this synthesis is cleavage of the primary amino acid arginine to yield ornithine, which is further decarboxylated to putrescine by ODC (Fig. 16).[94] One of the earliest changes in cells that are stimulated to grow is an increase in ODC activity, an event that foregoes DNA synthesis by several hours.[95] This rapid regulation of ODC is achievable due to the fact that the

molecule has an exceptionally short half-life of 10 minutes to one hour, which is indeed the shortest half-life of any known enzyme.[93, 96] The regulation of ODC is subject to feedback from polyamines and a unique inhibitor called antizyme (AZ).[94-96] High levels of polyamines are usually correlated with rapid proliferation, and elevated expression of ODC is a well-known feature of many forms of cancer, including gastric carcinoma.[97-100]

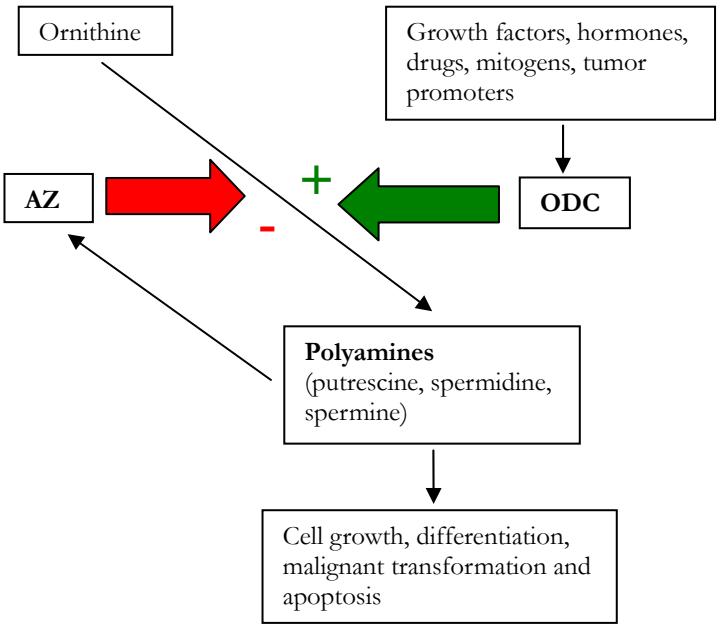
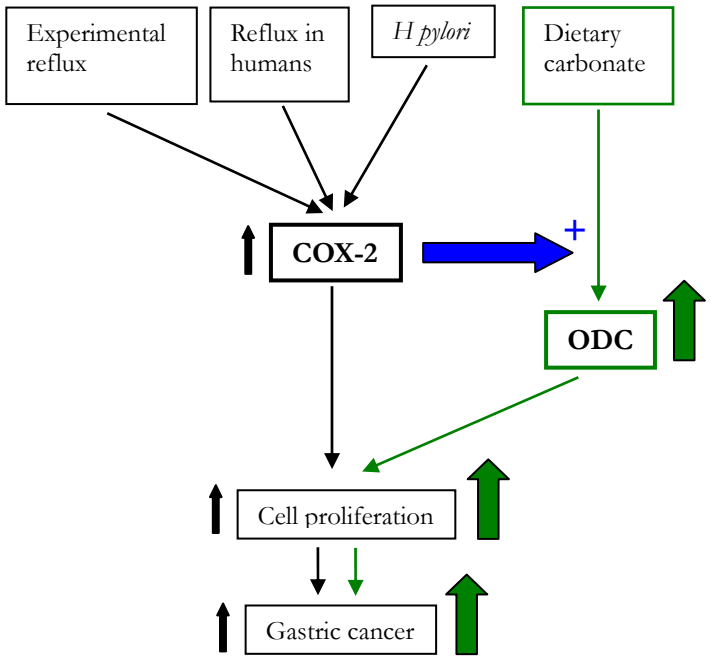


Figure 16. Schematic representation of the synthesis of polyamines.

It is firmly established that the constant high level of DGR following gastrojejunostomy results in the development of gastric tumors,[55, 60, 63, 65-67] but the pathogenesis underlying this event is not entirely understood. The investigation reported in paper III provides completely new information suggesting that the cancer-promoting effect of resection of the stomach and the subsequent reflux is related to an inflammatory reaction that is connected with increased expression of COX-2 in the gastric mucosa, in a manner similar to the way that *H. pylori* causes chronic inflammation and upregulation of COX-2.[86, 101] This evidence implies that both DGR and *H. pylori* infection induce a chronic inflammatory reaction and associated expression of COX-2, which leads to increased cell proliferation that in turn favors the development of gastric cancer.

I suggest that, in a situation involving reflux, it is the carbonate ions that initiate gastric carcinogenesis via upregulation of COX-2, and, notably, carbonate ions represent the chief component of the pancreaticoduodenal succus. Such malignant development is supported by my interesting finding that dietary supplementation of carbonate ions increased the expression of ODC in the non-transformed mucosa of rats, which is further indicated by our previous results showing that such addition to the diet markedly elevated the occurrence of gastric cancer. It has been reported that both COX-2 and ODC are involved in the genesis of premalignant lesions and cancer in the stomach.[89]



+ Required for stimulating effect of diet carbonate

Figure 17. Schematic diagram illustrating the roles of various factors involved in gastric carcinogenesis.

It has been suggested that ODC is located downstream of the growth-related protein COX-2.[92] However, the results described in Paper III seem to refute the view that such a relationship exists between the expression of COX-2 and ODC in rat gastric mucosa.

Initially, I discovered that DGR caused a prominent rise in the level of COX-2, whereas it did not provoke a significant increase in ODC at that time. However, after supplementation with carbonate ions, expression of ODC was distinctly elevated, but there was no additional increase in COX-2 expression. These results suggest that COX-2 is necessary for the rise in ODC. Furthermore, the hypothesis that COX-2 plays an important role in early stages of tumorigenesis agrees with an investigation conducted by Shin *et al.*[92] The enhanced ODC expression seen in animals receiving carbonate ions also had a stimulating effect on cell proliferation (measured as Ki67 levels) and tumor promotion (Fig. 17). Furthermore, the impact of carbonate ions on proliferation (Ki67) was not related to COX-2 expression, whereas it was associated with increased expression of ODC.

General discussion

What is carbonate?

Carbonate is an anion that has a charge of –2 and the empirical formula CO_3^{-2}, and it exists in three forms: as carbon dioxide (CO_2), as bicarbonate (HCO_3^-), or as carbonate (CO_3^{-2}). In biological systems, the enzyme carbonic anhydrase catalyzes the conversion between carbon dioxide and carbonate ions. In humans, the bicarbonate–carbonate ionic system acts as a buffer in blood.[102]

Carbonate occurs primarily in the form of a salt. This can involve a variety of cations, such us ammonium, potassium, and sodium, although the most common form is calcium carbonate or limestone. Calcium carbonate is found naturally in the following minerals and rocks: aragonite, calcite, chalk, limestone, marble, and travertine. Also, avian eggshells are composed of 95% calcium carbonate.[103]

Human use of calcium carbonate is seen primarily in the construction industry, either as a building material in its own right (e.g., marble) or as an ingredient of cement. Calcium carbonate is also employed as whiting in ceramics applications, in the form of a white powder that is added to many glazes. It is often simply called chalk, because it is the major component of blackboard chalk. It is also used in paint to enhance hardness and in plastics to improve impact strength. In addition, calcium carbonate increases resistance to wear and tear in rubber products, and it is used in the manufacture of soap to increase the volume of the product. Recently, calcium carbonate has also begun to replace kaolin in the making of glossy paper.[104]

Some examples of products containing calcium carbonate that are manufactured in the pharmaceutical, health-care, and food industries are described below.

Calcium carbonate in pharmaceutical and health-care products

Calcium carbonate is widely used in health-care products or in the pharmaceutical industry, as an antacid or as a supplement to calcium, potassium, magnesium, or zinc. It is also used as the base material in tablets and other products containing drugs.[105, 106]

Pharmaceutical lithium carbonate has been approved for clinical application in humans in the People's Republic of China, because it is said to have a beneficial effect on mental illness, mania in particular. Moreover, lithium carbonate can be used to produce lithium compounds for medicinal purposes, such as treatment of calculi (kidney and gall stones) and similar ailments. In addition, lithium carbonate can cure certain hormone imbalances and disorders like slow movement together with acute and chronic rheumatic arthritis. It is also used in production of vitamins (e.g., A, B, and C) and even as a component of substances such as synthetic cortical adrenaline, antihistamines, and antiseptics.[107]

Calcium carbonate in food processing

Calcium carbonate is widely used in food manufacturing, for instance to increase the nutrient content or to render products more attractive by making them taste better or have a more pleasing color. It is also employed in the making of tablets, beverages, and cereals, as well as in the refining of sugar.

A Japanese multinational company advertises and sells several calcium carbonate products that they claim can be used in various ways: *Collocalso-EX* consists of semi-colloid particles and is intended to improve powdered food products; *Collocalso-T* is made up of spindle-shaped particles that are said to efficiently neutralize acid, and it is widely used because it is believed to be a good calcium supplement and improve the quality of foods; *Collocalso-MG* comprises particles coated with glycerin fatty acid ester, and it is described as having a substantial effect on the palatability of food and is used extensively in seafood pastes; *Whiton-F* is composed of highly purified calcium carbonate and is used to enhance the nutritional value of various food products; *Porceal-N* consists of porous particles and exhibits anti-clumping characteristics when added to powdered products, and it can also be used to retain water or oil.[108]

Kansui[109] is an interesting product that has been used in Chinese cooking for over 2 000 years. Kansui originally consisted of water from Lake Kan in Inner Mongolian, which contains large amounts of potassium carbonate and sodium carbonate, as well as small amounts of phosphoric acid. Noodles made with Kansui have a yellowish hue and a characteristic flavor. Today, Kansui is mass produced synthetically from one or a combination of the mentioned compounds, and it is used in the making of noodles, wanton shells, and manju buns.[110] Kansui is also used in production of instant ramen, which is a Japanese noodle dish of Chinese origin,[103] and it has been estimated that about 80 billion meals of ramen are consumed worldwide each year.[110]

Soy milk or vegetable milk originates from China and is simply an extract obtained from whole soybeans. It has been used for human consumption since the Han Dynasty in 164 B.C., and the Japanese word for it is tofu. Today, many manufacturers enrich their soy milk products with calcium carbonate.[111, 112]

The vast majority of companies that are involved in processing calcium carbonate for various purposes are located in China, although some are also established in countries such us Malaysia, Thailand, India, Japan, and Vietnam. Furthermore, carbonate ingredients and additives are used in many Western nations, including the United States, the United Kingdom, and Germany, but in those cases mainly in the pharmaceutical industry.[113]

In closing, it should be mentioned that the countries that have significant dietary supplementation of carbonate also have the highest incidence of gastric cancer (see the section headed "Demographics" on page 13).

Epilogue

To the best of my knowledge, this thesis provides the first indication that COX-2 is responsible for the increased spontaneous incidence of gastric cancer that occurs in the rat model described here. In addition, the findings suggest that carbonate ions, given in dietary supplements, trigger elevated expression of ODC, which in turn enhances cell proliferation and subsequently increases the risk of gastric cancer. It is also noteworthy that carbonate is a major component of DGR into the stomach, and that such reflux is thought to be the main cause of gastric cancer.

Koek *et al.*[114] recently conducted an assessment using a combination of antroduodenal manometry and antral and duodenal Bilitec® measurements, and they demonstrated for the first time that DGR is a normal physiological event that takes place after the ingestion of a meal—a kind of postprandial phenomenon.[114] Moreover, they speculated that the duration and quantity of DGR might be affected by factors that are associated with the reflux, such as the amount and concentration of bile salts, secretion of bicarbonate from the pancreas and duodenum, gastric-duodenal motility, and the composition and amount of food consumed. In support of that suggestion, other investigators have shown that, compared to meals heavy in proteins, lipid-rich meals are connected with a higher reflux rate and more pronounced DGR.[115]

Patients with Barrett's esophagus show more evidence of DGR gastritis and more reflux of bile and other duodenal juice components than patients suffering from gastroesophageal reflux disease (GORD),[116] and those findings strongly implicate duodenogastroesophageal reflux (DGOR) in the pathogenesis of Barrett's esophagus.[116] Even experiments on rats have indicated that DGR has mutagenic effects that are involved in the development of esophageal adenocarcinoma.[61]

Considering all of the present results, along with the circumstances discussed above, I am convinced that carbonate ions represent the single most ominous constituent of DGR, because they can promote malignant transition in both the stomach and the esophagus, if the right microenvironment—reflux or *H. pylori* infection—is provided.

Conclusions

The results presented in this thesis demonstrate the crucial role of research conducted *in vivo* using laboratory animals. Without such an experimental design, it would not have been possible to discover that a food additive such as carbonate is involved in inducing gastric cancer.

The following important observations and conclusions have emanated from the current studies:

- In rats given a normal diet and subjected to gastric resection (gastrojejunostomy, BII), the incidence of gastric carcinoma increased from 0% to 25%.
- The incidence of gastric carcinoma was not altered by acetic acid given as a dietary supplement.
- Dietary supplementation with calcium carbonate caused an unexpected threefold increase in the incidence of gastric adenocarcinoma.
- Two independent series of experiments, performed a few years apart, demonstrated that carbonate ions are responsible for the observed rise in gastric cancer.
- In the present experimental model, it appears that an increased level of COX-2 is responsible for the higher incidence of gastric cancer. However, it also seems that the effects of carbonate ions on cell proliferation (measured as Ki67 staining) and tumor promotion are mediated by a significant rise in the level of ODC.

Populärvetenskaplig sammanfattning

Nästan en miljon nya fall av magsäckscancer diagnostiseras årligen världen över. Även om frekvensen har fallit dramatiskt de senaste åren så är magsäckscancern fortfarande den näst vanligaste orsaken till död i cancer sett ur ett världsperspektiv. Den geografiska variationen av denna cancertyp är anmärkningsvärd med den högsta förekomsten i asiatiska länder såsom Japan, Korea och Kina. Denna världsomspännande variation är sannolikt förknippad med riskfaktorer som går att förändra och de två riskfaktorer som dominerat debatten de senaste tio åren är: magsårsbakterien *Helicobacter pylori* och kostfaktorer.

Frekvensen av spontan magsäckscancer hos råtta är en raritet och detta har lett till att man med olika experimentella metoder försökt orsaka macksäckscancer. I vår första studie opererade vi bort nedersta delen av magsäcken för att skapa en reflux av de vätskor som finns i tolvfingertarmen, bukspottskörteln samt galla in i magsäcken och på så sätt utveckla spontan tumörbildning. I denna djurförsöksmodell studerades olika födoämnes-faktorers effekt på uppkomsten av magsäckscancer bland 256 manliga Wistar råttor. I den första omgången av studien fann vi överraskande att föda som tillförts kalciumkarbonat mer än trefalt ökade tumörförekomsten jämfört med kontroll gruppen (61% mot 17%). Genom att byta kalciumjoner mot natriumjoner kunde vi i en andra omgång av försöket visa att det var karbonatjonen som var ansvarig för den betydande ökningen av antalet cancrar (54% mot 12%). I både kliniska och experimentella studier är reflux av vätskor från tolvfingertarmen sammankopplad med utvecklingen av magsäckscancer. Dessutom är det visat att det är vätskan från bukspottskörteln och tolvfingertarmen snarare än gallan som är ansvarig för tumörutvecklingen och att dessa vätskor är särdeles rik på karbonatjoner.

I den sista studien undersökte vi uttrycket av olika cancer relaterade proteiner såsom COX-2 och ODC samt tillväxtaktiviteten med hjälp av Ki67 i normal magsäcksslemhinna från opererade råttor för att kunna bedöma betydelsen av karbonatjoner för cancerutvecklingen. Resultaten visar att om man oprerear bort nedersta delen av magsäcken ökar COX-2 uttrycket och tillväxtaktiviteten mätt med Ki67 stegras. Tillägg av karbonatjoner i födan ökar inte COX-2 nivåerna ytterliggare. Emellertid, bland opererande försöksdjur som erhållit kost med tillägg av karbonatjoner fann vi ett kraftigt förhöjt uttryck av ODC parallellt med en kraftigt stegrad tillväxtaktivitet. Det förefaller som om en enträgen kronisk inflammation med ökade COX-2 nivåer, förorsakad av till exempelvis reflux eller *H pylori* infektion, ökar risken att utveckla magsäckscancer. Föda med tillsats av karbonatjoner ökar påtagligt nivåerna av ODC i en COX-2-beroende miljö som i sin tur resulterar i en ytterliggare höljning av tillväxtaktiviteten och en ännu högre risk för utveckling av magsäckscancer.

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