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CHANGES OF STOMACH MICROENVIRONMENT AND GASTRIC CANCER DEVELOPMENT

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To my dearest family

ABSTRACT

While acknowledging the importance of microenvironment-related events, e.g. *Helicobacter pylori* (*H. pylori*) infection and atrophy, occurring during the gastric cancer (GC) progression, at present we have inadequate data to say to what extent these conditions could affect the further development of GC and what we should do for patients with these changes. In this thesis, with different focuses for each project, we aim to confirm the observed increasing trend of atrophic corpus gastritis (ACG) among young middle-age Swedes, to detect true carcinogenic *H. pylori* strains, and to quantify excess GC risks among endoscopy or appendectomized patients. With all the data obtained from our projects, a more accurate risk stratification for patients with potential probability of GC development would be a reality.

In **Study I**, based on Northern Sweden MONICA quinquennial population-based cross-sectional surveys conducted between 1990-2009, we randomly drew 5284 serum samples from all participants aged 35-64. Serologically defined ACG was determined by testing serum level of pepsinogen I (PG-I). The results revealed a surprising monotonic and significant upward trend in prevalence of PG-I-defined ACG (from 22 to 64 per 1000) in the youngest investigated age bracket 35-44 years. While in the oldest investigated age band (54-65 years), there was a clear and expected decline. Our further exploration, through the cross-sectional case-control analysis, indicated that CagA seropositivity was the strongest predictor of ACG presence (odds ratio [OR] =2.29). Further significant risk factors included diabetes, low education level and high body mass index (BMI). Interaction analyses indicated that the association between BMI and ACG was confined to the age category 35-44 years, where overweight and obesity, respectively, were linked to 2.8-fold and 4.7-fold increased odds of having ACG.

In **Study II**, a population-based case-control study, we measured antibodies against 17 *H. pylori* proteins using multiplex serology in sera from 268 cases and 222 frequency-matched controls. Unconditional multivariate logistic regression model was used for data analysis. In total, 15 proteins were significantly associated with increased GC risks, with the top three being CagA (OR=9.2), GroEL (6.6), HyuA (3.6). The excess risks were confined to non-cardia GC, but did not differ significantly between intestinal and diffuse subtypes. Using principal component analysis, we identified two significant factors among individuals without advanced chronic atrophic gastritis (AG): a CagA-dominant factor (antibodies against CagA, VacA, Omp as prominent markers), and a non-CagA factor (antibodies against NapA and Catalase as prominent markers). Strong dose-response relationships with non-cardia GC risks emerged for both antigen constellations: adjusted ORs (highest vs. lowest quartile) were 16.2 for the CagA-dominant factor, and 5.3 for the non-CagA factor.

In **Study III**, based on a nationwide cohort consisting of all patients (n=405,211) with gastric biopsies on non-malignant indications between 1979 and 2011 in Sweden, we quantified that crude annual incidence rates of GC were 20×10^{-5} for the normal group, 42×10^{-5} for those with minor changes, 59×10^{-5} for the gastritis group, 100×10^{-5} for the AG group, 129×10^{-5} for the

intestinal metaplasia (IM) group, and 263×10^{-5} for those with dysplasia. Compared to matching general Swedish population, the corresponding standardized incidence ratio (SIR) for overall GC was 1.0, 1.5, 1.8, 2.8, 3.4, and 6.5, respectively. The elevated GC incidence among precursor patients was stable throughout the follow-up period, and analysis restricted to the subset of participants with more than one biopsy suggested that the observed changes during re-evaluation(s) indeed have prognostic implications.

Similarly, in **Study IV**, we estimated the relative risks of GC among appendectomized patients in another nationwide register-based cohort study, using Swedish general population as reference. In total, 480,382 eligible patients received appendectomy were followed during the period of 1970-2009 for GC occurrence. The results indicated that no excess GC risk (SIR=1.00, 95% CI 0.93-1.07) was noted after appendectomy.

In conclusion, this thesis emphasizes that there is emerging evidence showing that GC, a fatal disease placing heavy burden on human health, might continue to be prevalent in the future. Given the large proportion of population affected by various stages of precancerous lesions, more accurate risk stratification is needed for optimizing the clinical management of these patients. *H. pylori* multiplex serology might be a useful tool for improving the overall cancer prediction capability. For patients biopsied on clinical indications, cost-effective endoscopy surveillance program needs to be developed for those with proven high-risk mucosal lesions (AG/IM/dysplasia).

LIST OF SCIENTIFIC PAPERS

I. Huan Song, Maria Held, Sven Sandin, Hilpi Rautelin, Mats Eliasson, Stefan Söderberg, Göran Hallmans, Lars Engstrand, Olof Nyrén, Weimin Ye. Increase in the Prevalence of Atrophic Gastritis Among Adults 35-44 y old in Northern Sweden Between 1990 and 2009. Clin Gastroenterol Hepatol. 2015 Apr 6. doi: 10.1016/j.cgh.2015.04.001. [Epub ahead of print]

II. Huan Song, Angelika Michel, Olof Nyrén, Anna-Mia Ekström, Michael Pawlita, Weimin Ye. A CagA-independent Cluster of Antigens Related to the Risk of Non-cardia Gastric Cancer: Associations between *Helicobacter pylori* Antibodies and Gastric Adenocarcinoma Explored by Multiplex Serology. Int J Cancer. 2014;134(12):2942-50.

III. Huan Song, Isabella Guncha Ekheden, Zongli Zheng, Jan Ericsson, Olof Nyrén, Weimin Ye. Gastric Cancer Incidence among Patients with Gastric Precancerous Lesions: An Observational Cohort Study in a Low-Risk Western population. BMJ 2015;351:h3867

IV. Huan Song, Christian C. Abnet, Åke Andrén-Sandberg, Anil Chaturvedi, Weimin Ye. Risk of Gastrointestinal Cancers among Patients with Appendectomy: A Large-scale Swedish Register-based Cohort Study during 1970-2009. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

ACG	Atrophic corpus gastritis
AG	Atrophic gastritis
BMI	Body mass index
Cag A	Cytotoxin-associated antigen A
CI	Confidence interval
CV	Coefficient of variation
ELISA	Enzyme linked immunosorbent assay
FFPE	Formalin-fixed and paraffin-embedded
GAC	Gastric adenocarcinoma
GC	Gastric cancer
H. pylori	*Helicobacter pylori*
HR	Hazard ratio
ICD	International Classification of Diseases
IM	Intestinal metaplasia
MFI	Median reporter fluorescence intensity
NRN	National registration number
NPR	National Patient Registry
OR	Odds ratio
PCA	Principal component analysis
PG	Pepsinogen
PPI	Proton pump inhibitor
SES	Socioeconomic status
SNOMED M	Systematized NOmenclature of MEDicine Morphology
SIR	Standardized incidence ratio

1 INTRODUCTION

It has been established that the occurrence of gastric cancer (GC), typically the intestinal type of non-cardia GC[1], develops through a series of well-recognized events (inflammation-metaplasia-dysplasia-carcinoma sequence, see Figure 1). This procedure, well known as Correa's cascade[2, 3], generally refers to a progression arising from normal mucosa, through chronic non-atrophic gastritis, atrophic gastritis (AG), intestinal metaplasia (IM), dysplasia, to ultimately GC. Despite declining incidence rates of GC have been documented in many developed countries, including Sweden, with disquieting reports indicating increasing presence of AG[4] and incidence of non-cardia GC[5] among young middle-age population, the changing tendency of GC incidence in the future is actually undetermined.

H. pylori infection is the most recognized risk factor for GC and its precursors, by triggering the evolving precancerous cascade. However, its etiological role on carcinogenesis is far from clear, especially considering that *H. pylori* is unlikely to continually survive or colonize in a typically hypochlorhydric environment of AG. This grants plausibility to the notion that *H. pylori* may create an environment for intestinal-type gastric carcinogenesis (atrophy and hypochlorhydria) rather than causing the cancer directly. In contrast, based on the preliminary studies on stomach microbial ecology in cancer patients, it is reasonable to postulate that other microenvironment- related factors might be involved in the development of GC.

Moreover, although the development of GC has been widely investigated and its underlying mechanisms are partly uncovered, in practice, the management of these pre-cancer events is still poor. A crucial reason is that, these earlier stages of the pathological changes commonly happen in a large proportion of the population, while an effective treatment for the later stages currently doesn't exist. Therefore, accurate risk assessments for patients at various stages can be of great public health importance.

This thesis focused on exploring the secular trend of AG in Sweden, detecting true carcinogenic *H. pylori* strains, and quantifying excess GC risks among endoscopy or appendectomized patients. We hope our findings could in turn help identify a subpopulation at extremely increased risk of GC, facilitating cost-effective and safe primary/secondary prevention for this deadly malignancy.

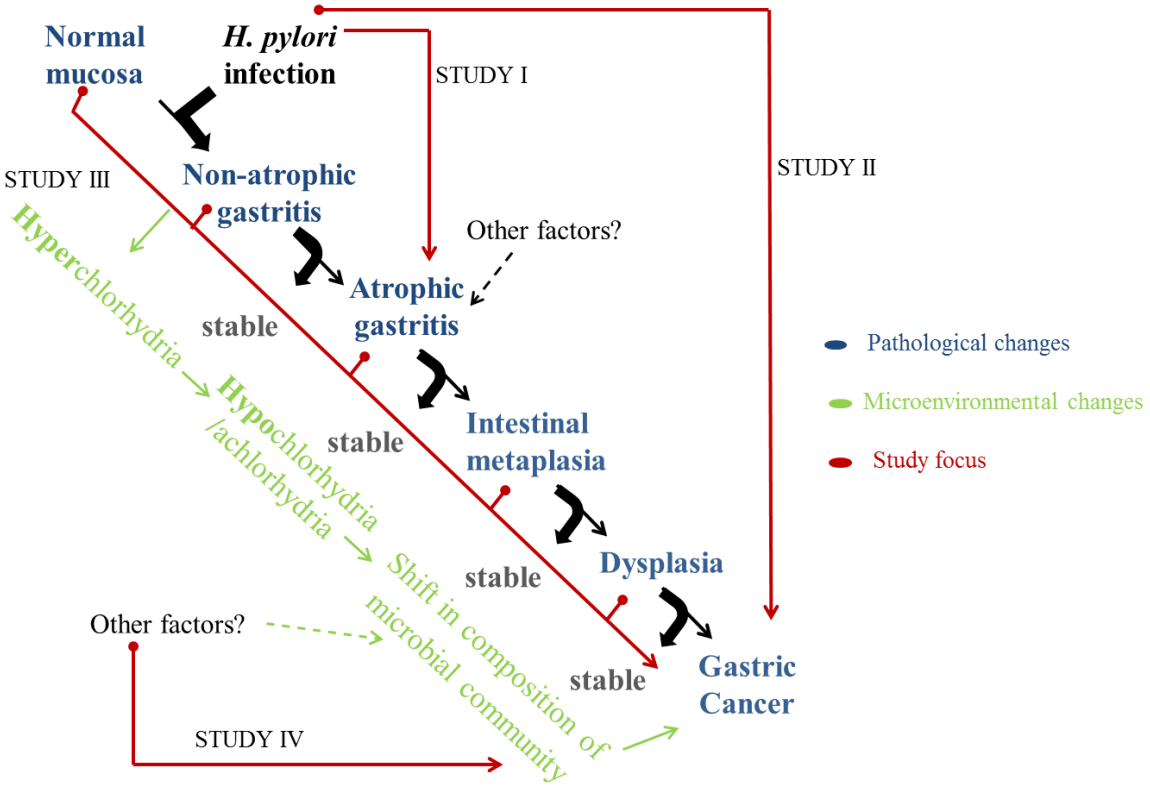


Figure 1. Changes of stomach microenvironment and gastric cancer carcinogenesis. Study focuses were marked

2 BACKGROUND

2.1 GASTRIC CANCER

2.1.1 Descriptive epidemiology

Despite the declining incidence rates in many developed countries, GC still ranks amongst the most common cancers in the world (the most common neoplasm in the first estimate of global cancer incidence in 1975, and the 5th in 2012)[6]. Also, this deadly malignancy contributes significantly to the overall mortality and accounted for 723,000 deaths (8.8% of the total) in 2012[6], which made it the third leading cause of cancer-related death for both sexes worldwide. The epidemiology of GC is characterized by its prominent geographic variation and male dominance, with age-standardized incidence rates ranging from 3.3 in western Africa to 35.4 in eastern Asia for men, and from 2.6 in western Africa to 13.8 in eastern Asia for women[6].

In Sweden, the downward trend of GC can be dated back to 1940. However, in recent years, the fall appears to have slowed down in both genders (Figure 2). While the annual reduction during 1992-2001 was 4.4% and 3.9% among Swedish men and women, respectively[7], it was 2.1% and 1.0% during 2002-2011[8]. In addition, besides an upward incidence trend for non-cardia GC which was recently reported among young US whites aged 25-39 years[5], our previous study observed a possible increasing trend of AG among 35-44 years adults in Sweden[4]. All of the emerging evidence suggests that the changing pattern of this malignancy in the future is uncertain.

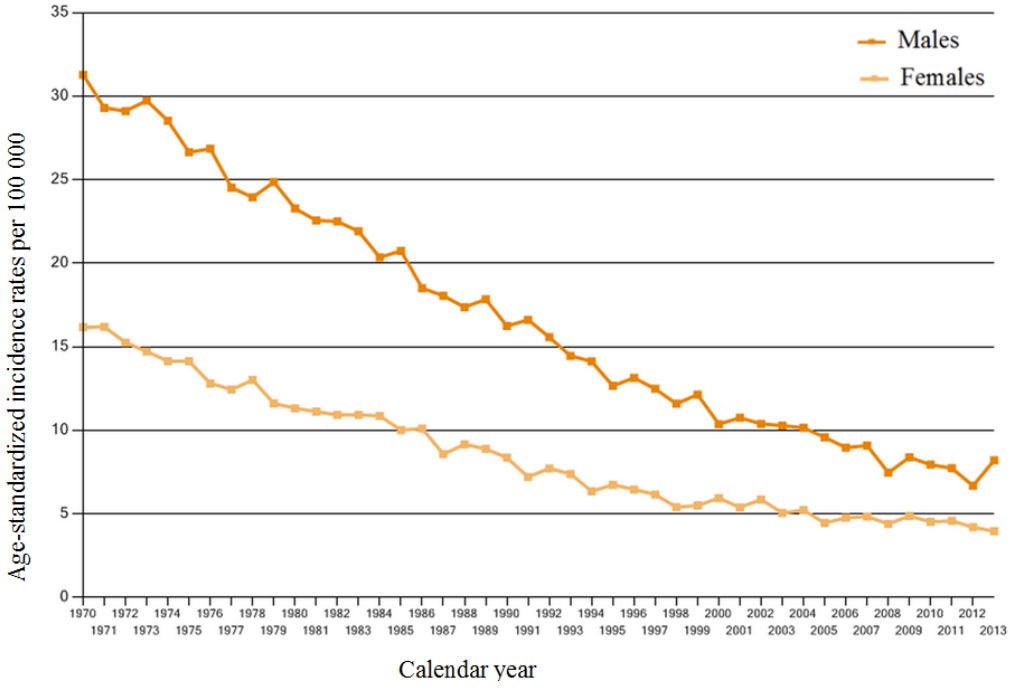


Figure 2*. Age-standardized incidence rates of gastric cancer in Sweden, stratified by gender (according to the population of Europe)

*This figure was generated by online tool provided by The National Board of Health and Welfare (Socialstyrelsen) in Sweden (http://www.socialstyrelsen.se/statistik/statistikdatabas/cancer)

2.1.2 Subtypes of gastric cancer

More than 95% of all GC cases are diagnosed as adenocarcinoma[9], indicating that the cancer originates from glandular epithelium of the stomach mucosa. Since GCs are overwhelmingly gastric adenocarcinomas (GAC), GAC is the main focus of this thesis. Other cancer types that can be found in the stomach include lymphoma, leiomycosarcoma, and neuroendocrine tumors.

2.1.2.1 Cardia and non-cardia gastric cancer

Anatomically, GC can be divided into cardia and non-cardia with respect to gastric subsite. Although this classification has been widely used in clinical/epidemiological studies, there is however no worldwide consensus on how to define this diagnostic category[10, 11]. In our studies, we applied a relatively conservative definition for cardia GC—tumor with its center located within 1 cm proximal and 2 cm distal to the gastroesophageal junction; while the others (those arising in fundus, body, antrum or pylorus of stomach, see Figure 3) were considered to be non-cardia (or distal) GCs[12].

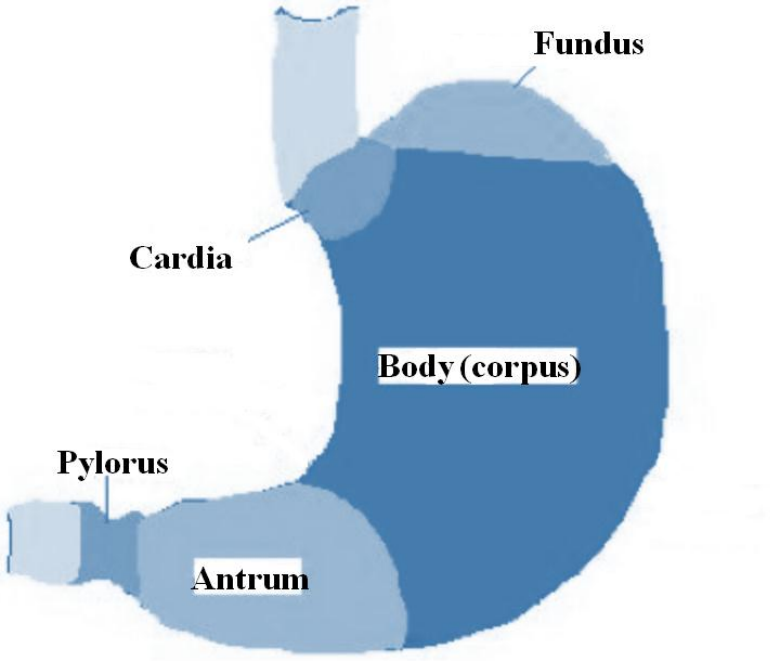


Figure 3. Anatomy of the stomach

Besides the distinction of origins, this classification may also indicate discrepancies in terms of epidemiology, etiology and prognosis. As opposed to the consistent finding of a declined incidence of non-cardia GC worldwide, the epidemiological evidence regarding the changing pattern of cardia GC incidence varied. A rising trend was shown in the US[11, 13] and Singapore[14], whereas investigations from other countries[12, 15, 16] reported a constant incidence. In addition, although cardia GC is histologically identical to most of the distal GC (adenocarcinoma), the occurrence of these two subtypes of GC seems to respond to different environmental and genetic determinants[17, 18]. Clinically, cardia GC tends to be more aggressive[9], thus with less favorable survival rate than that for non-cardia GC[19].

2.1.2.2 Intestinal and diffuse types

Two major histological subtypes of GAC, intestinal and diffuse, have been introduced by Dr. Laurén in 1965[1] based on morphological differences. The tumor cells in intestinal-type tumors adhere to each other, and tend to arrange themselves in the formation of tubules or glands (well-differentiated). This is very similar to adenocarcinomas arising elsewhere in the intestinal tract, and hence designated as intestinal-type. In contrast, in diffuse-type carcinomas, a defect of intercellular adhesion molecules allows individual tumor cells to grow and invade neighboring structures without any regular formation (undifferentiated).

With regard to the clinical characteristics, the intestinal type, usually having a long latent time, typically arises from a circumstance with precancerous lesions (e.g. IM or dysplasia), and prevails among the elderly; while the diffuse type is more likely to be with genetic abnormalities, and frequent in younger population. Although the former is thought to be more environment-related[20, 21] than the latter, both subtypes have been associated with *H. pylori* infection[9]. Additionally, numerous studies have failed to find a prognostic difference, though some have suggested a survival advantage for patients with intestinal-type tumors[22].

2.2 STOMACH MICROENVIRONMENT

2.2.1 Normal status of stomach microenvironment

In a normal condition, the healthy stomach, acts as a reservoir where ingested food gets preliminarily digested by being contracted and mixed with gastric juice (the admixture of fluids that secreted into the stomach).

The stomach, through gastric glands, secretes several factors which are necessary for maintaining its digestive functions. There are three types of glands located in different regions of the stomach. They can also be distinguished from each other by type of secretion[23] (Table 1).

Table 1. Different types of glands and their main secretions

Gastric glands	Location	Principle epithelial secretory cells	Secretion
Cardiac glands	Gastric cardia	mucus-secreting cells	Mucus
Fundic (oxyntic) glands	Fundus and body of stomach	Parietal (oxyntic) cells	Gastric acid (hydrochloric acid, HCl) and intrinsic factors
		Chief cells	Pepsinogens (I and II)
		Mucous neck cells	Mucus and pepsinogens (I and II)
Pyloric glands	Pylorus and antrum	Mucous neck cells and mucus-secreting cells	Mucus and pepsinogen II

The layer of mucus, acting in concert with $NaHCO_3$ which is secreted by surface mucous cells and serves as a neutralizing force, protects the stomach from the low PH and peptic conditions of the gastric lumen. This is known as the gastric mucosal barrier.

Pepsinogens (PGs) are the inactive precursor zymogens of pepsin (the principle enzyme of gastric juice). There are two immunochemically distinct types of PG: PGI and PGII. PGI is secreted merely by the chief and mucous neck cells of the corpus, whereas PGII is also

produced by pyloric glands in the pylorus and antrum. Regardless of the heterogeneity of origin, both PGI and PGII can be converted rapidly to an enzymatically active form in a normally low pH environment created by the acidity of gastric juice.

The secretion of HCI forms the typical acidic environment in the stomach. Although varied with the secretory rates, the normal human stomach (empty status) has a pH close to 2 (ranging from approximately 1-3). In addition to providing a state optimizing the proteolytic activity of pepsin, the strong acidity kills nearly all ingested pathogens, yet offers a perfect condition for the lonely survival of *H. pylori*.

2.2.2 Microenvironmental factors related to *H. pylori* infection

2.2.2.1 H. pylori infection

Pathology and changes in stomach microenvironment

H. pylori is a spiral bacterium colonizing in the human stomach. It was first described in 1874, and successfully cultured in 1982[24]. After colonization with *H. pylori*, essentially all infected persons develop chronic superficial gastritis, no matter whether or not there is any gastrointestinal symptoms. This inflammatory condition results in stimulation of gastrin release and impairment on structure and function of epithelial cells. The subsequent excess secretion of HCI (hyperchlorhydria) is a consequence of the elevated gastrin level.

For the majority of the patients, induced inflammation is mainly restricted to the antrum, and this hyperchlorhydria status can persist, sometimes for the lifetime of the host, at a mild and harmless level; while for others, pangastritis (gastritis of both the corpus and antrum) gradually develops, which is a pattern of inflammation with the potential for progression along the Correa's carcinogenic cascade[25].

Prevalence of *H. pylori* infection

Approximately half of the world's population has been infected by this microbe, rendering it one of the most common infections in humans[25]. The geographic distribution of *H. pylori* infection is strongly associated with socioeconomic conditions. With the observed declines owing to improved sanitation in recent decades, the prevalence of *H. pylori* infection now is estimated to be 70% in developing countries and 30%–40% in the industrialized countries[26].

Predictive value for the occurrence of GC

Although there is a consensus that the infection of *H. pylori* initiates the carcinogenic process in the stomach[25, 27], since most infected patients end up in the subclinical phase or with minor subsequent tissue damage, it's difficult to quantify the exactly causative role of *H. pylori* in the development of GC. The heterogeneity of infected *H. pylori* strains and host response have been proposed as the determinants for the discrepant pathogenic courses (i.e. gastritis, gastric ulcer, AG, etc.). Different strains could induce variations in complex immune response in human which might be reflected by specific antibody patterns. So far, antibody

against cytotoxin-associated antigen A (Cag A) is believed to be the most oncogenic biomarker of infection with high-risk *H. pylori* strain. However, the problem is that, the CagA-positive infection is also quite common — approximately 55%-89% even in a normal population[28]. With the development of multiplex serology, which will be capable of including CagA antigens from different strains (26695, G27, F32), the sero-positive rate of CagA could be even higher than in prior reports .This means that the existence of CagA alone provides gradually inadequate cues to predict the occurrence of GC. Further exploration on novel serum predictors is warranted.

2.2.2.2 Atrophic corpus gastritis

Pathology and changes in stomach microenvironment

Atrophic corpus gastritis (ACG), also referred as advanced AG, is mainly the consequence of persistent *H. pylori* infection, which pathologically suggests the presence of chronic inflammatory cells and the disappearance of normal glands. Consequently to the continuous loss of oxyntic glands and the replacement by intestinal glands, the gastric acid secretion decreases steeply; and by then the hypochlorhydric condition is established in atrophic mucosa, creating an environment allowing survival of other microbes, in parallel with a notable decline in density of *H. pylori.*

The onset of atrophy is of particular importance because it is well recognized as a risk factor for GC[29]. IM develops in the atrophic mucosa and is believed to constitute the background in which dysplasia and further intestinal adenocarcinoma occurs[30].

Prevalence of ACG

Generally, similar to the geographic distribution of GC incidence, consistently low ACG prevalence was reported from Scandinavian countries[31, 32] (in Sweden, about 6%-8%[33, 34]), in contrast to much higher prevalence proportions (20%-80%) observed in Asia and South America[34, 35]. But inconsistent data commonly appeared even within the same population, since the selection of the study population and the definition of ACG can be quite different between studies.

Additionally, the descriptive epidemiology of this potentially precancerous lesion with regard to its secular trend in the general population is less well studied, mainly due to its virtual lack of clinical symptoms. The few data available from Europe suggested an overall declining trend, without mentioning any age-specific trend[36]; whereas our previous cross-sectional analyses[4] of the population-based material in Sweden during 1990-1999 revealed an unexpected rise in the prevalence of ACG among 35-44 year-olds. Since ACG in the long term is tightly associated with the GC incidence, more in-depth studies for confirming such a trend are highly indicated.

Serological diagnosis

Clinically, ACG is diagnosed by histological assessment on gastric biopsies sampled during routine gastroscopy. However, as an invasive medical examination, this approach is practically infeasible for large-scale investigation with the objective of unveiling the prevalence rate among certain populations. Histology-based studies are usually limited by their biased sampling resources (e.g. hospital-based[37]) or small sample sizes. In contrast, serum pepsinogen concentration tests, which are less costly and invasive, have been widely accepted as a compromised choice in population-based screening surveys[38-40]. Validation studies[31, 41-43] demonstrated a reasonable accuracy along with a particular high specificity (95%-100%) of this serology method. However, inconsistencies in definition and cut-off values undermine the comparability of different serology-based studies.

2.2.3 Other possible factors that can affect the stomach microenvironment

2.2.3.1 Long term proton pump inhibitor use

Proton pump inhibitors (PPIs) are the most efficacious medication to reduce the secretion of gastric acid. Nowadays, in addition to short-term use, e.g. for eradication of *H. pylori* infection or management of gastroesophageal reflux disease, maintenance PPI therapy is also recommended, particularly for patients with erosive oesophagitis or/and Barret's oesophagus[44]. However, recently, the long-term use of this potent antisecretory medication has been suggested to be correlated to an altered ACG or GC risk[45], although this association has not been clearly established.

The PPI-related hypochlorhydria is the main concern for explaining the potential GC risks, especially for the patients with *H. pylori* infection. In subjects with suppressed acid secretion caused by maintained PPI use or any other mechanisms, *H. pylori* infect both the antrum and body of the stomach, instead of the antrum only. This unusual colonization pattern leads to corpus predominant gastritis[46], which subsequently results in the accelerated appearance of ACG — a major risk factor for the development of GC.

Non-*Helicobacter* bacterial overgrowth is another potential factor which can affect the development of gastric premalignant lesions under the condition of sustained low gastric acid level. In patients without long-term PPI use, it probably occurs after the formation of severe ACG. In PPI users, however, colonization of various microbes could happen at an earlier stage and might facilitate the further development of more severe gastritis[47]. Moreover, possible carcinogens produced by these microbes (e.g. nitrosamines and acetaldehyde) might also promote the development of precancerous lesions, or even carcinoma itself.

2.2.3.2 Appendectomy

The vermiform appendix is historically considered as a vestigial organ and the loss of the appendix is thought to have few, if any, long-term effects. Appendectomy is often performed after a clinical suspicion of appendicitis, because definitive test seems to be time-consuming and not beneficial given the low risk of appendix removal. Recent work suggests that the appendix may serve as a reservoir for the colonic microbiome[48]; and after biofilm-disrupting

events, such as use of antibiotics or gastroenteritis, the gastrointestinal microbiome initially emerges from the appendix. Therefore, removal of the appendix may disturb normal intestinal microflora, altering many aspects of human physiology including vulnerability to inflammation, extraction of nutrients, and ultimately playing a role in tumor formation, locally (colon cancer) or specifically (non-Hodgkin's lymphoma and other GI cancers).

A few previous studies have examined whether or not appendicitis and appendectomy are associated with altered risk of gastrointestinal cancers. A Swedish registry-based investigation has reported a significantly increased GC risk after appendectomy, although it's restricted to patients appendectomized under 20 years of age, and thus not well powered to examine individual cancer risk by the time it was analyzed[49]. A similar study from Denmark[50] also suggested an elevated GC risk. All of these findings favor the notion that removal of the appendix could somehow change the microenvironment of gut, including the stomach, which in turn, may play an important, as-yet-unknown role in the carcinogenesis at the site. More recently, a trial showed that uncomplicated acute appendicitis could be treated with antibiotics rather than appendectomy, highlighting the importance of assessing the effects of appendectomy[51].

3 AIMS

The overall aim of this thesis was to examine our hypothesis that the stomach microenvironment-related events, including *H. pylori* infection, AG, and the changes of stomach microbial composition, may play an important role in the development of GC.

- To gain an updated insight into epidemiological changes of ACG in Sweden, and search for the possible underlying explanations.

- To explore the novel serum antibodies of *H. pylori*, as well as their associations with the development of GC.

- To accurately measure the excess incidence of GC among endoscopy patients with pathological diagnosis of gastric precancerous lesions.

- To understand if other potential factors (e.g. appendectomy) could chang the stomach microenviroment, thereby affecting the occurrence of GC.

4 MATERIALS AND METHODS

4.1 SUBJECTS AND STUDY DESIGN

Study I

Age-stratified random samples of all 35-64 year old residents of Norrbotten and Västerbotten, two counties located in Northern Sweden, were drawn in 1990, 1994, 1999, 2004, and 2009 as part of MINOCA project (Multinational Monitoring of Trends and Determinants in Cardiovascular Disease)[52, 53]. In each year of survey, the samples were randomly selected (250 random samples were collected from each sex/10-year age stratum); and each selection round was carried out independently of the previous ones. Information about sociodemographic characteristics, anthropometric measures, life style factors, medical history, and broad dietary patterns was collected by questionnaires; and a blood sample was also acquired from each participant. The participation rate ranged from 69% to 81%, across different sampling years. Briefly, non-participants tended to be younger and mostly males, reported lower body mass index (BMI) and were more likely to be cigarette or snuff users compared to participants[54].

Based on the Northern Sweden MONICA project, **Study I** was conducted consisting of two parts. The first part has a cross-sectional design. From all participants aged 35-64 years, we drew a random sample consisting of 5284 individuals to assess the age- and calendar year-specific prevalence of serologically defined ACG. Then, in the second part, all serologically identified ACG cases together with their 1:3 randomly selected controls, who were frequency matched with cases by age group and year of sampling, were involved in a subsequent case-control study. Analyses were executed by combining lab results (the presence of antibodies against *H. pylori* and CagA) and questionnaire data.

Data (n=3266) from the first three occasions (1990/1994/1999) of this project have been analysed in 2002, and described in details in Maria Held's thesis[4]. Later, after performing serology tests on additional 2018 sera from the last two occasions (2004/2009), we merged the two datasets, and presented them together in paper I.

Study II

The second study is a population-based case-control study. The study base consisted of all native Swedish individuals between 40 and 79 years of age, living in 5 counties (distributed in 2 geographic areas with different incidence rates) in Sweden from February 1989 to January 1995[20]. Newly diagnosed patients with histologically confirmed GAC were identified as eligible cases; and in parallel with the case ascertainment, controls were randomly selected with an approximately 2:1 frequency matched to the cases by age and sex. Venous blood samples were required from study participants during the last few years of the study. In total, 298 case patients (participation rate was 100%) and 244 control subjects (participation rate was 70.5%) with blood samples (collected preoperatively for cases) were involved in our study. The crucial attributes that might affect the possibility of *H. pylori* infection, such as

age, sex, socioeconomic status (SES) in childhood, education level, as well as cancer type and site, were distributed equally among subjects in this serological sub-study and all subjects in the whole study[55].

Study III

Proceeding from computerized Swedish pathology registers, we established a cohort consisting of all patients who had gastric biopsies taken on non-malignant indications between 1979 and 2011. The 24 pathology departments provided the patients' national registration numbers (NRNs – unique identifiers for all Swedish residents) along with data on date, age, sex, and pathological-anatomical diagnosis using the Systematized NOmenclature of MEDicine Morphology (SNOMED M) codes.

In total, 405,211 eligible subjects were enrolled and followed for GC occurrence until the end of 2011.The cohort members were grouped by their SNOMED M diagnosis at baseline (first biopsy identified in the database). Our main focus was on Correa's cascade, with the groups (in order of progression): normal, minor mucosal changes, non-atrophic chronic gastritis, AG, IM and dysplasia. However, to prevent the possible effects of other clinical diagnoses on GC risk measurement, 66,300 patients whose main findings were of uncertain relevance to Correa's cascade lesions and to GC development were firstly identified and analyzed separately in a subcohort named 'other diagnoses subcohort'. For the remaining 338,911 patients (referred to as 'Correa's cascade subcohort' hereinafter) with normal gastric mucosa, minor changes, or lesions in Correa's cascade, baseline grouping was determined by the diagnostic code that indicated the most advanced lesion. Outcome and censoring information was collected from essentially complete health and demographic registers.

Study IV

The fourth study is a population-based cohort study that was conducted on the basis of National Patient Registry (NPR) of Sweden. A total of 501,160 patients who received appendectomy during January 1, 1970 to December 31, 2009 were identified by using Swedish surgery codes (4510, 4511, 4517,0058 during the time period 1970–1996, and JEA00, JEA01, JEA10 for 1997 and onwards). After exclusion of 20,778 patients with conflicting information (having emigration/death date recorded before the appendectomy date), the final cohort consisted of 480,382 eligible subjects. The dataset was then linked to the Swedish Cancer Registry, where all GC cases (presenting as International Classification of Diseases (ICD) version 7 code=151) were ascertained. Further cross linkages with Death Register, Emigration Register provided the necessary information for censoring follow-up.

4.2 LABORATORY METHOD

4.2.1 Enzyme linked immunosorbent assay (ELISA) test for pepsinogen (PG) level

In **Study I** and **II**, we measured serum concentration of PG I and II by using ELISA method according to the instructions of the manufacturer (Biohit Plc, Helsinki, Finland). The test is based on a sandwich enzyme immunoassay technique with a PGI/PGII specific antibody adsorbed on a microplate and a detection antibody labelled with horseradish peroxidase. When the enzyme reaction was finally terminated with stop solution, the intensity of the yellowish colour is directly related to the PGs concentration of the sample.

Quality control during testing

Whenever the test was performed, besides a positive control and a blank control provided by the producer, a pooling sample (produced by mixing sera donated by 5 healthy volunteers) was always added in duplicate in each plate to monitor the intra/inter-assay imprecision. Results indicated that the within-assay imprecision was consistently low (< 3%), and coefficient of variation (CV) for between-assay imprecision was, in **Study I**, 6.2% for PGI and 7.0% for PGII, in **Study II**, 7.0% for PGI and 10.8% for PGII. Additionally, to minimize the effect of between-assay variations on the results, samples collected from different survey occasions (**Study I**), or sera from cases and controls (**Study II**), were first mixed and then evenly placed in each plate. All tests were conducted continuously and finished within a short time period.

Cut-off points for atrophy diagnosis

Without agreement on the best cut-off values, various definitions were applied in different serology-based studies[56]. In **Study II**, we used the cut-off points which have been validated in Swedish population for AG diagnosis. With the criteria that PGI <25μg/l or the ratio PGI/PGII<3, the sensitivity and specificity of the markers for advanced AG were 71% (CI 68-74%) and 98% (CI 97-99%) respectively[33].

For **Study I**, however, since PG-I levels in 3266 serum samples from the first three occasions (1990/1994/1999) have been measured in 2002 by using a different commercial kit—immunoenzymometric assay (Gastroset PG-I, Orion Diagnostica, Espoo, Finland) which had gone out of production, we failed to use exactly the same analysis kit for all 5284 samples. The definition of the old kit (PG-I < 28 μg/L) offered a sensitivity of 81% and a specificity of 99% for the presence of ACG[31], with histology as the gold standard. Therefore, to maximize the consistency of these two kits, a specific cut-point (PG-I <45 μg/L) was selected for the new kit through comparison of the results measured by the two kits in a pilot validation study.

4.2.2 Immunoblot for *H. pylori* status

Part of the sera from **Study I** (for individuals recruited in the subsequent case-control analysis) were analysed for the presence of antibodies against *H. pylori* and CagA protein

with a commercially available immunolot assay (Helicoblot 2.1, Genelab Diagnostics, Singapore). The testing procedure and the result interpretation followed the instructions of the manufacturer. CagA was positive if 116,000-Mr band was present. *H. pylori* seropositivity was defined as presence of any of the following conditions: (i) a positive result for the 116,000-Mr band and one or more of the 89,000-, 37,000-, 35,000-, 30,000-, and 19,500-Mr bands together or along with the presence of the current infection marker (CIM); (ii) the presence of the 89,000-, 37,000-, or 35,000-Mr band; and (iii) the presence of both the 30,000- and 19,500-Mr bands. The sensitivity and specificity of this test for current *H. pylori* infection has been reported to be 95%-99% and 93%-98%, respectively, among American, Japanese and European patients[57-59].

4.2.3 *H. pylori* multiplex serology

In **Study II**, by collaborating with German Cancer Research Center, we applied *H. pylori* multiplex serology test to check presence of antibodies to 17 individual *H. pylori* proteins (Table 1). This multiplex method was developed based on a glutathione-S-transferase (GST) capture immunosorbent assay combined with florescent bead technology[60]. The quantity of antibodies bound to each antigen, defined as antibody reactivity, was measured by the median reporter fluorescence intensity (MFI) of at least 100 beads per bead set per serum. Antigen-specific cut-offs to determine dichotomous serostatus (positive/negative) were based on 46 additional sera within the same assay defined as *H. pylori* seronegative, calculating as the arithmetic mean MFI plus 3 SD after excluding positive outliers. Plate-to-plate variation was monitored via a standard sample on each plate, revealing low flux with median CV of 10.0% (range 5.2% to 17.7%). All sera were tested in one assay on the same day.

Table 2. Functions of *Helicobacter pylori* proteins expressed for multiplex serological test

Protein name	Full name	Biological function*
Cag A	Cytotoxin associated antigen A	may be necessary for the transcription, folding, export, or function of the cytotoxin.
GroEL	Chaperonin GroEL	Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions.
OMP	Outer membrane protein	Unknown.
Catalase	Catalase	Decomposes hydrogen peroxide into water and oxygen; serves to protect cells from the toxic effects of hydrogen peroxide.
VacA	Vacuolating cytotoxin	Induces vacuolation of eukaryotic cells. Causes ulceration and gastric lesions.
HcpC	Helicobacter cysteine-rich protein C	May hydrolyze 6-aminopenicillinic acid and 7-aminocephalosporanic acid (ACA) derivatives.
HP0305	Hypothetical protein	Unknown.
HpaA	*Helicobacter pylori* adhesin A	Putative neuraminyl-lactose-biding hemagglutinin homolog, which has been shown to mediate the binding of bacteria to sialic acid-containing host molecules expressed on the surface of gastrointestinal cells[61].
Cagδ	Cag pathogenicity island protein 3	Protein binding; might be a novel component of CagT4S outer membrance subcomplex[62].
CagM	Cag pathogenicity island protein 16	Unknown.
HyuA	Hydantoin utilization protein A	Hydrolase activity
Cad	Cinnamyl alcohol dehydrongenase ELI3-2	Nucleotide binding, oxidoreductase activity, and zinc ion binding.
HP0231	Hypothetical protein	Unknown.
NapA	Nutrophil-activating protein	Required for the survival in the presence of oxidative stress; also a virulence factor that activates neutrophils, mast cells and monocytes. It might have a role in the accumulation of neutrophils and monocytes at the site of infection. Induces superoxide anion generation, adhesion and chemotaxis of neutrophils, through a pertussis toxin-sensitive pathway involving MAP kinases.
UreA	Urease alpha subunit	Ammonia produced by ureolysis increases the gastric pH thereby providing an environment permissive for colonization of the stomach.
BabA	Blood group antigen-binding adhesin	Devoted to the attachment to human gastric epithelium, leading to higher colonization densities and augmented nonspecific immune response ,and ,subsequently, more aggressive gastritis
HomB	Helicobacter outer membrane B	Unknown.

* The information is from Swiss-Prot database (http://www.expasy.org/sprot) except where noted otherwise.

4.3 STATISTICAL ANALYSES

For case-control analyses (the second part of **Study I** and **II**), the relative importance of each factor was expressed as the odds ratio (OR), with two-sided 95% Wald confidence intervals (CIs), of having target disease (ACG /GC) among exposed relative to a reference category (typically, unexposed). Owing to the frequency matching design, unconditional logistic regression model was applied for both of these studies. Multivariate models included the frequency matching factors, as well as potential confounders—with biological/statistical importance or selected using the Akaike statistic. Potential interaction effects between exposures were also assessed in multiplicative model where a cross-product term represented the interaction between the two variables.

In **Study II**, since high correlation and multicollinearity between original MFI values of the studied *H. pylori* antibodies have been noted, we didn't add these variables directly into multivariate model. Instead, principal component analysis (PCA) was implemented. Briefly, this method is executed to supply a low-dimensional picture by viewing the correlated original variables (serum levels of assorted *H. pylori* antibodies in this case) from its most informative viewpoint. Original variables (MFI values of tested antibodies) were first restructured by B-spline transformation. Then, using the PCA method, significant components (or factors) were determined with eigenvalue >1.0 as the criterion. Lastly, stratified by quartiles of controls' factor scores, risk levels of non-cardia GC among score-graded subgroups were estimated by unconditional logistic regression.

For population-based cohort study (**Study III** and **IV**), we calculated the standardized incidence ratio (SIR; the ratio of the observed to the expected number of newly diagnosed GC cases) with its 95% CIs to estimate the relative risk for each exposure group, using the general Swedish population as reference. The expected number of cases was calculated by multiplying the observed number of person-years by age- (5-year strata), sex-, and calendar year-specific incidence rates derived from the entire Swedish population. Particularly, a peak of cancer incidence was invariably observed shortly after patient enrollment. This is principally due to the symptomatic yet undetected cancers and closer follow-up in exposed cohort, reflecting possibly selection or surveillance bias. Thus, the first one (**Study IV**) or two (**Study III**) years of follow-up, as well as the corresponding events happening during that period, were disregarded from main analysis.

In **Study III**, the associations between pathological changes and risk of GC were then evaluated by hazard ratios (HRs) with 95% CIs, derived from the Cox proportional hazards regression model. We used attained age as underlying time-scale, and adjusted for sex, and stratified by pathology units. Assumption of proportional hazards for involved variable was checked graphically and by the method of Schoenfeld's partial residuals; and neither of them revealed indication of violation. We also applied the Nelson-Aalen method to estimate the cumulative incidence of total GC among subjects with different baseline diagnoses.

A *P* value less than 0.05 was considered to be statistically significant. All statistical analyses were carried out using SAS 9.2-9.4 software (Cary, NC).

5 RESULTS

5.1 STUDY I

5.1.1 Pilot study of consistency of results from the two PG kits

In the pilot study, 894 sera from 900 randomly selected participants in the first three survey rounds, already analyzed with the old PG-I kit, were re-tested using the new kit (sera from 6 selected participants had been used up). A strong correlation between paired measures of PG-I concentration was noted (Pearson correlation coefficient = 0.85; Intraclass correlation coefficient=0.77). Plots of Bland-Altman analysis (Figure 4) revealed that the new kit could be of practical use, although a small upward adjustment is needed (18 units higher than the old kit). For sero-positivity, taking the old results as a 'gold standard', best agreement (*kappa*=0.80) was reached when PG-I <45 µg/L was selected as cut-off point for the new kit.

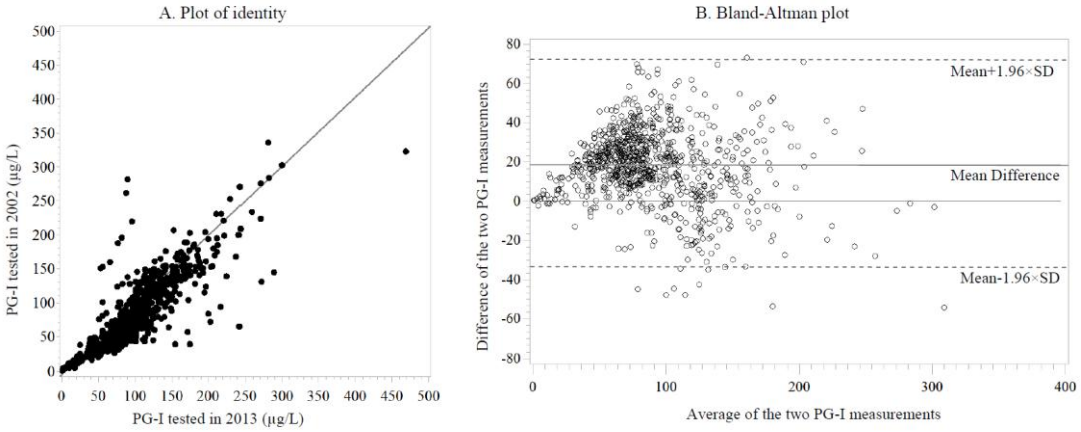
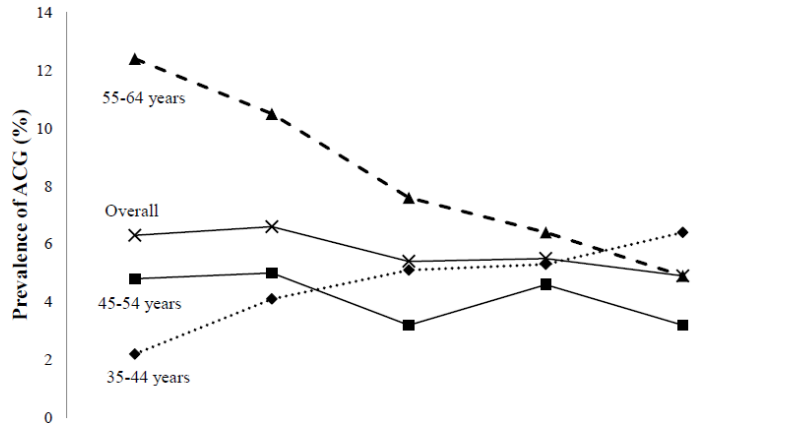


Figure 4. Bland-Altman plot for comparison of pepsinogen I (PG-I) obtained in 2002 using Gastroset PG-I assay and PG-I measured in 2013 using PG-I ELISA Kit (n=894)

Since serum PG II value was also suggested to be correlated with a severe, highly active gastritis and high incidence of gastric cancer [63], we evaluated PG-II levels among these sera using PG-II ELISA (Biohit Oy, Helsinki, Finland). However, correlation test indicated only moderate agreement between these two definitions (old PG-I-defined ACG vs. PG-I/PG-II-defined atrophic gastritis, *kappa*=0.59).

5.1.2 Prevalence of PG-I-defined functional ACG



	1990	1994	1999	2004	2009
Age group	Positive/all (%)	Positive/all (%)	Positive/all (%)	Positive/all (%)	Positive/all (%)
35-44 years	8/367 (2.2)	15/367 (4.1)	15/294 (5.1)	17/321 (5.3)	19/297 (6.4)
45-54 years	18/377 (4.8)	20/400 (5.0)	11/344 (3.2)	16/350 (4.6)	11/311 (3.5)
55-64 years	43/348 (12.4)	42/400 (10.5)	28/368 (7.6)	25/393 (6.4)	17/346 (4.9)
Overall	69/1092 (6.3)	77/1167 (6.6)	52/1006 (5.4)	58/1064 (5.5)	47/954 (4.9)

Figure 5. Observed prevalence of PG-I-defined functional atrophic corpus gastritis (ACG)* across different survey occasions, stratified by age group (10-year strata)

*Defined by serum pepsinogen I (PG-I) concentration <28 µg/L (measured by Gastroset PG-I assay, Orion Diagnostica, Finland) for samples from 1990/1994/1999 survey occasions, and <45 µg/L (measured by PG-I ELISA Kit, Biohit Oy, Helsinki, Finland) for samples from 2004/2009 survey occasions. In a validation study, the agreement of the two kits was proved to be high (kappa=0.80).

PG concentrations were measured in sera from 5283 subjects. One serum from 1994 was missing. Overall, 305 subjects (58 per 1000; 95% CI 51–64 per 1000) were considered to be with significant ACG according to their PGI concentrations. Slightly higher prevalence was observed among women relative to men (62 versus 53 per 1000; *P*=0.17). Figure 5 illustrates the age-specific prevalence rates across the 5 sampling years. Among the entire population aged from 35 to 64, although having a tendency to descend, the changing trend over time was non-significant (the slope of the regression line indicated a prevalence reduction of -0.8 per 1000 per year of observation; 95% CI -1.8 –+0.1, p=0.08). Notably, a significant fall appeared among 55-64 years old subjects (from 124 to 49 per 1000 between 1990 and 2009, corresponding to a slope of -4.0 per 1000 per year of observation; 95% CI -5.8 –-2.0, p <.0001), along with surprisingly increased prevalence rates which were continually observed among 35-44 year-old participants. In this subgroup, prevalence rates of ACG rose from 22 to 64 per 1000 in last 20 years, attaining a significant trend assessed both by Mantel-Haenszel Chi-square test (p= 0.0073) and by the slope of the regression curve (+2.0 per 1000 per year of observation; 95% CI +0.5 –+3.5, p=0.0073). Sub-analysis confined to non-smokers (n=4236) revealed a very similar age-specific changing pattern (data not shown).

5.1.3 Risk factors for the presence of PG-I-defined ACG

In total, 305 ACG cases and 915 matched controls were included. With the exception of one serum which has been used up, *H. pylori* serology test was successfully performed on all

other 1219 sera. The distribution of age, sex, and other main characteristics that might affect ACG occurrence among cases and controls, along with their ORs estimating the relative probability of having ACG in various exposure strata are displayed in Table 2 (detailed characteristics can be found in paper I).

Table 2 The distribution of studied factors and their associations with risk of having PG-I-defined atrophic corpus gastritis

Factors	Cases /all* (%)	Controls/all* (%)	Simply adjusted model OR (95% CI)†	Best fitted model OR (95% CI)§
Age group				
35-44 years	74/305 (24.3)	222/915 (24.3)	-	-
45-54 years	76/305 (24.9)	228/915 (24.9)	-	-
55-64 years	155/305 (50.8)	465/915 (50.8)	-	-
Sex				
Female	167/305 (54.8)	453/915 (49.5)	Reference	Reference
Male	138/305 (45.2)	462/915 (50.5)	0.81 (0.62-1.05)	0.75 (0.56-1.02)
Education level				
≤12 years	254/302 (84.1)	690/908 (76.0)	Reference	Reference
>12 years	48/302 (15.9)	218/908 (24.0)	0.59 (0.41-0.83)	0.62 (0.42-0.90)
***H. pylori* serostatus**				
Negative	145/305 (47.5)	544/914 (59.5)	Reference	-
Positive	160/305 (52.5)	370/914 (40.5)	1.74 (1.31-2.29)	-
CagA serostatus				
Negative	98/305 (32.1)	451/914 (49.3)	Reference	Reference
Positive	207/305 (67.9)	463/914 (50.7)	2.26 (1.69-3.02)	2.25 (1.66-3.06)
BMI				
Normal (BMI<25 kg/m^2)	97/305 (31.8)	391/915 (42.7)	Reference	‡
Overweight (BMI 25~30 kg/m^2)	135/305 (44.3)	376/915 (41.1)	1.47 (1.09-1.99)	‡
Obesity (BMI≥30 kg/m^2)	73/305 (23.9)	148/915 (16.2)	2.04 (1.42-2.93)	‡
Diabetes				
No	285/303 (94.1)	886/910 (97.4)	Reference	Reference
Yes	18/303 (5.9)	24/910 (2.6)	2.35 (1.25-4.42)	2.05 (1.05-4.00)
Smoking status				
Never smoker	167/301 (55.5)	466/909 (51.3)	Reference	Reference
Former smoker	97/301 (32.2)	270/909 (29.7)	1.00 (0.75-1.36)	1.02 (0.75-1.40)
Current smoker	37/301 (12.3)	173/909 (19.0)	0.59 (0.40-0.88)	0.57 (0.37-0.87)
Hard liquor consumption				
Never	98/304 (32.2)	231/912 (25.3)	Reference	Reference
A few times per year	126/304 (41.5)	432/912 (47.4)	0.68 (0.50-0.93)	0.73 (0.52-1.02)
Once per month or more	80/304 (26.3)	249/912 (27.3)	0.75 (0.53-1.07)	0.89 (0.60-1.30)
Fruit consumption				
Quintile1 (≤0.50 times/day)	59/305 (19.3)	202/915 (22.1)	Reference	-
Quintile2 (0.51-0.98 times/day)	49/305 (16.1)	177/915 (19.3)	0.95 (0.62-1.46)	-
Quintile3 (0.99-1.50 times/day)	65/305 (21.3)	183/915 (20.0)	1.22 (0.81-1.84)	-
Quintile4 (1.51-2.22 times/day)	69/305 (22.6)	180/915 (19.7)	1.32 (0.88-1.98)	-
Quintile5 (>2.22 times/day)	63/305 (20.7)	173/915 (18.9)	1.26 (0.83-1.90)	-

* Total number varied due to missing information.

† Odds ratios and 95% confidence intervals derived from logistic regression models including the variable in question together with the frequency matching factors, age group (10-year strata) and survey year.

§ Odds ratios and 95% confidence intervals derived from a best fitted logistic regression model including CagA serostatus, smoking status, sex, body weight, education, diabetes, and hard liquor consumption, along with the frequency matching factors, age group (10-year strata) and survey year.

‡ The effects of body mass index were not shown in this table due to their significant interaction with age groups. These results can be found in Table 3.

As expected, current tobacco use, but not former use, showed a negative linkage with having a significantly low PGI level. No such relationship was observed among current snuff users. Assessed by a simple logistic regression model that only adjusted for the frequency matching

variables, OR among *H. pylori* seropositive subjects, as opposed to *H. pylori* seronegative ones, was 1.7 (95% CI 1.3-2.3). A stronger, but unimpressive, association was observed between CagA serostatus and ACG risk (OR=2.3; 95% CI 1.7-3.0). With regard to sociodemographic factors, sex and county of residence seemed to be irrelevant to the occurrence of PGI-defined ACG, while overweight or obesity, and lower education level (<12 years) were significantly linked to increased odds of having this disease. The importance of diabetes was marked by its elevated OR, which indicated that diabetes patients had 2.4-fold increased odds of developing ACG, compared to the ones without a history of diabetes. Recent PPI use was only reported in 1.6% (19/1220) of all studied subjects. Despite a slightly higher prevalence among cases than controls, we observed no significant association of PPI use with ACG. All types of alcohol intake, notably wine and hard liquor consumption, exhibited a protective tendency against ACG, with ORs lower than unity. No apparent impact was noted regarding coffee, vegetable, and fruit intake.

Moderate correlations were detected within four groups of variables: CagA/ *H. pylori* sero-status, wine/beer/hard liquor consumption, fruit/vegetable intake, and snuff/ cigarette use. Therefore, in fully adjusted multivariate model, only the most significant one from each group was included. The best fitted model that obtained the lowest AIC included CagA serostatus, smoking status, sex, obesity, education, diabetes, and hard liquor consumption. Briefly, all tested factors acquired almost identical results from these two multivariate models. Compared to results derived from simply adjusted model, the association of CagA serostatus with ACG remained essentially unchanged after adjustments for additional covariates, with an OR of 2.3(95% CI 1.7-3.1). The same situation was found for education. After full adjustment, subjects who had more than 12 years of education had 38% lower odds of having PGI-defined ACG, compared with the ones having lower education level. Partly attenuated by the further adjustment, the association between self-reported history of diabetes and ACG occurrence still attained the level of significance, with point estimated OR of 2.1 and 95% CI 1.1-4.0 in the best fitted model.

Particularly, interaction between age groups and BMI levels on ACG was observed, indicating that the effect of BMI varied in different age groups (Table 3). Among participants aged 35-44 years, the presence of higher BMI levels entailed increasing odds for ACG—subjects who were overweight and obese exhibited 2.8-fold (95% CI 1.5-5.4) and 4.7-fold (95% CI 2.1-10.5) increased odds, respectively, than the ones having lower BMI value. While for the two other age groups, no such relation could be conferred.

Table 3. Relationship of overweight/obesity with PG-I-defined functional atrophic corpus gastritis by age group

Age group	BMI level[*]	Cases (%)	Controls (%)	OR (95% CI)[†]	OR (95% CI)[§]
35-44 years	Normal	24 (32.4)	131 (59.0)	Reference	Reference
	Overweight	31 (41.9)	69 (31.1)	**2.84 (1.49-5.40)**	**2.78 (1.47-5.27)**
	Obesity	19 (25.7)	22 (9.9)	**4.73 (2.13-10.46)**	**4.59 (2.09-10.09)**
45-54 years	Normal	28 (36.8)	96 (42.1)	Reference	Reference
	Overweight	28 (36.8)	95 (41.7)	1.05 (0.60-1.97)	1.06 (0.57-1.97)
	Obesity	20 (26.4)	37 (16.2)	1.32 (0.63-2.79)	1.32 (0.63-2.76)
55-64 years	Normal	45 (29.0)	164 (35.3)	Reference	Reference
	Overweight	76 (49.0)	212 (45.6)	1.29 (0.83-2.00)	1.27 (0.82-1.97)
	Obesity	34 (22.0)	89 (19.1)	1.28 (0.74-2.20)	1.26 (0.73-2.16)

[*] BMI: body mass index; Normal=BMI<25 kg/m^2; overweight=BMI 25~30 kg/m^2; obesity=BMI≥30 kg/m^2.

[†] Odds ratios and 95% confidence intervals derived from a fully adjusted logistic regression model, including CagA serostatus, smoking status, sex, county of residence, body weight, education, diabetes, PPI use, coffee, hard liquor, and fruit consumption, along with the frequency matching factors, age group (10-year strata) and survey year.

[§] Odds ratio and 95% confidence intervals derived from a best fitted logistic regression model, including CagA serostatus, smoking status, sex, body weight, education, diabetes, and hard liquor consumption, along with the frequency matching factors, age group (10-year strata) and survey year

In further confirmatory analyses, restricting to non-smokers only or using birth cohort clustered multilevel regression models, almost identical strength of associations were detected for each risk factor (data not shown).

5.1.4 Changes in exposure prevalence 1990-2009

Analysis of the case-control investigation indicated that similarly descending tendencies were observed in both case (subjects with PGI-defined ACG) and control groups (data not shown). Nevertheless, a tendency for converged curves revealed that the *H. pylori* prevalence dropped more sharply among 55-64 years old subjects, relative to the younger ones aged 35-44 (Figure 6, panel A)—in the former the slope was -14.3 (95% CI -20.3 – -8.2, P < 0.001) per 1000 per year while in the latter, it was -7.2 (95% CI -14.3 – -0.1, P=0.05) per 1000 per year (P for difference between slopes = 0.16). The convergence of curves was more clear for the prevalence of CagA seropositivity (Figure 6, panel B) —in the oldest age band, the slope was -18.8 (95% CI -24.4 – -13.2, P<0.0001) per 1000 per year, while the curve for the youngest category was almost flat (slope: -5.0, 95% CI -13.3 – +3.3, P=0.24) (P for difference between slopes = 0.006).

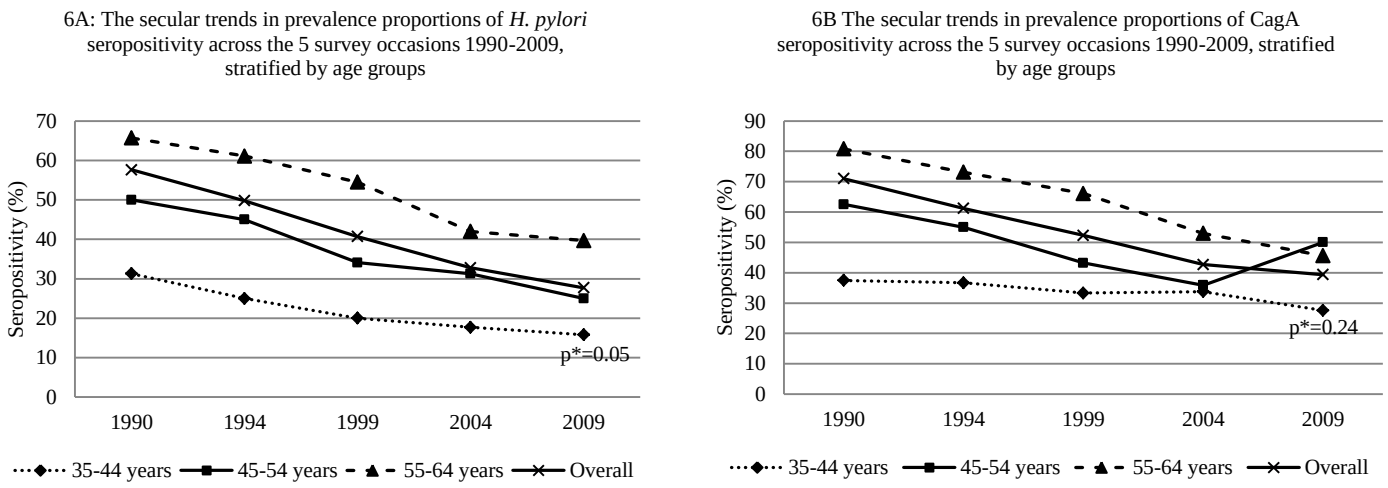


Figure 6. Age-specific secular trends in prevalence proportions of *H. pylori* and CagA seropositivity among subjects involved in the case-control analysis (n=1219) across the 5 survey occasions 1990-2009, determined by immunoblot kit

* The trend across sampling years was examined by Mantel Haenszel chi-square test

The changing patterns of other risk factors were then examined among the entire studied population (n=5283). The prevalence of obesity experienced an obvious raise during the study period, from 11% (1990) to 22% (2009), among all 35-64 years olds; whilst in the youngest age category it almost quadrupled (5.5% to 21.1%). PPI users were only documented since 1999; and no considerable gradient regarding the proportion of PPI use was found across the following sampling years. For diabetes, the prevalence increased from 2% to approximately 4% during the last 20 years in the studied population. This was mainly due to the elevated prevalence rates among 55-64 year olds (from 3% to 7%), while for 35-44 year olds, the prevalence rate remained low, around 1% for each year of observation. Also, the proportion of highly educated subjects remarkably increased over years, from 16% in 1990 to 31% in 2009. Age-stratified analysis indicated that the upward trends were undifferentiated between age groups: the 35-44 years old group changed from 20% to 37%, the 45-54 group from 16% to 32%, and the 55-64 group from 9% to 27%.

5.2 STUDY II

5.2.1 Evaluation of the associations between each serological *H. pylori* antibody and the presence of GAC

The distribution of age, sex and other basic characteristics that might affect the possibility of *H. pylori* infection among 268 cases (48 of them with cardia, 220 with non-cardia GAC which were further classified as intestinal and diffuse subtypes according to Laurén's Classification) and 222 controls are listed in Table 4.

Table 4. Basic characteristics of the study population

	Cardia GAC, n (%)	Non-cardia GAC, n (%)			Control n (%)
		All	Intestinal	Diffuse	
Age at interview (years)					
40-49	5(10.4)	12(5.5)	1(0.9)	11(13.6)	13(5.9)
50-59	6(12.5)	26(11.8)	9(7.9)	14(17.3)	11(5.0)
60-69	17(35.4)	62(28.2)	32(28.1)	24(29.6)	96(43.2)
70-79	20(41.7)	120(54.5)	72(63.2)	32(39.5)	102(46.0)
Sex					
Male	40(83.3)	136(61.8)	80(70.2)	46(56.8)	159(71.6)
Female	8(16.7)	84(38.2)	34(29.8)	35(43.2)	63(28.4)
Smoking status					
Never-user of tobacco	16(33.3)	75(34.3)	38(33.6)	27(33.3)	89(40.1)
Ex-user of tobacco	10(20.8)	55(25.1)	30(26.6)	21(25.9)	80(36.0)
Current user of tobacco	22(45.8)	89(40.6)	45(39.8)	33(40.7)	53(23.9)
Socio-economic status					
Unskilled manual workers	17(35.4)	125(56.8)	57(50.0)	49(60.5)	71(32.0)
Skilled manual workers	18(37.5)	46(20.9)	26(22.8)	17(21.0)	63(28.4)
Non-manual workers	9(18.8)	27(12.3)	14(12.3)	11(13.6)	62(27.9)
Self-employed persons	1(2.1)	9(4.1)	7(6.1)	2(2.5)	6(2.7)
Farmers	3(6.3)	13(5.9)	10(8.8)	2(2.5)	20(9.0)
Level of fruit and vegetable consumption					
Low	27(56.3)	110(50.2)	61(54.0)	36(44.4)	83(37.4)
Median	14(29.2)	75(34.3)	34(30.1)	32(39.5)	82(36.9)
High	7(14.6)	34(15.5)	18(15.9)	13(16.1)	57(25.7)
Area of residence					
South	32(66.7)	122(55.5)	61(53.5)	49(60.5)	122(55.0)
North	16(33.3)	98(44.5)	53(46.5)	32(39.5)	100(45.0)
Number of siblings					
1-2	12(25.0)	38(17.3)	22(17.5)	14(17.3)	66(29.7)
3-4	13(27.1)	49(22.3)	25(21.9)	18(22.2)	55(24.8)
5-6	12(25.0)	51(23.2)	24(21.1)	23(28.4)	54(24.3)
≥7	11(22.9)	82(37.3)	45(39.5)	26(32.1)	47(21.2)
Total subjects in each group	48	220	114	81	222

Table 5 depicts the prevalence proportions of specific antibodies against 17 *H. pylori* proteins determined by multiplex serology and their associations with different subsites/subtypes of GAC. It was apparent that the associations were confined to non-cardia GAC, but showed no important difference between intestinal and diffuse types. Except for BabA and HP0305, adjusted ORs for non-cardia GAC were all above unity and were all significant, with the top four being CagA (OR=9.2; 95%CI 5.3-15.8), GroEL (OR=6.6; 95% CI 3.7-11.7), HyuA (OR=3.6; 95% CI 2.3-5.6), and VacA (OR=3.5; 95% CI 2.3-5.5).

A correlation (*P* value <0.0001 in Pearson correlation test) was demonstrated between most of the 17 antibodies. Further we found moderate multicollinearity by collinearity diagnosis—the largest condition index was 16.3 for continuous MFI values and 12.0 for dichotomous sero-status variables.

Table 5. The seroprevalence of antibodies against each of the 17 *H. pylori* proteins and their associations with sub-sites/ subtypes of gastric adenocarcinoma (GAC)

	All controls (n=222)		All GAC (n=268)			Cardia GAC (n=48)			Non-cardia GAC								
									All (n=220)			Intestinal (n=114)			Diffuse (n=81)		
	n	%	n	%	OR*(95%CI)	n	%	OR*(95%CI)	n	%	OR*(95%CI)	n	%	OR*(95%CI)	n	%	OR*(95%CI)
VacA +	95	42.8	173	64.5	2.5(1.7-3.8)	18	37.5	0.8(0.5-1.5)	155	70.5	3.5(2.3-5. 5)	83	72.8	3.7(2.1-6.5)	56	69.1	3.9(2.1-7.4)
HyuA+	105	47.3	190	70.9	3.0(2.0-4.5)	24	50.0	1.4(0.7-2.8)	166	75.5	3.6(2.3-5.6)	84	73.7	2.9 (1.7-5.1)	60	74.1	4.0 (2.1-7.6)
GroEL +	132	59.5	228	85.1	4.2(2.6-6.8)	30	62.5	1.2 (0.6-2.5)	198	90.0	6.6(3.7-11.7)	102	89.5	5.3(2.6-10.9)	73	90.1	9.0 (3.7-22.2)
HcpC+	77	34.7	130	48.5	1.8 (1.2-2.7)	20	41.6	1.5(0.7-2.9)	110	50.0	1.8 (1.2-2.8)	54	47.4	1.4 (0.9-2.4)	47	58.0	2.8 (1. 6-5.2)
CagA +	108	48.7	215	80.2	4.6 (2.9-7.1)	19	39.6	0.7 (0.4-1.5)	196	89.1	9.2(5.3-15.8)	98	86.0	6.0 (3.2-11.4)	75	92.6	20.6(7.6-55.7)
Omp+	116	52.3	190	70.9	2.0 (1.3-3.0)	23	47.9	0.8 (0.4-1.6)	167	75.9	2.5(1.6-3.9)	85	74.6	2.1 (1.2-3.7)	61	75.3	2.9 (1.5-5.5)
NapA+	111	50.0	191	71.3	2.6 (1.7-4.0)	23	47.9	1.0(0.5-2.0)	168	76.4	3.4(2.1-5.4)	85	74.6	2.8(1.6-4.8)	61	75.3	3.7(1.9-7.0)
HP0231+	101	45.5	179	66.8	2.4 (1.6-3.6)	23	47.9	1.2(0.6-2.3)	156	70.9	2.9 (1. 9-4.6)	80	70.2	2.5(1.4-4.2)	55	67.9	2.9(1.6-5.3)
HpaA+	83	37.4	127	47.4	1.7(1.1-2.5)	21	43.8	1.4(0.7-2.8)	106	48.2	1.7 (1.1-2.6)	54	47.4	1.6(0.9-2.6)	40	49.4	1.9(1.1-3.4)
HP0305+	83	37.4	132	49.3	1.4(0.9-2.0)	19	39.6	1.1 (0.5-2.2)	113	51.4	1.4 (0.9-2.2)	55	48.3	1.3(0.7-2.1)	41	50.6	1.4(0.7-2.5)
HomB+	75	33.8	116	43.3	1.4(0.9-2.1)	16	33.3	1.1(0.5-2.2)	100	45.5	1.6(1.0-2.4)	50	43.9	1.5(0.9-2.4)	37	45.7	1.6(0.9-2.9)
Catalase+	62	27.9	129	48.1	2.2(1. 5-3.3)	16	33.3	1.4(0.7-2.8)	113	51.4	2.4 (1.6-3.7)	56	49.1	2.1(1.2-3.6)	41	50.6	2.6(1.4-4.7)
UreA +	114	51.4	180	67.2	1.9 (1.3-2.9)	28	58.3	1.3 (0.7-2.6)	152	69.1	2.1 (1.4-3.3)	77	67.5	1.8(1.0-3.0)	58	71.6	3.0(1.6-4.7)
Cagδ+	66	29.7	101	37.7	1.5 (1.0-2.3)	14	29.2	1.1 (0.5-2.4)	87	39.6	1.6 (1.0-2.5)	41	36.0	1.5(0.9-2.5)	37	45.7	2.3(1.3-4.2)
BabA+	35	15.8	58	21.6	1.5(0.9-2.5)	14	29.2	2.4(1.1-5.3)	44	20.0	1.3 (0.8-2.3)	22	19.3	1.2 (0.6-2.4)	18	22.2	1.8(0.9-3.7)
Cad+	58	26.1	97	36.2	1.8(1.1-2.7)	14	29.2	1.3(0.6-2. 7)	83	37.7	1.9(1.2-2.9)	45	39.5	2.0(1.2-3.5)	26	32.1	1.4 (0.8-2.7)
CagM+	52	23.4	76	28.4	1.5(0.9-2.3)	9	18.8	0.9(0.4-2.1)	67	30.4	1.6 (1.0-2.6)	36	31.6	2.0 (1.1-3.5)	22	27.2	1.5(0.8-3.0)

* Relative risks are estimated by odds ratio (OR) and 95% confidence interval (CI), derived from unconditional logistic regression model with adjustments for age, sex, area of residence, SES, use of tobacco, level of fruit and vegetable consumption, and number of siblings. Reference group was always the unexposed group (the group with negative sero-status of specific *H. pylori* protein)

5.2.2 GAC risk by the number of positive *H. pylori* antibodies

Subjects being seropositive for 4-9 and ≥10 proteins exhibited, respectively, 7.2-fold (95% CI 3.5-14.9) and 13.0-fold (95% CI 6.3-26.9) higher odds than subjects with 0-3 positive proteins. This positive, number-dependent relationship was further aggrandized when we specifically focused on individuals classified as uninfected by the *H. pylori* IgG ELISA test. In this stratum, subjects with more than 10 positive antibodies were unexceptionally from GC case group. Among subjects classified as infected, the gradient could not be investigated due to zero non-cardia GAC cases in the reference group.

5.2.3 Associations between *H. pylori* antibodies and non-cardia GAC stratified by AG status

Based on PG tests (PGI< 25μg/l or PGI/PGII< 3), 50.0% (134/268) cases and 20.3% (45/222) controls were confirmed to be with serologically defined AG. Stratified analysis by different AG status (Table 6) revealed that the associations between *H. pylori* antibodies and non-cardia GAC were substantially attenuated or even reversed in subjects with AG diagnosis; conversely, in the non-AG stratum, strength of most associations was enhanced.

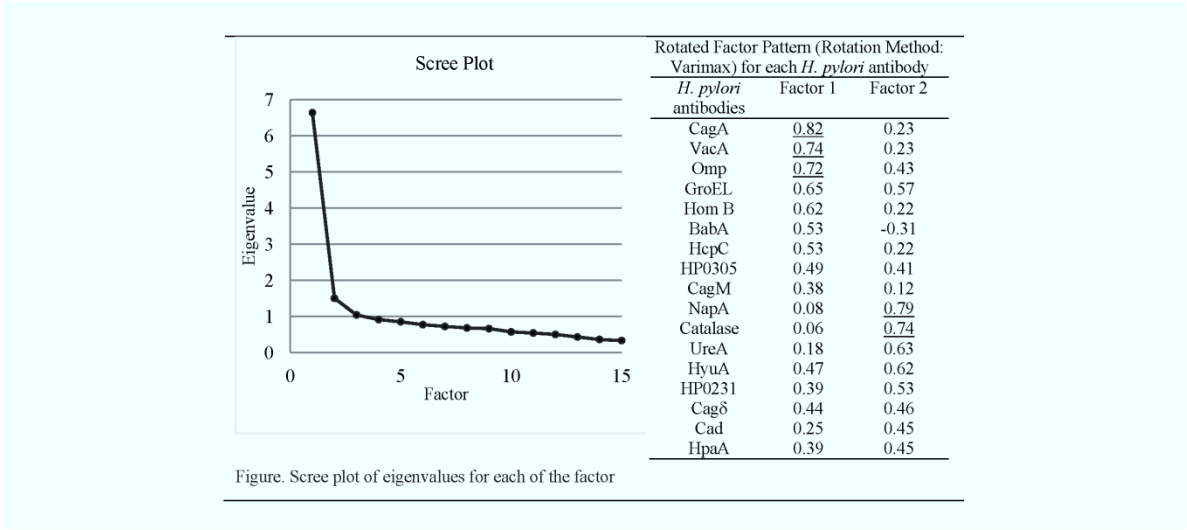
Table 6. Association between different antibodies against *H. pylori* antigens and non-cardia gastric adenocarcinoma (GAC) stratified by serologically defined atrophic gastritis (AG)

	AG Group (n=167, non-cardia GAC /control=122/45)			Non-AG Group (n=275, non-cardia GAC /control=98/177)		
	NCGAC	Control	OR(95%CI)*	NCGAC	Control	OR(95%CI)*
	n (%)	n (%)		n (%)	n (%)	
VacA+	83(68.0)	35(77.8)	0.6(0.2-1.6)	72(73.5)	60(33.9)	7.5(3.9-14.6)
HyuA+	89(72.9)	29(64.4)	1.4(0.6-3.2)	77(78.6)	76(42.9)	6.5(3.4-12.7)
GroEL +	111(91.0)	39(86.7)	1.5 (0.5-5.3)	87(88.8)	93(52.5)	9.4 (4.2-21.1)
HcpC+	56(45.9)	25(55.6)	0.6(0.3-1.4)	54(55.1)	52(29.4)	3.1(1.7-5.6)
CagA +	108(88.5)	35(77.8)	2.2(0.8-6.3)	88(89.8)	73(41.2)	17.3(7.6-39.7)
Omp+	84(68.9)	33(73.3)	0.7(0.3-1.5)	83(84.7)	83(46.9)	7.4(3.6-15.5)
NapA+	92(75.4)	34(75.6)	0.9(0.4-2.3)	76(77.6)	77(43.5)	5.8(3.0-11.3)
HP0231+	85(69.7)	30(66.7)	0.8 (0.4-2.0)	71(72.5)	71(40.1)	5.3(2.8-10.0)
HpaA+	59(48.4)	25(55.6)	0.7(0.3-1.6)	47(48.0)	58(32.8)	2.2(1.2-3.9)
HP0305+	53(43.4)	23(51.1)	0.6(0.2-1.3)	60(61.2)	60(33.9)	2.6(1.4-4.7)
HomB+	50(41.0)	23(51.1)	0.6(0.3-1.3)	50(51.0)	52(29.4)	2.5 (1.4-4.5)
Catalase+	59(48.4)	18(40.0)	1.4(0.6-3.0)	54(55.1)	44(24.9)	3.2(1.8-5.7)
UreA +	84(68.9)	31(68.9)	0.9 (0.4-2.2)	68(69.4)	83(46.9)	2.7 (1.5-4.9)
Cagδ+	43(35.3)	19(42.2)	0.7(0.3-1.6)	44(44.9)	47(26.6)	2.3(1.3-4.1)
BabA+	24(19.7)	8(17.8)	0.9(0.3-2.6)	20(20.4)	27(15.2)	1.5 (0.7-3.2)
Cad+	84(68.9)	33(73.3)	0.9 (0.4-2.0)	37(37.8)	42(23.7)	2.9(1.5-5.6)
CagM+	30(24.6)	14(31.1)	0.7(0.3-1.7)	37(37.8)	38(21.5)	3.4(1.8-6.5)

* OR adjusted for age, sex, area of residence, SES, use of tobacco, level of fruit and vegetable consumption, and number of siblings.

5.2.4 Selection of significant *H. pylori* antibodies for non-AG subjects using Principal Component Analysis

For 275 subjects without cardia GAC and AG, the MFI values of 17 *H. pylori* antibodies were integrated and analyzed using PCA method. Figure 7 illustrates a scree plot displaying the eigenvalues of computed components. The factor loading of each variable is also listed. Factor1 and 2, with eigenvalues >1.0 (6.6 and 1.5, respectively), were considered as acceptable components describing most of variation in the data. Along with the loading values annotated variables' contribution to each factor, the two significant components could be summarized as: (1) A CagA-dominant factor, with reasonable high loadings (>0.7) from antibodies against CagA, and VacA, and Omp, and (2) a non-CagA factor that antibodies against NapA and Catalase loaded highly.



Rotated Factor Pattern (Rotation Method: Varimax) for each *H. pylori* antibody

H. pylori antibodies	Factor 1	Factor 2
CagA	0.82	0.23
VacA	0.74	0.23
Omp	0.72	0.43
GroEL	0.65	0.57
Hom B	0.62	0.22
BabA	0.53	-0.31
HcpC	0.53	0.22
HP0305	0.49	0.41
CagM	0.38	0.12
NapA	0.08	0.79
Catalase	0.06	0.74
UreA	0.18	0.63
HyuA	0.47	0.62
HP0231	0.39	0.53
Cagδ	0.44	0.46
Cad	0.25	0.45
HpaA	0.39	0.45

Figure. Scree plot of eigenvalues for each of the factor

Figure 7. Principal component analysis of antibodies against 17 *H. pylori* proteins among subjects without serologically defined atrophic gastritis (patients with cardia GAC were excluded)

Independent factor scores for newly-defined principal components were used in further regression models. The comparisons of non-cardia GAC risks (Table 7) among ordinal groups graded by the quartiles of controls' factor scores indicated a dose-response manner for both factors—higher score levels seemed to be associated with an increased probability of having non-cardia GAC, with adjusted ORs (highest vs. lowest quart) 16.2 (95% CI 4.8-54.9) for the CagA-dominant factor, and 5.3 (95% CI 2.1-13.3) for the non-CagA factor.

Table 7. Comparison of non-cardia gastric adenocarcinoma risk among subgroups classified by factor scores of principle component analysis*

Categories defined by Factor Scores (quartile)		Noncardia GAC cases (n=98)		Controls (n=177)		Noncardia GAC cases *vs* controls
		n	%	n	%	OR (95% CI)†
Factor 1:	Lowest level (< 1st quartile)	6	6.1	44	24.9	Reference
	Lower level (1st -2nd quartile)	5	5.1	45	25.4	1.0(0.2-4.1)
	Higher level (2nd-3rd quartile)	31	31.6	44	24.9	4.4(1.3-15.0)
	Highest level (≥ 3rd quartile)	56	57.1	44	24.9	16.2(4.8-54.9)
Factor 2:	Lowest level (< 1st quartile)	18	18.4	44	24.9	Reference
	Lower level (1st -2nd quartile)	7	7.1	45	25.4	1.6(0.5-5.6)
	Higher level (2nd-3rd quartile)	24	24.5	44	24.9	2.3(0.9-6.0)
	Highest level (≥ 3rd quartile)	49	50.0	44	24.9	5.3(2.1-13.3)

* Analysis restricted to individuals without chronic atrophic gastritis.

† OR adjusted for age, sex, area of residence, SES, use of tobacco, level for fruit and vegetable consumption, and number of siblings

5.3 STUDY III

5.3.1 Characteristics

After excluding the first 2 years of follow-up, we identified 288,167 patients in the 'Correa's cascade subcohort'. They accrued 2,381,032 person-years at risk (Table 8). Correspondingly, in the 'other diagnoses subcohort', 54,130 patients remained after the 2-year lag, accumulating 381,603 person-years at risk.

Table 8. Characteristics of patients enrolled in the stomach biopsy cohort, first two years of follow-up excluded

Mucosal status at baseline*	Number of subjects	Age at entry, mean (yr) ±SD†	Sex (% male)	Follow-up duration, mean (yr) ±SD†	Accumulated person-years§
Correa's cascade					
Normal	81,174	47.7±19.9	40.5%	9.7±5.7	621,359
Minor mucosal change	11,571	57.5±18.5	43.8%	10.2±6.6	95,437
Gastritis	167,521	58.3±18.2	46.5%	10.7±6.2	1,463,788
AG	14,285	60.3±18.5	41.3%	10.1±6.3	115,583
IM	11,530	66.1±14.7	47.8%	7.9±4.6	68,122
Dysplasia	2,086	65.5±14.9	51.3%	10.0±6.0	16,743
Overall	288,167	55.7±19.3	44.5%	10.3±6.1	2,381,032
Other diagnoses					
Cellular degeneration/ infiltration or abnormal karyotype	826	60.1±18.8	50.9%	9.4±5.7	6,120
Reactive gastropathy	9,130	53.2±17.5	37.3%	5.9±2.9	35,699
Atypia	5,124	63.9±14.5	54.3%	11.4±6.8	48,254
Hyperplasia or hypertrophy	12,392	60.9±16.5	40.8%	9.1±5.3	87,853
Metaplasia (not IM)	9,883	65.2±14.7	49.4%	10.3±5.7	82,399
Polyps	13,935	61.7±14.3	36.3%	9.3±5.5	101,289
Benign tumor	2,840	62.3±16.1	35.2%	9.0±5.6	19,989
Overall	54,130	60.9±16.1	41.8%	9.0±5.5	381,603

* Baseline was defined as the first biopsy identified in the database. When multiple diagnoses were present, the most severe one was selected.

† SD: standard deviation.

§ Calculated after exclusion of the first two years of follow-up.

AG: atrophic gastritis; IM, intestinal metaplasia

5.3.2 Risk assessments for different exposure groups

Table 9 displays the distribution of GC cases across mucosal histology groups, along with crude incidence rates and SIRs. In the Correa's cascade subcohort, the incidence of total GC in the 'normal' group was 19.5 × 10-5 year^{-1} (95% CI 16.2-23.3), which was equal to the expected incidence in the age-, sex- and calendar period-matched Swedish population (SIR=1.0). Starting from chronic non-atrophic gastritis, excess risks were observed which then increased monotonically with steps in Correa's cascade. For the dysplasia group, the incidence of GC reached 262.8×10-5 year^{-1} (95% CI 190.9-352.8), corresponding to a 550% excess in relation to the matched Swedish population (SIR=6.5).

For the other diagnoses cohort, except the 'reactive gastropathy' group, excess risk of GC was observed in all other lesion groups, with the top three being 'atypia' (SIR=4.2), 'metaplasia' (3.1), and 'benign tumor' (2.7). The excess risk was mainly confined to non-cardia GC, but patients with a diagnosis of 'atypia' had 2.7-fold increased incidence also for cardia GC (Table 9).

Using Cox regression model, adjusted HRs were obtained for describing the association between gastric mucosal histology groups and the occurrence of GC, with the 'normal' group as reference. Elevated HR was linked with more severe exposure stratum. This trend was essentially the same as the one observed in SIR calculation. Compared to the normal group, significantly excess GC risks were found for minor mucosal change (HR 1.8, 95% CI 1.2-2.5), gastritis (HR 2.6, 95% CI 2.2-3.2), AG (HR 4.5, 95% CI 3.5-5.8), IM (HR 6.2, 95% CI 4.7-8.2), and dysplasia group (HR 10.9, 95% CI 7.7-15.4), after being adjusted for sex and stratified by pathology units. Similar but slightly higher HRs were observed when analysis was restricted to non-cardia GC only.

Table 9. Observed number of gastric cancers (GC), crude incidence rate per 100,000 person-years, and standardized incidence ratios (SIRs) with 95% confidence intervals (CIs) by mucosal status at baseline

Mucosal status at baseline[*]	Cardia GC			Non-cardia GC			All GC		
	Observed cases[†], n	Crude incidence rate[§]	SIR (95% CI)[‡]	Observed cases[†], n	Crude incidence rate[§]	SIR (95% CI)[‡]	Observed cases[†], n	Crude incidence rate[§]	SIR (95% CI)[‡]
Correa's cascade									
Normal	24	3.9	1.0 (0.7-1.5)	97	15.6	1.1 (0.9-1.3)	121	19.5	1.0 (0.9-1.3)
Minor mucosal change	5	5.2	1.0 (0.3-2.3)	35	36.7	1.6 (1.1-2.3)	40	41.9	1.5 (1.1-2.0)
Gastritis	120	8.2	1.3 (1.1-1.6)	744	50.8	1.9 (1.8-2.1)	864	59.0	1.8 (1.7-1.9)
AG	12	10.3	1.6 (0.8-2.8)	104	90.0	3.0 (2.5-3.7)	116	100.4	2.8 (2.3-3.3)
IM	12	17.6	2.3 (1.2-4.0)	76	111.6	3.7 (2.9-4.6)	88	129.2	3.4 (2.7-4.2)
Dysplasia	5	29.9	3.8 (1.2-8.8)	39	232.9	7.1 (5.1-9.8)	44	262.8	6.5 (4.7-8.7)
Other diagnoses									
Cellular degeneration/ infiltration or abnormal karyotype	0	0	-	4	65.4	2.5 (0.7-6.3)	4	65.4	2.0 (0.5-5.1)
Reactive gastropathy	1	2.8	0.8 (0.1-4.3)	3	8.4	0.7 (0.1-2.0)	4	11.2	0.7 (0.2-1.8)
Atypia	10	20.7	2.7 (1.3-4.9)	76	157.5	4.5 (3.6-5.7)	86	178.2	4.2 (3.3-5.2)
Hyperplasia or hypertrophy	7	8.0	1.4 (0.6-2.8)	45	51.2	2.1 (1.5-2.8)	52	59.2	1.9 (1.4-2.5)
Metaplasia (not IM)	6	7.3	1.0 (0.4-2.1)	97	117.7	3.6 (2.9-4.3)	103	125.0	3.1 (2.5-3.7)
Polyps	7	6.9	1.2 (0.5-2.5)	52	51.3	2.1 (1.6-2.8)	59	58.2	1.9 (1.5-2.5)
Benign tumor	2	10.0	1.6 (0.2-5.9)	16	80.0	3.0 (1.7-4.8)	18	90.0	2.7 (1.6-4.3)

[*] Baseline was defined as the first biopsy identified in the database. When multiple diagnoses were present, the most severe one was selected.

[†] The first two years of observation and corresponding events were excluded.

[§] Per 100,000 person-years

[‡] Observed to expected number of GC cases, based on age- (5-year strata), sex-, and calendar year (5-year strata)-specific incidence data in the total Swedish population. Ninety-five percent CIs of SIRs were calculated by assuming that observed cancer occurrence followed a Poisson distribution.

AG: atrophic gastritis; IM, intestinal metaplasia

The temporal pattern of GC occurrence by mucosal histology group (in Correa's cascade subcohort) was illustrated in the Nelson-Aalen cumulative incidence plot shown in Figure 8. Since 2-year lag time was applied, time '0' stands for day '730' after the initial gastric biopsy. The curves separated out by group as expected right after time '0', and the elevated incidence appears to have been rather stable throughout the follow-up period.

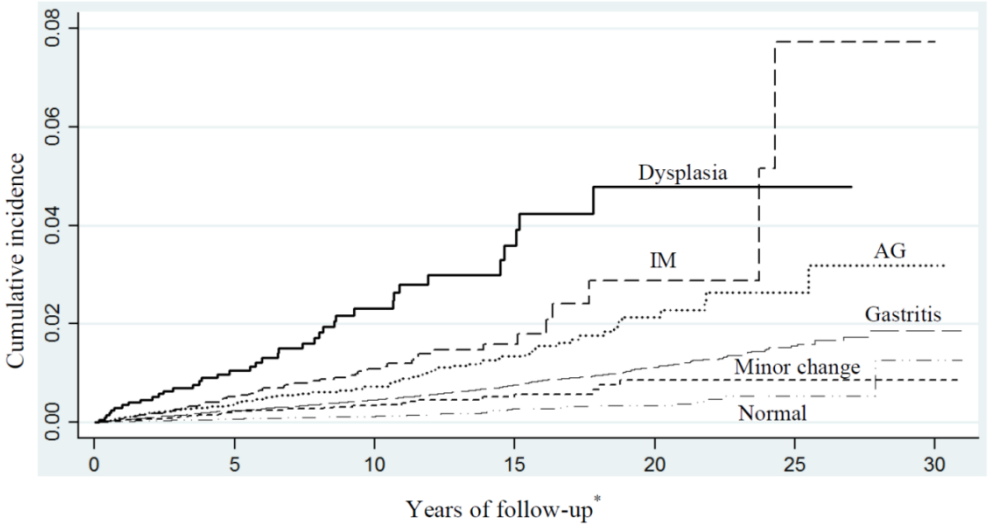


Figure 8. Cumulative incidence of gastric cancer among patients with different baseline diagnoses
AG, atrophic gastritis; IM, intestinal metaplasia
*The first two years of follow-up were excluded.

Figure 9 exhibits that, for patients with repeated endoscopy data, the SIRs were determined by both baseline diagnosis and subsequent histopathological evolution—SIRs increased with more advanced baseline mucosal lesions; and within each baseline mucosal status group, progression was consistently associated with a higher incidence of non-cardia GC than 'no change'/ 'regression'.

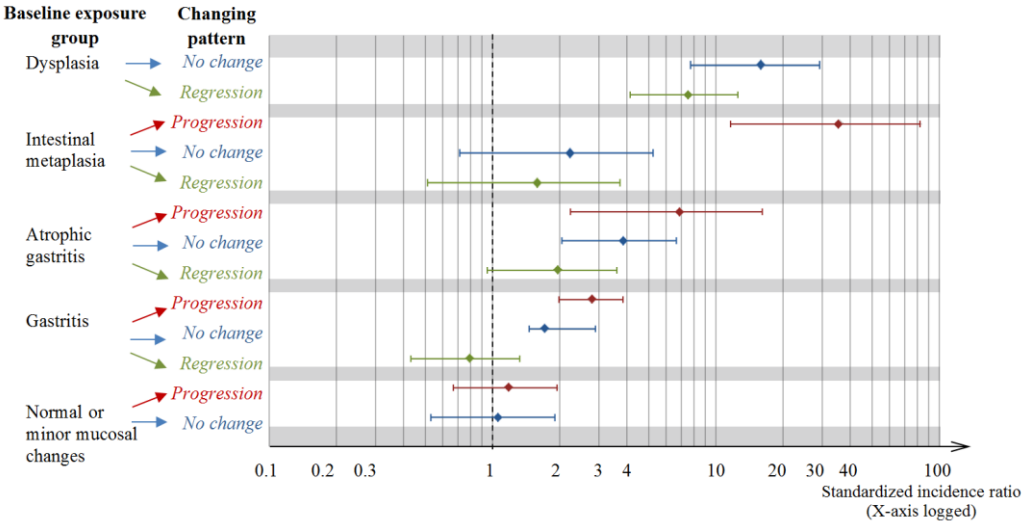


Figure 9. Standardized incidence ratios (SIRs) and 95% confidence intervals (CIs)* for non-cardia gastric cancer in patients with multiple biopsy records (n=55,621), categorized by baseline† exposure group and following changing pattern.
* The first two years of observation and corresponding events were excluded.

† Baseline was defined as the first biopsy identified in the database. When multiple diagnoses were present, the most severe one was selected.

5.4 STUDY IV

5.4.1 Characteristics

The main characteristics of the study population are listed in Table 10.

Table 10. Description of appendectomies in Sweden during 1970-2009, grouped by underlying diagnoses

	All appendectomy cohort	Appendicitis subcohort*	Other diagnoses subcohort†
Number	480,382	327,496	152,886
Age, mean± SD (years)	32.5±19.6	30.2±18.7	37.4±20.6
Age group, n (%)			
0-19 years	157,024 (32.7)	120,807 (36.9)	36,217 (23.7)
20-39 years	169823 (35.3)	120,488 (36.8)	49,335 (32.3)
40-59 years	95527 (19.9)	53,841 (16.4)	41,686 (27.3)
60 years and above	58008 (12.1)	32,360 (9.9)	25,648 (16.7)
Sex, n (%)			
Men	217,710 (45.3)	180,172 (55.0)	37,538 (24.6)
Women	262,672 (54.7)	147,324 (45.0)	115,348 (75.4)
Calendar year of appendectomy, n (%)			
1970-1979	113,499 (23.6)	62,270 (19.0)	51229 (33.5)
1980-1989	139,977 (29.1)	88,966 (27.2)	51011 (33.4)
1990-1999	124,677 (26.0)	92,167 (28.1)	32510 (21.3)
2000-2009	102,229 (21.3)	84,093 (25.7)	18136 (11.8)
Duration of follow-up			
mean± SD (years)	18.6± 10.9	17.5± 10.6	20.8±11.2
n (%)			
0-4 years	66,417 (13.8)	46,989 (14.4)	19,428 (12.7)
5-14 years	127,550 (26.6)	99,038 (30.2)	28,512 (18.6)
15-24 years	134,484 (28.0)	92,666 (28.3)	41,818 (27.4)
>=25 years	151,931 (31.6)	88,803 (26.1)	63,128 (41.3)

* includes patients with perforated appendicitis (diagnoses coded as ICD-8:54000–54003, 54302, or ICD-9: 540A, 540B or ICD-10: K35.0, K35.1, K38.3) or acute non-perforated appendicitis (diagnosis codes of ICD-8: 54090–54208, or ICD-9): 540X, 541, 542, or ICD-10: K35.9, K36, K37).

† includes patients with entirely negative appendectomy (patients ended up with a diagnosis of mesenterial adenititis or unspecific abdominal pain, and without any other surgical procedure appeared in the same hospitalization record), or incidental appendectomy (patients without appendicitis diagnosis but having additional surgical procedure performed at the same time as the appendectomy).

5.4.2 GC risk among appendectomy patients

SIRs together with their 95% CIs were calculated for all appendectomy patients, as well as subgroup patients with different medical diagnoses (appendicitis/other diagnosis), after excluding the first year of follow-up (Table 11). In general, no excess GC risk was observed. Sensitivity analyses that restricted to data during 1987-2009 revealed similar results as in the main analysis (with similar point estimates, yet wider CIs).

Table 11. Standardized incidence ratios (SIRs) and 95% confidence intervals (CIs) for gastric cancers in appendectomy patients with different discharge diagnoses

	All appendectomy cohort		Appendicitis		Other diagnoses	
	Observed cases*	SIR (95% CI) §	Observed cases*	SIR (95% CI) §	Observed cases*	SIR (95% CI) §
Gastric cancer, all	831	1.00 (0.93-1.07)	496	1.01 (0.92-1.10)	335	0.98 (0.87-1.09)
Non-cardia gastric cancer	685	0.99 (0.92-1.07)	393	0.99 (0.90-1.10)	292	0.99 (0.88-1.11)
Cardia cancer	146	1.06 (0.89-1.25)	103	1.10 (0.91-1.35)	43	0.95 (0.69-1.28)

*The first year of observation and corresponding events were excluded.

§ Observed to expected number of GC cases, based on age- (5-year strata), calendar year- (5-year strata) and sex-specific incidence data in the total Swedish population. 95 % CIs of SIRs were calculated by assuming that observed cancer occurrence followed a Poisson distribution.

In further stratified analysis by time period under surveillance (1-4 /5-14 /15-24 /≥25 years), appendectomy was associated with an increased cardia GC risk (SIR 1.63, 95% CI 1.12-2.31) within the first observation period (1-4 years). However, this excess risk quickly disappeared 4 years after appendectomy. In long-term observation, no excess risk was noted, compared to the general population.

6 DISCUSSION

6.1 METHODOLOGICAL CONSIDERATIONS

6.1.1 Study design

6.1.1.1 Case-control study

In **Study I** and **II**, the associations between exposure and outcome were explored by using case-control analysis, which means the exposure statuses were assessed at the time/after the diagnosis of disease. Although this study design is thought to be efficient and worthwhile when studying a rare outcome (e.g. cancer), distortion caused by errors from retrospectively collected information is possible. Questionnaire data with erroneous recall may lead to misclassification of exposure (recall bias). Further, blood sampled after cancer diagnosis may not necessarily reflect the real condition before cancer onset — during disease progression, spontaneous disappearance of the causative *H. pylori* strains and/or immunologic anergy may occur [64-66], followed by an unclear serological status. Both of these conditions can invalidate the estimates, and thereby should be kept in mind when interpreting study results.

The population-based design and the feasibility of modeling with potential confounding factors imply good internal validity for the case-control analyses. However, given the awareness of considerable geographical variations in GC incidence and *H. pylori* prevalence, whether or not these findings can be generalized to other relevant population should be further investigated in future studies.

6.1.1.2 Cohort study

In **Study IV** (a retrospective cohort), exposure assessment was timely enough since the loss of appendix happened just at the time point when appendectomy was performed, whereas in a cohort like that in **Study III**, there exists an 'unobserved' period before the capture of exposure status which needs caution. This means that individuals are recruited as 'exposed' or 'unexposed' at the time of initial diagnosis (exposure measured), instead of the time at onset of the condition (exposure started). Such left truncation of observation matters the most for the exposure groups with mild changes (i.e. gastritis group), which correspond to the stages naturally asymptomatic or mildly symptomatic. Considering the facts that our **Study III** was limited to patients who underwent gastroscopies with biopsies on clinical indications, and the left-truncation was impossible to be fully estimated or predicted by other covariates, the study results cannot be readily generalizable to non-patients (or healthy persons subjected to screening). To be precise, the effective exposure here is pathological diagnosis of the gastric mucosal lesion (derived from spontaneous medical visits and a biopsy performed on certain clinical indication), not the lesion itself. Other issues that can affect the external validity of a cohort study (i.e. **Study III** and **IV**) to relevant populations include the similarity between populations, and the comparability of clinical practices regarding the indications and diagnosis criteria.

Moreover, another challenge in cohort studies is the selection bias. This is especially true when the possibility of exposure diagnosis, namely the cohort entry, is related to the risk of the studied outcome. For instance, in **Study III**, the diagnosis of severe mucosal lesion might be due to the symptoms caused by an emerging malignant lesion (but not yet detected). Thus, ignoring such bias can lead to overestimation of the exposure effect.

Study III and **IV** are population-based cohort studies conducted on the basis of Swedish health care system (i.e. the Swedish pathology register and Swedish patient register). Such study design, while facilitating the complete enrollment and follow-up, cannot provide all detailed information for specific research question which prevents us from accurate evaluations. For example, in **Study III**, since biopsies were performed merely for clinical intentions, conditions about concomitantly lesion or the status of *H. pylori* infection might not be fully recorded. Also, only limited personal data could be extracted from biopsy records. Consequently, some important confounders, such as family history of GC[67] and environmental factors[68], could not be considered during modeling. However, further molecular investigation capable of combining clinic data with genetic/epigenetic information obtained from preserved formalin-fixed and paraffin-embedded (FFPE) biopsy samples could be a potential solution to conquer this limitation.

6.1.2 Possible bias

6.1.2.1 Selection bias and surveillance bias

Generally, selection bias occurs when the selection probabilities are influenced by exposure or target disease status, resulting in 'the study population does not represent the target population'[69]. According to the different sources of selection bias, it can affect both internal and external validity of a study.

The population-based case-control design with its relatively high response rates reduced the possibility of having selection bias in **Study I** and **II**. It was further verified by additional comparisons regarding the main characteristics between participants and non-participants[26, 54], which revealed even distributions for most of those studied variables.

As described above, the major concern of selection bias in these projects is for **Study III**, where the likelihood of patient enrollment might be increased by the symptoms related to the studied outcome (GC). Its effect can be visualized by the 'interval based method'[70] presented in Figure 11, which illustrates the calculated SIRs for several disjoint time intervals close to the time of entry (baseline biopsy). High SIRs which appeared in earlier intervals indicated that concentrated outcome events (GC) happened during the first 24 months of follow-up, especially the first 6 months. However, these extremely high SIRs actually mean nothing with regards to the purpose of exploring the association between studied gastric lesions and GC. It's probably more due to mentioned selection bias[71], rather than truly carcinomatous evolution. In addition, abnormally increased SIRs were also noted for other exposure strata (e.g. normal/minor change group) with inherently low risk of GC, which confirmed the

general effects of selection bias. Thus, in our main analysis of **Study III**, the first two years of observation and outcomes detected during the same period were discarded.

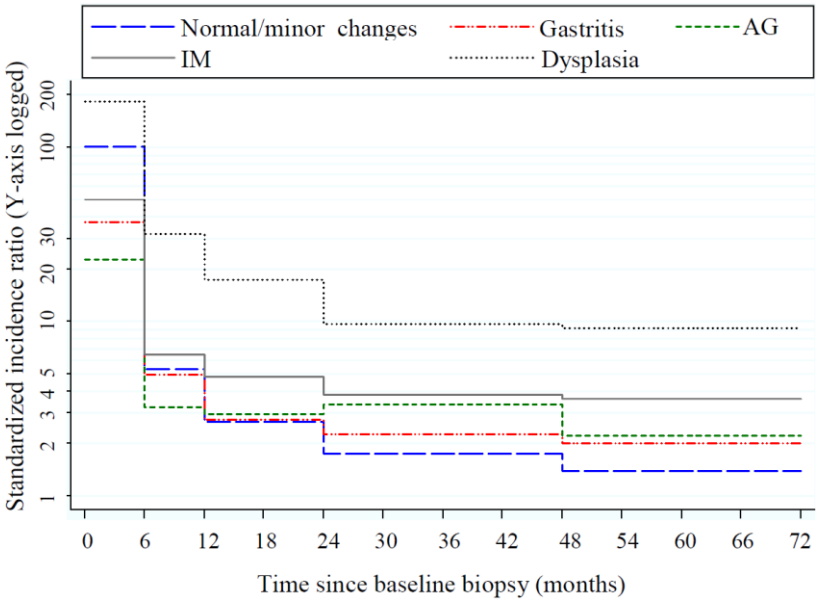


Figure 10. The standardized incidence ratios (SIRs) of gastric cancer among biopsied patients with different pathological lesions in the stomach, by follow-up duration (n=338,911, using interval-based method, in which SIRs were calculated separately for different time intervals:<6 months, 6-<12 months, 12-<24 months, and 24-72 months) AG: atrophic gastritis; IM, intestinal metaplasia

It's notable that this period with concentrated events can be found in any register-based retrospective cohort study where participants are identified from inpatient registers, even when the exposure and the outcome are notoriously irrelevant. This situation is caused by surveillance bias, which occurs when one group (usually the exposure group) is followed more closely than the other group (in the case of SIR calculation, the general population). In other words, the outcome gets a chance to be diagnosed more often because of initially unrelated medical visits, but not because it truly happens more often in this group. Ignoring the surveillance bias can lead to overestimation of exposure effects. Therefore, we always used a lag time (time interval between index hospitalization and start of follow-up) to wash out the effect of such bias on estimates.

6.1.2.2 Misclassification of exposure

The misclassification of exposure can introduce bias to a study. In practice, it can happen in many different ways, and thus have diverging effects on the studied association. Major possible misclassifications in this thesis include:

ACG defined by serologic PG test (Study I and II)

As mentioned in the 'Method' section, serologic PG test is the only pausible alternative to histopathological examiation for ACG diagnosis, and has been widely used in epidemiology studies. However, variations in definition and cut-off values brought difficulties in comparing results from different studies. Both the decrease of PG-I and PG-I/PG-II ratio have been suggested to be closely correlated with grade and extent of ACG [72]. But PG-II and the PG-I/PG-II ratio start to significantly change already during the non-atrophy gastritis stage [73, 74],

contrary to PG-I which is exclusively affected by atrophy occurring in the corpus. Therefore, the choice of specific cut-off point is a joint reflection of the severity of atrophy the study concerned and the preferred balance between specificity and sensitivity. Anyway, reasonable definition should rely on validation studies conducted applying the same testing method (ideally, exactly the same commercial kit) in the same (or at least similar) population. In a single study, the misclassification owing to serological definition should be randomly assigned (non-differential) in participants with different characteristics, e.g. case/control (**Study II**,), or recruited at different time points (**Study I**).

Another drawback of PG-based diagnosis is that it could be influenced by factors that modify serum PG levels by other mechanisms than ACG. It has been reported that smokers have higher PG-I levels than non-smokers[75], which therefore tends to underestimate the prevalence of PG-I-defined ACG among smokers. Nevertheless, the sensitivity analysis we have done by restricting to non-smokers (n=4236) confirmed the identified age-specific changing patterns.

***H. pylori* antibodies detected by multiplex serology (Study II)**

Previous studies aiming to measure the effects of *H. pylori* on GC occurrence have always faced with difficulties since a hypochlorhydric environment characterizes the intermediate state (AG) in the possible causal pathway. It leads to the disappearence of *H. pylori* . Consequently, with sera sampled after GC diagnosis, we might underestimate the virtual association because along with the progression of ACG the reduction in antibody titer can be expected[66]. This speculation has been further confirmed in our analysis, which showed the associations between non-cardia GC and most seromarkers were severely attenuated in the presence of serologically confirmed advanced AG. Under this condition, we chose to perform PCA for novel biomarker identification only among subjects with no serologically detected AG. Notwithstanding the missclassification of AG may exist (as disscussed above), this approach, to some extent, allays concerns about serious underestimation.

Pathological diganosis for biopsy samples (Study III)

In a large, nationwide cohort that heavily relied on data collected from pathological departments lasting over a span of 30 years, inconsistencies or changes in clinical practice, in terms of biopsy taking and pathological diagnosis, might influence the consistency of exposure assessment. Fortunately, although the inter- and intra-observer variation was inevitable, the practice guidelines for the standardized pathological diagnosis have been set up by the Swedish Society for Pathology, and didn't experience any big changes during the study period. For biopsy-taking, no widespread and generally accepted guidelines existed during the whole study period, so the practice could differ by time. For example, in the early part of the study period, when gastroscopies were more commonly performed by surgeons, biopsies were normally taken only when visible abnormalities were seen. In a more recent era, when gastroscopies were done mainly by gastroenterologists, routine biopsies from the antrum and corpus were commonly taken also when there was no visible pathology. Given

that our strategy is to group according to the most severe diagnosis, the deviation caused by various biopsy practices (i.e. whether or not sampling from normal tissue) should be limited.

Sampling errors, which are especially common when sampling patchy precancerous lesions (atrophy or IM)[76], should be another possible source of misclassification. Inability to capture the stomach's most advanced lesion leads to some overestimation of the GC incidence in groups classified as having less advanced lesions, accompanied by an underestimation in groups denoted as having more advanced lesions. However, in our analysis, the incidence of GC in the 'normal' group was equal to the unity that was expected in the matching population; and a change downward in Correa's cascade indeed was followed by a lower GC incidence. These reassuring observations indicate that sampling error did not overwhelm our results.

6.1.3 Confounding

A confounder is a third variable that is associated with the exposure of interest and is potentially an independent cause of the outcome of interest. In other words, the apparent effect of the exposure is distorted as the effect of extraneous factors (confounders) is mistaken for, or mixed with, the actual exposure effect (which can be null)[77]. In our **Study I** and **II**, where detailed information acquired from interview and biological sample testing are available, we controlled for a range of potential confounding factors at analysis. In our register-based studies (**Study III** and **IV**), however, the lack of data on potential confounders is a concern. Further analysis of archived biological samples (**Study III**) on molecular level (e.g. measure smoking exposure by immunohistochemical quantitation of related DNA adducts) is a possible way to address this limitation. Future efforts in other study settings might also consider to collect additional information to accomplish more accurate assessment. In **Study IV**, the impact of suspected confounding factors was 'visualized' by additional risk assessment on its most relevant cancer type. Such as for smoking, SIR for lung cancer indicates no over-presented smokers in our appendectomy cohort as opposed to the general population (SIR 1.03, 95% CI 0.98-1.09).

6.2 INTERPRETATION OF THE FINDINGS

6.2.1 The temporal pattern of ACG prevalence

In **Study I**, our analyses, based on population-based quinquennial cross-sectional surveys during 1990-2009 in two northern counties in Sweden (a low incidence country with world age-standardized incidence rates in men 6.3×10^{-5}, women 3.2×10^{-5})[78], revealed a surprising monotonic and significant upward trend in prevalence of PG-I-defined functional ACG in the youngest investigated age bracket (35-44 years); whilst a decline in prevalence was expectedly observed among people aged from 55 to 64 years.

Contrary to our results, both a serology study from Japan, based on two cross-sectional measurements (1989 and 1996) in a selected population[79], and an endoscopy-based study from Finland, involving consecutive series of outpatients from one single hospital in 1977,

1985 and 1992[80], found suggestive evidence of a particularly marked decline of AG in the young age group (20-39 years). And an overall significant drop in the number of diagnoses of AG was reported in a nation-wide biopsy cohort conducted in the Netherlands during 1991-2005[36], although age-specific trends were not provided. Nevertheless, our finding was in line with a report based on cancer registration data from the US, showing that the incidence of non-cardia gastric cancer increased significantly among Whites aged 25 to 39 years[7]. With considerations to differences in terms of study design, time period, population, and disease definition, the discrepancies between these results do not necessarily invalidate one another. Admittedly, the 'two-batch' measurements conducted using different PGI kits can be an arguable issue as interpreting our outcomes, but the comparability of them has been well validated in a pilot study. Moreover, although point prevalence estimations could be very sensitive to the choice of cut-off points, secular trends were probably not seriously distorted because the deviation, if any, from the "true" value was most likely constant over time. This can be further demonstrated by the expected decreasing trend observed in the oldest age category, which could be viewed as a "negative control". Therefore, it's unlikely that the age-related differences in secular trends could be explained by disparity between testing kits.

Another notable result of our study is that in 2009 the prevalence of PG-I-defined ACG was 30% higher in the younger group than that in the older group (64 versus 49 per 1000). This has never been reported previously. The interpretation might be that this is just a temporary, instantaneous pattern caused by the crossed age-specific trends of ACG prevalence. If this trend continues, we expect that the prevalence curve for the elderly should go up sharply in next 20 years since it has been theoretically reflected by current middle-age curve.

With the strengths of our study, including its clear basis in the source population, the fairly large sample size, the richness of background information, and multiple observation points over a 20-year span, we have the reason to believe the observed upward trend among young middle-aged people in the prevalence of serologically defined ACG does exist. Assuming a constant increase (+2.0 per 1000 per year) and the same increasing rate with age observed today, a clear increasing trend of ACG among the whole population could be anticipated within the coming 10 years; and after 20 years, the number of ACG cases is expected to rise by approximately 29%. Given the importance of ACG to GC development, this finding might predict that the declining GC incidence curve may reach a nadir in the next 30-40 years in the studied population, followed by a new upward trend.

6.2.2 GC risk assessment

6.2.2.1 Among patients with H. pylori infection

The heterogeneous outcomes of *H. pylori* infection imply that there exists a substratum with a risk that is considerably higher than that observed among all *H. pylori* infected. In **Study II**, we specifically focused on 17 potentially oncogenic biomarkers which are essentially antibodies against different *H. pylori* proteins. The highlight of this study is that we proposed a new approach to identify genuinely novel discriminative patterns of *H .pylori* antibodies.

First, multiplex serology test succeeds to illustrate the co-variances among those *H. pylori* proteins, which is compulsively sealed by surveys concentrating on a single protein. Then, PCA was applied as a means to cope with between-antibody correlations and entailing dependencies that complicate statistical modeling.

On the basis of our results, we confirmed that CagA and other studied biomarkers were strongly associated with the occurrence of non-cardia GC. However, the associations could be severely attenuated in the presence of serologically-defined advanced AG, which causes the disappearance of *H. pylori* owing to low acid secretion. This corroborates previous results stated by others[64, 65, 81]. Furthermore, among patients without advanced AG, PCA identified two significant factors: (1) A CagA-dominant factor (antibodies against CagA, VacA, and Omp as prominent markers) appeared to be largely driven by CagA. Thus, its covarying antibodies could contribute essentially little to the discriminatory ability, although they might serve the purpose of decreasing misclassification of CagA-positive infection and thus strengthen the predictive ability. (2) The second constellation of antibodies, with NapA and Catalase as prominent markers and was theoretically independent of the CagA constellation, corresponded to a moderately strong increase in the odds of having non-cardia GC. Although this is not confirmative evidence indicating the direct oncogenic actions of NapA and Catalase — their coding genes may just happen to be adjacent to the truly causative gene(s) or the proteins may just be somehow intrinsically correlated to other more important proteins, further research on the possible oncogenic mechanisms of NapA/Catalase factor is warranted. Also, the reproducibility of this result in other populations needs further examination; and if it has been proved to be generalizable, it's reasonable to believe that the overall cancer-predictive ability of *H. pylori* serology might be improved by detecting individuals highly exposed to the NapA/Catalase factor among those with CagA-negative infection. Nonetheless, given the relatively small proportion of variance attributable to this factor, its clinical value may be limited. More studies aiming at identifying additional oncogenic virulence factors of *H. pylori* are necessary.

6.2.2.2 Among endoscopy patients with stomach biopsy

Our data, based on the largest nationwide stomach biopsy cohort reported so far, confirmed that among biopsy patients with clinical indications, the diagnosis of these studied precursors (gastritis, AG, IM, and dysplasia) indeed predicted a GC risk level above the general population average. And in line with results reported previously[82, 83], the elevation of risk levels ranked in a way just as it's described in Correa's cascade: dysplasia patients experienced the highest relative risk (about 6.5-fold) of GC, followed by 3.4-fold, 2.8-fold, 1.8-fold for IM, AG, gastritis patients, respectively. Among individuals who underwent endoscopy with biopsies taken but received diagnosis of normal histology, no excess risk of GC was observed (SIR=1.0), relative to general population.

Despite the well-established neoplastic cascade, results from previous follow-up studies on absolute risks associated with having these precancerous lesions are inconsistent[30, 36, 84, 85]. Besides the geographic differences, interpretations for these variations can be: different study

designs, limited sample sizes or follow-up period, and various approaches to deal with the concentrated GC events which were commonly observed after the first few years of cohort entry[82, 86]. One similar large biopsy cohort conducted in the Netherlands suggested an emphasis on the first year after index biopsy by stating that more than 25% patients from high-graded dysplasia group received their GC diagnosis within 1 year[82]. In our analysis, since we particularly focused on long-term risks, a 2-year lag time was applied during which a large peak in incidence was observed. After 2 years, the peak was passed, so our current estimates cannot be blurred by the initially overlooked prevalent cases. The annual incidence of total GC was assessed to be19.5×10^{-5} (95% CI 16.2-23.3) for the 'normal' group, which then increased monotonically with each step in Correa's cascade, and reached the highest level (262.8×10^{-5}, 95% CI 190.9-352.8) for the group with 'dysplasia' diagnosis. These numbers can be approximately translated into a 20-year GC risk of 1 in 85 with gastritis, 1 in 50 with AG, 1 in 39 with IM, and 1 in 19 with dysplasia among patients who undergo gastroscopies with biopsies on clinical indications. The elevated incidence was rather stable, and the gaps between cumulative incidence curves grew continuously throughout the whole follow-up period. Intriguingly, further analysis with repeated endoscopy data indicated that if reiterated biopsies showed a change – upward or downward along the Correa cascade – compared to the initial grouping, this seemed to have prognostic significance.

In addition, our study also quantified GC risks among patients with some other gastric mucosal conditions. Patients with atypia diagnosis presented a notably increased GC risk (4.2-fold higher than general population), which was numerically beyond that for IM patients. This might be partly attributable to the ongoing debate on the definitions of 'atypia' and 'dysplasia' within the field of pathology[87, 88], since under microscopy, it can be a great challenge to distinguish atypia from dysplasia. Moreover, previous histological studies found that the strictly-defined atypia indeed highly co-occurred (49%-78%) with gastric cancers[89, 90], and thereafter concluded it as a neoplasic lesion and renamed it as 'pit dysplasia'. Consistent with prior reports, other gastric pathological conditions with potentially high cancer risks were benign tumor[91], metaplasia, polyps[87, 92] and hyperplasia[93]. Rather, for reactive gastropathy (an abnormality caused by chemical injury), no excess GC risk was noted[94].

6.2.2.3 Among appendectomized patients

The possible linkage between GC risk and appendectomy has been suggested previously[49, 50], with unclear explanation. Possible mechanisms proposed included low SES, which is linked to both high risk of appendectomy[95] and *H. pylori* infection during childhood[96]. Moreover, although no evidence indicated that *H. pylori* infection could directly affect the pathogenesis of appendicitis[97], the unspecific abdominal pain caused by *H. pylori*[98] might potentially increase the likelihood of having appendectomy. In addition, the so-called 'safe house' theory [99,100] indicated that removal of the appendix might disturb normal gastrointestinal microflora[101], which in turn might have an effect on the carcinogenesis in the stomach.

However, physiologically, there is no clue showing that such micro-environmental dysbiosis, mainly occurs in the large intestine, could induce subsequent changes in foregut microbiota.

Contrary to previous findings, our register-based cohort study (the largest one reported so far), found no evidence to support the supposition that the appendectomies, performed for either appendicitis or other indications, could alter the risk of GC occurrence. It's notable that our analysis, essentially an extension of Cope's research[49], with longer follow-up period and without age restriction, overturned the result in the previous report. With the consideration that the oldest participants in Cope's study were only 48 years old at the time of analysis, the limited GC cases (n=8) identified there could be driven by extreme etiology (e.g. genetic reason) rather than the exposure of interest. Alternatively, it's possible that the elevated SIR for GC was just a chance finding. When we limited our analysis to participants aged below 20, no such excess risk of GC could be seen anymore (data not shown). On the other hand, the marginally significant excess risk of GC in the study from Denmark[50] didn't exist among patients with more than 10 years of follow-up. This contradicts with the long precancerous period required for GC development when it's triggered by environmental factors[102], and therefore substantially undermines the possibility of causal relationship between appendectomy and GC.

7 CONCLUSIONS

- The observed increasing prevalence of ACG among 35-44 years Swedes, together with other emerging evidence, predict that the declining GC incidence curve may reach a nadir in the next 30-40 years in the studied population, followed by a new upward trend.

- Although CagA remains the strongest risk indicator, there are other serum antibodies against *H. pylori* proteins associated with noncardia GAC risk. With principal component analysis, we unveiled two essentially independent constellations of antibodies (the CagA-dominated cluster and a novel cluster with NapA and Catalase as prominent markers) that both predicted risk for noncardia GAC. The addition of CagA co-varying seromarkers may decrease exposure misclassification of CagA-positive infection; and detection for NapA/Catalase factor among those with CagA-negative infection may further improve the overall cancer-predictive ability of *H. pylori* serology.

- Among endoscopy patients with biopsies on clinical indications, approximately 1 in 256 with normal mucosa, 1 in 85 with gastritis, 1 in 50 with AG, 1 in 39 with IM, and 1 in 19 with dysplasia will develop GC within 20 years. The elevated GC incidence among precursor patients is stable throughout the follow-up period; and the observed changes during re-evaluation(s) indeed have prognostic implications.

- Previous evidence regarding the association between appendectomy and GC is only suggestive. Based on our large nationwide cohort with a fairly long follow-up period, we concluded that no excess GC risk could be observed among patients who underwent appendectomy.

8 FURTURE STUDIES

Despite that we have put a lot of efforts into attempting to improve GC risk prediction, there still remain many challenges. For instance, we aimed to explore the *H. pylori* antibodies and their associations with GC occurrence. However, it has been reported[64-66] that the disease progression, notably severe AG, changes the conditions in the *H. pylori* habitat to the extent that the causative strains may disappear or get displaced by other more adapted strains. Hence, with current case-control study design with blood samples collected after cancer diagnosis, we cannot rule out the possibility that our findings might be affected by disease-related changes. Fortunately, emerging new techniques might provide the possibility of analyzing genetic profile of the microbe by using old FFPE samples[103-105]. This new approach, along with our nationwide stomach biopsy cohort (introduced in **Study III**) where long-term archived samples have been collected many years ahead of GC diagnosis, offers a unique opportunity to expand current knowledge about carcinogenicity-driving specificities of colonizing *H. pylori* strains, as well as the role of microbe-environment interactions. Our future studies, therefore, will include the exploration of the relationship between *H. pylori* genetic profile (detected among biopsy patients with gastritis changes) and development of GC (diagnosed at least 10 years after initial biopsy) by using a nested case-control design.

The fact that *H. pylori* colonize the atrophic stomach poorly, and in intestinal metaplasia hardly at all[106], leads to a hypothesis that some other bacteria may be attributable to the development of GC. However, so far there is no longitudinal analysis exploring the association between the microbial ecology in atrophic mucosa and the risk of GC. Therefore, based on our stomach biopsy cohort, we plan to conduct a nested case-control study on human stomach microbiota in mucosa and GC risk among subjects with moderate to severe AG.

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