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Risk Factors for Esophageal Squamous Cell Carcinoma

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Stockholm 2013

To my dearest family

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ABSTRACT

Aims: The etiology of esophageal squamous cell carcinoma (ESCC) in the high risk areas is largely unknown and a few environmental risk factors which have been identified do not explain its oddly high incidence in esophageal cancer belt. The aims of this thesis were to investigate the association of opium/tobacco consumption, serologic gastric atrophy, gastric mucosa-associated microbiota and contact with farm animals with the risk of ESCC.

Methods: The population of Golestan Province in northeastern Iran has very high rates of ESCC. From 2003 to 2007, we administered a validated structured questionnaire to 300 incident ESCC cases and 571 controls. Controls were matched to cases for neighborhood of residence, age ($\pm$ 2 years), and sex. We measured serum pepsinogen I and II among 293 incident cases and 524 matched controls. Conditional logistic regression models were applied to calculate odds ratios (ORs) and 95% confidence intervals (CIs) adjusted for potential confounders. Furthermore we obtained a frozen gastric tissue biopsy from subjects with ESCC, esophageal squamous dysplasia, mid esophagus esophagitis, and age/sex-matched endoscopy clinic controls with healthy esophagus. To characterize bacterial lineage present in gastric mucosa, we performed a multiplex sequencing with GS-FLX Titanium targeting 16S rRNA.

Results: Risk of ESCC was increased in those who used opium only (OR = 2.12, 95% CI: 1.21 - 3.74), and in those who used both tobacco and opium (2.35, 95% CI: 1.50 - 3.67).   All forms of tobacco use (cigarettes, hookah, and nass) were associated with higher ESCC risk.

Gastric atrophy (defined by a validated criterion, pepsinogen I <55 μg/dl) was associated with a two-fold increased risk (OR = 2.01, 95% CI: 1.18 - 3.45) of ESCC in the absence of non-atrophic pangastritis (defined as pepsinogen II < 11.8 μg/dl). Sequencing of 16S rRNA in gastric biopsy samples resulted 2075 operational taxonomic units (OTUs). Conditional logistic regression model based on principal coordinate analysis (PCoA) showed a marginal variation in pattern of gastric microbiota using Unifrac (p = 0.004) and weighted Unifrac distances (p = 0.018) between subjects with esophageal cancer or dysplasia and controls. No such difference between subjects with mid-esophagitis and controls was observed.

Among four groups of farm animals (equines, ruminants, domestic canine and poultry) contact with ruminants was associated with an 8-fold increase in risk of ESCC. This association stayed stable when duration and level of contact were considered.

Conclusions: Though opium and tobacco consumption are associated with the risk of ESCC in the study area, they do not explain the extreme high incidence in northern Iran. Changes in gastric environment might be linked to ESCC risk as fundal atrophy may increase the risk for ESCC and pattern of gastric microbiota differs in patients with esophageal squamous dysplasia (and ESCC) from subjects with normal esophagus. The observed relationship between lifelong contact with ruminants and ESCC needs further investigation.

Key words: Esophageal cancer, opium, tobacco, atrophic gastritis, dental health, oral hygiene, pepsinogen, squamous dysplasia, microbiota, animal contact.

LIST OF PUBLICATIONS

I.   Nasrollahzadeh D, Kamangar F, Aghcheli K, Sotoudeh M, Islami F, Abnet CC, Shakeri R, Pourshams A, Marjani HA, Nouraie M, Khatibian M, Semnani S, Ye W, Boffetta P, Dawsey SM, Malekzadeh R. Opium, tobacco, and alcohol use in relation to esophageal squamous cell carcinoma in a high-risk area of Iran. Br J Cancer. 2008: 3;98(11):1857-63

II. Nasrollahzadeh D, Malekzadeh R, Aghcheli K, Sotoudeh M, Merat S, Islami F, Kamangar F, Abnet CC, Shakeri R, Pourshams A, Semnani S, Boffetta P, Dawsey SM, Ye W. Gastric atrophy and esophageal squamous cell carcinoma: possible interaction with dental health and oral hygiene habit. Br J Cancer. 2012:21; 107(5):888-94.

III. Dariush Nasrollahzadeh, Reza Malekzadeh, Alexander Ploner, Masoud Sotoudeh, Ramin Shakeri, Anders F Andersson, Björn Winckler,Weimin Ye. Variations of gastric microbiota are associated with early esophageal squamous cell carcinoma and squamous dysplasia. *Manuscript*

IV. Dariush Nasrollahzadeh, Reza Malekzadeh, Ramin Shakeri, Masoud Sotoudeh, Shahin Merat, Farhad Islami, Farin Kamangar, Christian C. Abnet, Paolo Boffetta, Sanford M. Dawsey, Weimin Ye. Animal contact and risk of esophageal squamous cell carcinoma. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

ESCC	Esophageal squamous cell carcinoma
H. pylori	*Helicobacter pylori*
PG	Pepsinogen
DMFT	Decayed, missing, and filled teeth
cag	Cytotoxin-associated gene
OR	Odds ratio
CI	Confidence interval
OTU	Operational taxonomic unit

1 THESIS SUMMARY

The incidence of esophageal cancer ranks 8th worldwide (1). Its incidence varies between geographical areas with high rates in east Africa, South America, central and eastern Asia, and some parts of Europe. North eastern Iran and north central China are two ends of the "esophageal cancer belt" with peak incidence rates. Squamous cell carcinoma and adenocarcinoma are two main histology types of esophageal cancer. The predominant form of esophageal cancer observed in high risk area is squamous cell carcinoma. While smoking and alcohol drinking are two major known risk factors for esophageal squamous cell carcinoma (ESCC) in low risk areas, their contribution to the risk in the "esophageal cancer belt" is minor. The objective of this thesis is to investigate associations of some environmental factors with esophageal squamous cell carcinoma in the extreme-risk region of north eastern Iran.

2   BACKGROUND

2.1   DESCRIPTIVE EPIDEMIOLOGY

Based on estimated new cancer cases in 2008, incidence of esophageal cancer ranks 6th among men and its mortality ranks 5th and 8th among men and women worldwide (1). Wide variation in its incidence and distinct geographical hotspots are main epidemiologic features of esophageal cancer. Highest incidence rates have been reported from the "esophageal cancer belt", an area that stretches from northern Iran to northern China (2) (Figure 1).

The earliest report of the high incidence of esophageal cancer in northern Iran goes back to early 1970s which showed that in the eastern Caspian sea littoral (Gonbad area) incidence rates among men and women were 109/100 000 and 174/100 000 person-years respectively (3, 4). Beside slightly higher female to male ratio in the high risk area of Northern Iran, the wide variation of incidence rates within relatively small geographic area is still evident as in regions a few hundred kilometers apart, its incidence dropped to 5/100 000. Albeit esophageal cancer incidence has dropped in this area, recently-developed cancer registry in the eastern Golestan province has confirmed that the rates are still high in eastern part of the province (5, 6).

Estimated age-standardised incidence rate per 100,000
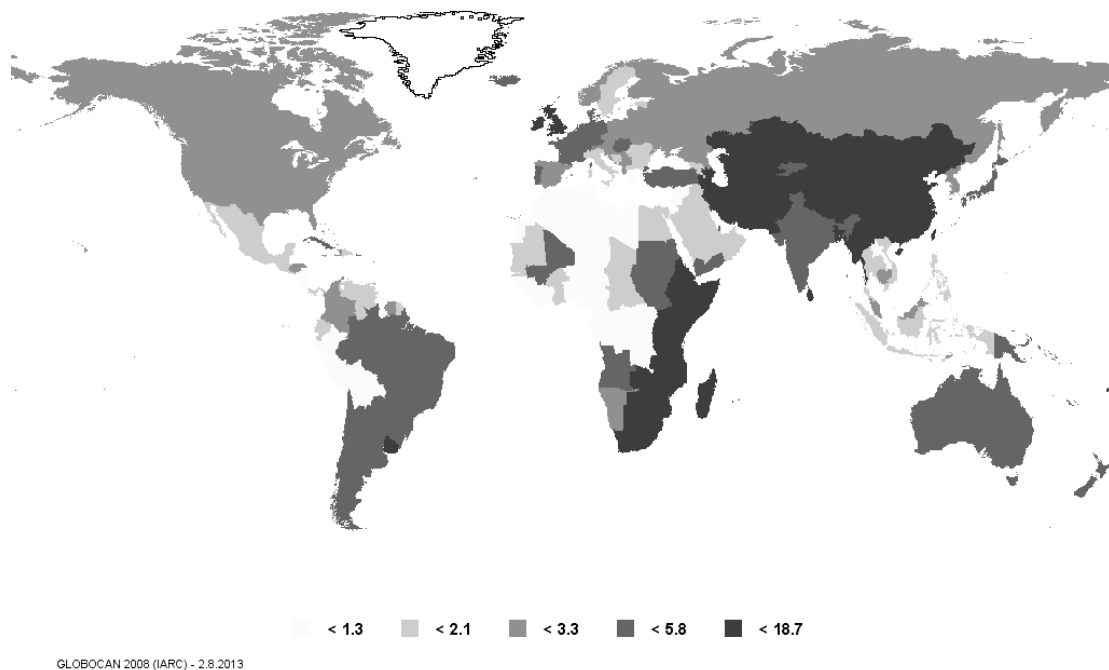Esophagus: both sexes, all ages

GLOBOCAN 2008 (IARC) - 2.8.2013

Figure 1. Map of esophageal cancer incidence rate (source: Globocan 2008, IARC)

2.2   RISK FACTORS STUDIED IN THIS THESIS

In the high risk areas the prominent histologic type is squamous cell carcinoma (approximately 90%) (7). Esophageal squamous cell carcinoma (ESCC) is difficult to detect early, and almost always associated with poor outcome. So far no set of risk factors could fully explain its high incidence in northern Iran.

2.2.1   Opium

Studies conducted by the International Agency for Research on Cancer (IARC) and Institute of Public Health Research (IPHR) of Tehran University in the 1970s suggested that opium use may have a link to ESCC high incidence rate in Northern Iran(8).

An ecologic study that compared people living in high-risk areas for ESCC with those living in lower risk areas found that 50% of the adult residents in the high-risk areas, compared to 11% of the residents in the reference group, tested positive for urine morphine metabolites. In another study among rural inhabitants, the prevalence of the urinary morphine metabolites with level of equal to or higher than 1 μg/ml was 6-fold higher among residents of high-risk compared to low-risk areas (9, 10).

Opium consumption is still common among the old generation living in this region (11) and it is used a traditional medicine to treat pain, diarrhea, and insomnia. Whereby neither crude opium nor its refined products were proven to be mutagenic (12). However, inhaling the smokes from consumption of crude and refined opium might expose the individual to the potential carcinogens, including polycyclic aromatic hydrocarbons (PAHs). Histologic examination of non-tumorous esophageal tissues from ESCC cases and controls showed higher severity of anti-PAH antibodies staining in the same population (13). Considering that the link between this observation and exposure to opium was justified as an exposure secondary to cancer. Limited observations have shown that smoke condensates from opium and morphine cause mutations in *Salmonella typhimurium* (12, 14), and morphological transformations in cultured Syrian hamster embryo cells (15) whereas crude opium itself is not a mutagen (12, 14). Whereby at acute high doses, morphine-- as the major alkaloid in opium-- may alter the first pass hepatic clearance of the carcinogens i.e. *N*-nitrosodiethylamine (16, 17). The mechanisms responsible for the association which has been found between opium consumption and elevated ESCC risk need to be investigated.

2.2.2   Tobacco and alcohol

Heavy alcohol consumption and cigarette smoking are major causes of ESCC in most parts of the world (18-21). These two habits are correlated behaviorally. Based on questionnaire data, alcohol consumption was rare in Golestan Province during 1970s, especially among rural people, and therefore was unlikely to be an important cause of ESCC in this area(8), recent study confirmed the low prevalence of alcohol consumption in this population (22). Cigarette smoking appeared to be associated with a 2-fold increased risk of ESCC which was not as strong as the observed 3-5 fold elevated risk in low risk area (19, 20).

Tobacco consumption in the Golestan province is not restricted to cigarette. Habit of chewing nass which is a combination of tobacco, ash and lime was a relatively common habit among men living in the rural area (8). Smoking Hookah (water-pipe) is an additional but uncommon habit of using tobacco in this area (8, 9, 11).

Nass exposes users to both carcinogens in unburned tobacco (e.g., nitrosamines) and the carcinogens in ash (e.g., PAHs). Association between chewing tobacco and esophageal cancer has been shown through several studies(23). Smoking Hookah exposes users to the smoke from both tobacco and burning charcoal (24). Among other types of tobacco use, IARC considered chewing betel quid -which includes tobacco- as a risk factor for esophageal cancer (25). The association between hookah or chewing nass and ESCC has been minimally studied (26, 27).

2.2.3 Gastric environment

Impact of gastric environment on esophageal carcinogenesis has been addressed through observational studies (28, 29). As a link for this impact, gastric fundal atrophy has been shown to be associated with ESCC risk (30). Early studies have observed an overgrowth of bacteria in hypochlorhydric stomach through culturing bacteria in gastric juice (29). Recently sequencing-based methods showed lower bacterial diversity associated with higher gastric pH (31).

2.2.3.1 *Gastric mucosal atrophy*

Loss of appropriate glands in gastric mucosa is considered atrophy. Atrophic transformations might be in two forms: shrinkage or disappearance of mucosal glands and/or replacement of native glands by metaplastic ones (32). Long-standing *Helicobacter pylori (H. pylori)* infection and pernicious anemia are two medical conditions linked to gastric atrophy. In pernicious anemia, changes mainly affect gastric corpus while *H. pylori* infection results in multifocal atrophic gastritis. The association between gastric atrophy and a variety of malignancies was described in 1870s through post-mortem examination (33).

Gold standard method for diagnosing atrophy is histologic evaluation of gastric biopsy specimens. In large epidemiologic studies, serologic biopsy of gastric function through measurement of biomarkers such as pepsinogens and gastrin has been utilized as a feasible diagnostic method (34). Pepsinogens are the inactive forms of pepsins; the proteinases in gastric juice responsible for digestion of protein. Two distinct immunologic types of pepsinogens are synthesized and stored in adult stomach mucosa; type I is mainly produced in the chief cells of corpus and type II synthesized in most part of gastric mucosa and also part of duodenum (35). Small proportion of pepsinogen enters the blood in correlation with the amount which is secreted into gastric lumen(36), its measurement has been long time applied as a marker for stomach secretary tissue mass (37). Pepsinogen I (PGI) decreases in parallel with progression of atrophy in corpus and pepsinogen II (PGII) changes or remains unchanged (35). Whereby decrease in PGI to PGII ratio (PGI/PGII) has been shown to be valuable in detecting fundic atrophy. The outcome of interest and the method of laboratory assessment vary among studies which aimed to evaluate diagnostic ability of pepsinogens. Implementing different cutoff values for defining fundic atrophy among different populations (38) have been mainly aimed at diagnostic ability of pepsinogens in gastric cancer screening. PGI <70 µg/l and PGI/PGII ratio<3, measured by radioimmunoassay, it the most common-used the threshold to distinguish at risk population for gastric adenocarcinoma (39).

H. pylori infection is considered as an early step in the process which includes fundic atrophy (40) while gastritis caused by *H. pylori* affects serum level of pepsinogen (41).

It is likely that outcome of *H. pylori*-induced gastritis would differ among different populations. In the majority of infected individuals inflammation remains limited to the antrum (42) but in some infected individuals, inflammation extends from the antrum to the corpus and results in pangastritis (43) and it might be a step in the progression from gastritis toward fundic atrophy and gastric carcinogenesis (44). This might result in different proportion of pangastritis among infected population (45).

Epidemiologic studies have shown an association between gastric atrophy and ESCC risk. An observed association between pernicious anemia and ESCC in a register based study was linked to gastric atrophy (46) plus a population-based case control study found that serologic marker of gastric atrophy was associated with an increased risk of ESCC (30). Association between gastric atrophy and ESCC has been studied in studies with different designs since then. However the magnitude of the reported measurement of risk varied (34, 47-50) among them, positive association, (34, 51, 52) no association (53) and an association with esophageal squamous dysplasia (54) have been reported.

The underlying mechanism linking gastric atrophy to esophageal cancer has not been explained. Bacterial overgrowth in achlorhydric stomach was proposed as an explanation for this link (30). In an atrophic stomach, hypochlorhydria allows bacterial overgrowth (29) and may produce potential carcinogenic substances such as nitrosamines (55, 56), although no dose-response relation with severity of atrophy has been reported (48).

Through gastric pH monitoring it was found that ESCC is often accompanied with a hypochlorhydric stomach even in conditions that atrophy could not be detected clinically (28, 57). Based on bacterial cultivation of gastric juice it is evident that an increase in gastric pH is followed by harboring a bacterial profile characterized by high production of nitrosamines (58, 59).

2.2.3.2   *Gastric mucosa-associated microbiota*

Human stomach was considered an inhospitable environment until the recognition of *H. pylori* (60). *H. pylori* is considered as a definite carcinogen (61) and associated with gastric adenocarcinoma (62). In some areas within the esophageal cancer belt, serologic studies showed majority of *H. pylori* strains contain *cag* pathogenicity island (63), however the same stomach might host both CagA positive and negative strains (64). Among *H. pylori* virulence factors, CagA antigen induces longer immune response (65). Association between *H. pylori* seropositiveness and ESCC has been evaluated in several case-control and cohort studies (30, 49, 63, 66-70) with little evidence of association. Results on CagA seropositivity and risk of esophageal cancer are controversial.

Gastric acidity is believed to be an important factor preventing other bacteria from colonizing in the stomach, although 16S rRNA sequencing of gastric mucosa revealed a diverse bacterial community colonizing human stomach (71). More than one hundred phylotypes have been detected in stomach mucosal biopsy of which 50% were derived from uncultivated bacteria (71). Although the role of *H. pylori* in the development of chronic inflammation of stomach is evident (72), little is known about other members of gastric microbiota community (73). *H. pylori* negative stomach showed high abundance of *Streptococcus* and *Prevotella* (74) among them certain *Streptococcus* species are resistant to low pH (75). In the presence of inflammation (antral gastritis) microbiota changes in favor of *Firmicutes* phylum and under-representation of

Proteobacteria phylum (74). Animal studies have shown that increase in abundance of certain members in *Firmicutes* phylum altered the pathogenicity of *H. pylori* (76). Furthermore long-term intragastric colonization of *H. pylori* decreases the abundance of *Lactobacilli* in Mongolian gerbils (77). Yet little is known about the role of gastric mucosa-associated microbiota in the progression of inflammation and production of carcinogens.

2.2.4 Poor orodental hygiene

Poor orodental hygiene, characterized by the number of tooth loss and low frequency of tooth brushing, has been associated with higher risk of esophageal cancer (78-82). The association between tooth loss and risk of esophageal cancer showed dose-response relationship in most studies from the high risk areas. Some studies showed that number of decayed and filled teeth was associated with esophageal cancer risk after controlling for common risk factors including tobacco and alcohol consumption (80, 82). Furthermore this association appears to be valid even in esophageal squamous dysplasia as the only known ESCC precancerous status (83).

In general, tooth loss might be an indicator of a less healthy lifestyle. Having in mind that low socioeconomic status links with poor oral health, there are several suggested hypotheses:

(1) Poor oral hygiene may result in changes of the oral bacterial flora (84), causing periodontal diseases, and increased production of acetaldehyde and nitrosamines. A study showed that endogenous nitrosamine formation in oral cavities with poor hygiene is 8-fold higher than in those with good hygiene (85). The rich oral microbiota and frequent or chronic episodes of inflammation during several decades of life, maintain a low-grade chronic inflammation with inflammatory mediators which might reach esophageal mucosa through salivary flow (86).

(3) Poor dentition could alter dietary habit due to reduction in masticatory ability (87). Edentulous people less likely consume fiber and vegetables (88).

2.2.5 Animal contact

Contact with animals has been suggested as a potential risk factor for ESCC in high risk areas based on the following ecologic data, veterinary workups and occupational cohort studies:

(1) Geographical distribution of cancer belt is well-matched to the nomadic lifestyle with close contacts with equines, ruminants, canines and poultry among inhabitants of the eastern Caspian Sea littoral. This type of life style is disappearing as socioeconomic status has improved during the last decades. Shared environmental resources between farm animal and human might be an explanation for this association in ecologic observations. Another explanation could be exposure to farm animal products without industrial processing which is more common among villagers who keep animals within household. An early nutritional study showed higher consumption of sheep's and goat's milk in Northern Iran compared to the lower risk area (89).

(2) Further suggestive observations stem from reports of high incidence of concurrent occurrence of esophageal malignancies in endemic areas among autopsied chickens in Linxian, China (90) and cattle in Kenya (91).

(3) Studies on occupational exposure among butchers (92) and workers in poultry (93) or ruminant abattoirs' plans (94) showed inconsistent results of increased risk of

mortality due to esophageal cancer based on low number of cancer cases. A study on a cohort of workers in slaughtering plans of ruminants showed a standardized mortality ratio (95% CI) of 1.6 (1.0-2.4) based on 23 esophageal cancer cases (94). The same group reported an excess relative risk (95% CI) of esophageal cancer among poultry workers with 4.7 (1.1-22.5) based on 6 cases of esophageal cancer (93).

Based on occupational studies, animal viruses were proposed as predisposing factors for cancer development. A group of farm animal viruses have already been defined to cause cancer of the alimentary tract (bovine papilloma viruses) (95) or upper aerodigestive tract (bovine nasal adenocarcinoma) (96). Although 58% of human pathogens are zoonotic (97), it is not clear whether these viruses are transmissible to human or within human population and whether they could cause cancer in human.

2.2.6 Other risk factors

Among environmental factors, possible risky habits include drinking or eating hot (hot tea) (98), Maté (99), and pickled vegetables (100). Recognized nutritional deficiencies consisted of low intake of fresh fruit and vegetables (19, 89), selenium and zinc deficiency (101, 102). High content of dietary haeme has been suggested as another dietary factor associated with a higher risk of ESCC (103). Known medical condition associated with ESCC risk are achalasia (104), pernicious anemia (46) and probably celiac disease (105). Among infectious agents, human papilloma virus (106) has been suggested as a risk factor however evidences are inconclusive yet (106-109). Poor socioeconomic status is consistently associated with a higher risk of esophageal cancer (110).

3 AIMS

- To study the associations between opium and tobacco consumption and risk of ESCC.

- To study the association between serologic gastric fundal atrophy and risk of ESCC.

- To study the pattern of gastric mucosa-associated microbiota in subjects with ESCC, esophageal squamous dysplasia and mid-esophagitis compared with gastric microbiota in subjects with normal esophagus.

- To study the association between farm animal contact and risk of ESCC.

4 SUBJECTS AND METHODS

4.1 SUBJECTS (STUDIES I, II, IV)

The study area included eastern part of Golestan province. Golestan province is located in eastern Caspian littoral. To ascertain incident cases, a specialized clinic (Atrak) for performing research and diagnosis of upper gastrointestinal tract cancers was established in the largest city of catchment area, Gonbad. Atrak was the only specialized clinic for upper gastrointestinal cancer at the time of study. Efforts were made to inform public health system workers and physicians about the research and asked them to refer their new patients suspected of having upper gastrointestinal tract cancers to Atrak clinic.

After a year of pilot study, from December 2003 to June 2007 cases were recruited. Including criteria were: giving consent to participate in the study, presence of histopathologic evidence for   ESCC, being 18 years old and more, residing in the study area at the time of registration, and having no history of cancer. All the patients underwent upper gastrointestinal endoscopy. Biopsy samples were examined by experienced pathologists at Tehran University Digestive Disease Research Center (DDRC).

For each case subject, we attempted to select two control subjects matched to the case for neighborhood of residence or village, age (± 2 years), and sex. Though, for approximately 10% of cases, only one matched control could be found. In both rural and urban areas, the annually- updated family health census was used to identify eligible controls. In each village, two controls were randomly selected from the list of eligible controls to interview. If potential control could not be interviewed, the next person on the list was invited, and so forth. In the cities, a list of the eligible controls was ordered by nearness of the addresses to the case's residence, with the list starting from the person living closest to the case's residence.

4.2 SUBJECTS (STUDY III)

For all subjects who were referred to Atrak clinic upper gastrointestinal endoscopy was proposed. At least 9 biopsies from the normal-looking esophagus and stomach were taken. Esophageal chromoendoscopy with 2% Lugol solution was performed and biopsies were taken from unstained mucosa. Unstained lesions were further examined for the presence of squamous dysplasia.

For this study three sets of subjects were considered: early ESCC, esophageal squamous dysplasia, and mid-esophageal esophagitis. Those with squamous dysplasia and no evidence of cancer were included as squamous dysplasia subjects. Histopatholgically confirmed esophagitis in a biopsy sample from the mid-esophagus and without severe dysplasia or neoplasia were considered as subjects with mid-esophagitis. Cancer cases included histopathologically confirmed ESCC with early stage (I, II). Healthy esophagus group consisted of endoscopy room patients who were referred to Atrak clinic with normal esophagus in all biopsies and without any malignancies.

4.3 DATA COLLECTION

4.3.1 Questionnaire data

Trained team of interviewers including a nurse, a physician, and a nutritionist administered structured general questionnaire and validated food frequency questionnaire (111). During the pilot phase from March 2002 to November 2003, interviewers were trained and questionnaires were tested for reliability and validity. To diminish inter-individual variation, a limited number of staff conducted the interviews. Interviews were conducted face-to-face interviews for cases and controls. No proxies were used for interview, though nutrition questionnaire was responded by a person who usually cooked at the home. Interview of case subjects was planned on the day of diagnostic upper gastrointestinal endoscopy at Atrak clinic. For each control individual, interview was done in the health houses or centers. Selected controls from Gonbad city were invited to a research facility established by DDRC for enrollment and follow up of a cohort study on upper gastrointestinal tract cancers and were interviewed (22).

The general questionnaire covered questions on demographic variables, socioeconomic status, place of residence and its change, medical history and anthropometric measurements. For study I, we extracted data from questions on life-long history of tobacco use (cigarettes, hookah, and nass). A detailed history was obtained including starting and stopping ages. Any change in amount and type of tobacco use was recorded. Ever users were those who had smoked, used, or chewed cigarettes, hookah, or nass at least weekly for a period of 6 months. Ever-tobacco-users were individuals who had done any of the above.

The general questionnaire included questions on opium use. In Golestan province opium products included crude opium, refined opium extract, opium dross, and heroin. Opium users were defined as subjects who had consumed opium at least once per week for a minimum of 6 months. Users were then asked about type(s) of opiate used, the route of administration, the age they started and stopped using, and the amount they consumed. Any change in amount or type was recorded.

Based on a cohort study and questionnaire data, alcohol consumption in the study area was uncommon (11). Subjects were asked about alcohol use and the amount and frequency of use as well.

Trained physicians examined each patient's oral cavity and teeth. Number of decayed, missing, and filled teeth (DMFT score) in addition to frequency of tooth brushing were recorded (80).

Additionally, data on life-long history of exposure to four groups of domestic animals, equines, ruminants, canines and poultry was recorded during interview. A detailed history was obtained including starting and stopping ages and level of contact. Also any change in type of animal or level of contact was recorded. Level of contact was graded to 4 levels. Occasional contact with animals that were kept within 200 meters of residence or work place was graded as level one. Second level was defined as less than daily and once every 2 weeks contact with animals kept in the neighborhood or workplace. Daily contact with animals kept in residence place or neighborhood considered third level. Fourth level was graded for those who were responsible for jobs including feeding, cleaning or slaughtering animals on daily basis.

The general questionnaire also collected detailed information on potential confounders of interest.

4.3.2 Serology data

Prior to endoscopy, 12 ml venous blood sample from each case subject was collected and its serum was stored serum at -80°C. Collected blood samples from matched controls were transferred on ice in a cooler box (~ 4°C). The time between collection and processing of neighborhood control samples was less than 12 hours. For those controls who were interviewed at the Golestan Cohort Center, sample processing and storage were performed immediately.

Serum pepsinogen I and II were measured using enzyme linked immunosorbent assays (ELISA) (Biohit, Finland). The conduction of serology was blinded toward case control status. Whereby matched sets (generally one case and 2 matched controls) were always placed on the same plate for ELISA. Aliquots from a pool of serum samples of healthy individuals were distributed among assay plates (2 repeated samples per plate). *H. pylori* serology was evaluated qualitatively with Western Blot assay (Helico Blot 2.1, Singapore). *H. pylori* infection was considered positive if (1) both 19.5 and 30 kDa bands were present or (2) any of the 35, 37, or 89 kDa band was present. CagA was positive if 116 kDa band was present. (Figure 2)

Figure 2. CagA band categorization in the immunoblot test. The first band from right is CagA. Three strips from above to down includes: positive CagA band, faint band, and negative CagA band.

We used validated cut-off points for diagnosis atrophy in the study population. The optimal cutoff value for serologic diagnosis of gastric fundal atrophy was PGI<55 µg/l with sensitivity and specificity of 61.9% and 94.8%, respectively. At cutoff concentration of 11.8 µg/l, PGII demonstrated 84.2% sensitivity and 45.4% specificity to distinguish non-atrophic pangastritis (45).

4.3.3 16S rRNA sequence data

DNA was extracted from equal length of frozen gastric tissue (5-6 mm) using DNeasy Blood & Tissue Kit (Qiagen, Inc., Valencia, CA). UV lamp exposure (for an hour with 15-20 cm distance from source) and centrifuge filtering through Amicon membrane (Millipore Co., Billerica, MA) were the method of decontamination. Mechanical and chemical lysis methods were applied to facilitate human cell and bacterial cell wall breakage. Tissues were treated with filtered-lysozyme in lysis buffer (Tris-EDTA-Triton) and overnight incubation at 56 °C in buffer AL (Qiagen, Inc., Germany) and proteinase K. Tissues were blended 100 µg glass beads (Tactum Lab, Sweden) with 0.1, 0.5 and 1 mm diameter for 1 minutes in Bullet Belender (BBX24, Next Advance, Inc., NY). Mixture was incubated with RNase (Qiagen, Inc., Valencia, CA). Tubes containing only beads, lysozyme, lysis buffer, and extraction kit substances were included as negative controls.

Small subunit of ribosomal RNA (16S rRNA) gene was amplified from extracted DNA by using a primer targeting region V3-V4. Forward primer (Bekt_341F: 5'-CCTACGGGNGGCWGCAG) and reverse primer (Bekt_805R: 5'-GACTACHVGGGTATCTAATCC) carried 454-adaptor sequences A and B (112). Unique 7 nt barcode for each sample was included in reverse primer in a way that barcodes differ in 2 nucleotides. Polymerase chain reaction (PCR) included a mix containing 10ul 5X PCR buffer HF, 0.65 µl Phusion high fidelity DNA polymerase (New England Biolab Inc., MA), 1 µl PurePeak deoxyribonucleoside triphosphates (200 µM, ThermoScientific, Milwaukee), and 2.5 µl of each primer (10 µl, MWG eurofins, Germany). 1–2 µl of template DNA was added to this mix with total volume of 50 µl. GeneAmp PCR system 9700 cycler (Applied Biosystems, CA) was used with cycling parameters of initiation (95°C for 5 min), 30 cycles of denaturation (95°C for 40 s), annealing (58°C for 40 s), and elongation (72°C for 1 min) with a final extension at 72°C for 7 min. Each sample was put in triplet with one negative control. Amplified products for each sample were pooled and verified by gel electrophoresis using 1% Agarose in TBE buffer. PCR products were purified by Agencourt AMPure XP magnetic beads (Beckman Coulter, Inc.,) with size selection through modifying PEG concentration to obtain 400-500 nt template. DNA concentration in each sample was measured using Qubit 2.0 fleurometer (Invitrogen Inc., UK). An amplicon pool was formed by pooling equimolar amounts of all DNA libraries for a minimum of 20 ng/dl. Pool amplicon was subsequently sent to SciLifeLab (Stockholm), where pooled DNA amplified in PCR-mixture-in-oil emulsions and sequenced on whole PicoTiterPlate (PTP) on GS-FLX Titanium XLR-70 system (Roche Diagnostics Co., Sweden) . Plate was divided into 2 lanes and duplicate samples were run on both lanes.

4.4 DATA ANALYSIS

4.4.1 Studies I, II, IV

For each of the exposures studied, average intensity, total duration, and cumulative use (average intensity multiplied by duration of use) were calculated. For study I, median amount in the control subjects was considered as the threshold to categorize tobacco and opium users as none, low use and high use. Age of first use was also categorized in a similar fashion.

For study IV, total duration, average level of contact and intensity for each animal group (equines, ruminants, dogs, and poultry) were categorized into quartiles based on cut point levels among controls. Because exposure to more than one animal type was common, duration of contact was divided by the number of animal types.

Conditional logistic regression models were used to calculate unadjusted and adjusted odds ratios (ORs) and 95% CIs. By design, case and control subjects were matched for age, sex, and place of residence. For study I, results were adjusted for education (as a marker of socioeconomic status) and ethnicity (Turkmen versus non-Turkmen). Additionally ORs (95% CIs) for different types of tobacco and opium use adjusted for each other, and for education, ethnicity, and total intake of fruit and vegetables was calculated.

For study IV, results were adjusted for education (as a marker for socioeconomic status), ethnicity (Turkmen versus non-Turkmen), opium and tobacco use (four categories), fruit and vegetable use, alcohol consumption, source of drinking water (piped-water versus otherwise) and use of animal waste as fuel.

All statistical analyses were done using Stata Software (StataCorp, College Station, TX).

In study II, PGI and PGII were analyzed as dichotomous variables using validated cutoff points. There was no *a priori* DMFT cutoff value. We used median DMFT among controls as a cutoff value for dichotomizing. Interaction on additive scale between dichotomous variables of gastric atrophy and poor dental health or oral hygiene habit was evaluated and the relative excess risk due to interaction, attributable proportion and synergy index were calculated.

4.4.2 Study III

4.4.2.1 *Sequence data processing*

A pipeline developed by BILS (Bioinformatics Infrastructure for Life Sciences) on Uppmax (Uppsala Multidisciplinary Center for Advanced Computational Science) computational platform was used for computations. Standard flowgram files (*.sff) from each lane were split to individual samples and then samples with equal barcodes were joined. To filter noisy reads from sequences, AmpliconNoise v1.25 with its default filtering setting (PCR noise precision= 30, pyro noise precision= 0.6) was used. Chimeric sequences were removed applying Perseus based on comparing abundance of each chimeric sequence with its parents (113). All noise-free individual sequences were combined and clustered by applying complete linkage clustering algorithm in FCluster. Newly-shaped unique cluster of sequences were remapped to operational taxonomic unit (OTU) at similarity level of 93, 95, and 97% using nearest neighbor algorithm. At this stage those samples with lower than 1000 sequences were removed before subsampling of OTU matrices. Taxonomy hint(s) of each OTU cluster was identified using lowest common ancestor algorithm implemented in SINA aligner(114). Aligner was run against SILVA 111 reference database (115) through importing to ARB (116).

4.4.2.2 *Statistical analysis*

Estimate of microbial diversity was measured and compared between groups using species richness' indices (Chao1 and Shannon). Squamous dysplasia and early esophageal cancer were treated as one disease group to improve the power of analysis. Assigned OTUs were compared individually between study groups using conditional logistic regression, and false discovery rate (FDR) control was used to correct for multiple comparisons. A minimum of eight discordant pairs was requested to include any OTU in the conditional logistic regression model. Beta diversity was estimated using Unifrac and weighted Unifrac distance based on abundance of OTUs. Community structure based on Unifrac distance was utilized to generate hierarchical dendograms to cluster individual samples. Principal coordinate analysis (PCoA) was applied to ordinate dissimilarity matrices. Conditional logistic regression model was used to compare first 10 coordinates between groups of study subjects. Akaike information criterion (AIC) was used to select a best-fit model. Calculations and statistical analysis were performed using R (www.r-project.org) and phyloseq package (117, 118).

4.5 VALIDATION STUDY

Definitive gastric atrophy diagnosis through gastro-endoscopy and histology examination requires invasive clinical intervention. Yet, histology examination of tissue biopsies may be subject to error due to patchy nature of atrophy and the limitation in the number of biopsies (119). There was no previously validated threshold for serologic diagnosis of gastric fundal atrophy in our study population. To study gastric atrophy association with ESCC, a valid criterion for assessment of exposure was needed to minimize misclassification of atrophy.

We performed a study to evaluate the validity of serologic diagnosis of fundic atrophy by examining the levels of PGI, PGII, PGI/PGII ratio versus histology among patients who were visited endoscopy clinics in the eastern Golestan province (45).

All dyspeptic patients older than 50 years old visiting consecutively the endoscopy clinics located in Gonbad city between April 2007 and August 2008 were enrolled in the study. Patients with a history of malignancies were excluded. Before performing endoscopy and after an overnight fast, five ml blood was taken and serum aliquots were kept at -80°C. Five biopsy specimens were taken from the greater curve and posterior wall in mid-antrum and mid-corpus plus *incisura angularis* and were submitted to the Digestive Disease Research Center laboratory for histologic examination using updated Sydney System criteria (120).

Inflammation, intestinal metaplasia, and atrophy were assessed and graded as mild, moderate or marked. We grouped combined moderate or marked atrophy as atrophic group and mild or no atrophy was grouped as non-atrophic. We classified marked or moderate gastritis as pangastritis when biopsies from corpus and antrum or corpus and incisura angularis were diagnosed with non-atrophic gastritis. Blind to histology results we measured serum PGI and PGII by using ELISA (Biohit, Finland) at the Swedish Institute for Infectious Disease Control (SMI).

 "Receiver operating characteristic (ROC) curves were constructed using different combinations of sensitivity and specificity, and the areas under the curves (AUCs) and their 95% CIs were calculated. Youden index was calculated to choose the optimal cutoff value that confirmed the diagnosis of fundic atrophy with a FPF ≤ 20% - 30%."(45)

Among 309 enrolled subjects, the best cutoff values for serologic diagnosis of fundic atrophy were PGI < 55 μg/l and PGI/PGII ratio < 5. Due to the high prevalence of pangastritis which might affect pathology gold standard, we restricted study samples to those without serologic pangastritis (PGII < 11.8 μg/l). The sensitivity of using PGI < 55 μg/l to define fundic atrophy improved to 83.3% (95% CI: 51.6 - 97.9) among those with PGII < 11.8 μg/l (Table 1).

4.6 SENSITIVITY ANALYSIS

In study II, we observed an elevated risk of ESCC associated with pepsinogen II. From validation study we learned that presence of pangastritis could decrease the diagnostic accuracy of PGI. We linked this association to the possibility of gastritis secondary to ESCC. Gastric biopsy for majority of neighborhood controls was not available as less than 10% of them visited our clinic afterwards. The best that we could do was to compare gastritis in the histology of ESCC cases to endoscopy room controls and observed a 2-fold elevated risk of ESCC among those with pangastritis. From validation study we learned that presence of pangastritis could decrease the diagnostic

accuracy of PGI for atrophy detection and possibly cause misclassification. To quantify the magnitude of such bias, we estimated the classification probabilities using external validation study (45). Effects of the range of PGII levels (as a marker of pangastritis) on diagnostic accuracy of PGI and ORs for the association with ESCC were tested. We found that when proportion of cases and controls with pangastritis differs, the estimate would be exaggerated if mostly ESCC cases have pangastritis and it would be if pangastritis would be dominant among controls.

Table 1. Screening characteristics of pepsinogens for diagnosis of fundic atrophy and non-atrophic pangastritis.

Cutoff value	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)
Fundic atrophy			
PGI < 55 µg/l	61.9 (38.4 - 81.9)	94.8 (91.6 - 97.1)	0.78 (0.68 - 0.89)
PGII > 11.8 µg/l	40.0 (19.1 - 63.9)	34.0 (28.6 - 39.8)	0.37 (0.26 - 0.48)
PGI/PGII < 5	75.0 (51.0 - 91.3)	91.0 (87.0 - 94.0)	0.83 (0.73 - 0.93)
PGI < 55 and PGI/PGII < 5	60.0 (36.1 - 80.9)	97.2 (94.6 - 98.8)	0.79 (0.68 - 0.90)
Non-atrophic pangastritis			
PGII > 11.8 µg/l	84.2 (75.6 - 90.7)	45.4 (38.5 - 52.5)	0.65 (0.60 - 0.70)

PG: pepsinogen, AUC: area under curve. Table is reproduced with modifications from a published article by the author (45)

4.7   ETHICAL CONSIDERATION

The study was approved by the Ethical Committee of the Digestive Disease Research Centre of Tehran University of Medical Sciences, Iran and the Institutional Review Board of National Cancer Institute, US and the Stockholm Regional Ethics Vetting Board, Sweden.

5 RESULTS

5.1 SUBJECTS

A total of 300 ESCC case subjects were enrolled in this study. Of these, 271 had two matched controls and 29 had one matched control (Table 2).

Table 2. Demographic characteristics of study subjects (table content was reproduced from a previously-published paper (26) by author with permission from publisher)

	ESCC cases (%)	Matched controls (%)
Total	300 (100)	571 (100)
Age (years)		
≤ 50	32 (11)	61 (11)
51 – 60	81 (27)	144 (25)
61 – 70	86 (29)	177 (31)
> 70	101 (34)	189 (33)
Gender		
Male	150 (50)	278 (49)
Female	150 (50)	293 (51)
Place of residence		
Urban	82 (27)	150 (26)
Rural	218 (73)	421 (74)
Ethnicity		
Turkmen	171 (57)	312 (55)
Non-Turkmen	129 (43)	259 (45)

ESCC: Esophageal squamous cell carcinoma

5.2 STUDY I

In total 30% of ESCC cases and 18% of the matched controls reported that had ever used opium. Opium use was less among women as 27% of men and 11% of women had ever used opium. Approximately 70% of opium users were living in rural areas.

Table 3 shows the results of variables for opium use when study subjects were limited to those who started using opium earlier than one year prior to diagnosis, and the adjusted OR (95% CI) for ever use of opium was 1.92 (1.30 - 2.84).

The habit of ever smoking cigarettes was associated with a 1.47 (95% CI = 0.98 – 2.21) elevated risk of ESCC. Grouping cigarette smokers, hookah and nass users as tobacco users resulted in an OR of 1.85 (95% CI= 1.27 – 2.68) for ESCC risk. The nearly-significance risk was resulted when adjustment for opium was done (OR=1.50, 95% CI = 0.99 -2.28). In total, 7% of ESCC cases reported ever smoking hookah and among them, those who smoked hookah more than 3 times per day showed a 3-fold elevated risk for ESCC. Habit of chewing nass was reported by 15% of ESCC cases and in a model which included hookah and cigarette use, chewing nass was associated with 3-fold increase in ESCC compared to tobacco-non users. Adjustment for opium decreased the risk to 2-fold.

In all 21 subjects, 7 cases (2%) and 14 controls (2%) reported to have ever used alcohol for six months or more. Due to the small number of exposed subjects- based on questionnaire- we did not detect any risk associated with alcohol.

When we examined any tobacco or opium use, alone or together, compared to using neither (Table 4), tobacco use alone, opium use alone, and a combination of the two were all significantly associated with higher ESCC risk.

Table 3. Opium consumption and risk of ESCC (after exclusion of those who had started tobacco or opium use a year before diagnosis)

	Unadjusted OR (95% CI)	Adjusted[1] OR (95% CI)	Adjusted[2] OR (95% CI)
Opium use			
Never	Referent	Referent	Referent
Ever	1.92 (1.30 - 2.84)	1.96 (1.32 – 2.91)	1.69 (1.08 - 2.65)
Duration			
Never used	Referent	Referent	Referent
≤ median	1.03 (0.54 - 1.97)	1.06 (0.55 - 2.03)	1.02 (0.52 - 2.00)
> median	2.44 (1.57 - 3.80)	2.52 (1.61 - 3.96)	2.17 (1.29 - 3.64)
Amount			
Never used	Referent	Referent	Referent
≤ median	1.32 (0.76 - 2.32)	1.33 (0.75 - 2.34)	1.25 (0.70 - 2.26)
> median	2.46 (1.51 - 4.00)	2.61 (1.59 - 4.30)	2.25 (1.26 - 4.01)
Intensity			
Never used	Referent	Referent	Referent
≤ median	1.36 (0.80 - 2.32)	1.35 (0.79 - 2.31)	1.24 (0.70 - 2.20)
> median	2.56 (1.54 - 4.26)	2.74 (1.63 - 4.61)	2.38 (1.31 - 4.33)
Start age			
Never used	Referent	Referent	Referent
≤ median	2.60 (1.68 - 4.04)	2.73 (1.74 - 4.28)	2.43 (1.45 - 4.08)
> median	0.74 (0.35 - 1.54)	0.74 (0.35 - 1.56)	0.70 (0.32 - 1.51)

[1]Adjusted for education and ethnicity.

[2]Adjusted for education, ethnicity, total intake of fruit and vegetables, alcohol consumption, and tobacco use.

ESCC: Esophageal squamous cell carcinoma.

Table 4. Opium and tobacco consumption and risk of ESCC (we excluded those who had started tobacco or opium use a year before diagnosis)

	Unadjusted OR (95%CI)	Adjusted[1] OR (95% CI)
Opium and tobacco		
Used neither tobacco nor opium	Referent	Referent
Used tobacco but not opium	1.67 (1.03 - 2.68)	1.67 (1.03 - 2.71)
Used opium but not tobacco	2.12 (1.11 - 4.04)	2.02 (1.05 - 3.88)
Used both tobacco and opium	2.17 (1.36 - 3.47)	2.31 (1.43 - 3.72)

[1]Adjusted for education, ethnicity, total intake of fruit and vegetables, and alcohol consumption.

ESCC: Esophageal squamous cell carcinoma.

5.3 STUDY II

Study subjects were the same as study I. Serum samples were available for 293 (98%) cases and 524 (92%) controls. For those cases with available serum samples, at least one matched control existed. Demographic characteristics of the study participants and potential confounders are summarized in the Table 5. No substantial differences were observed between those who provided serum and those whose serum samples were not available.

Table 5. Characteristics of ESCC cases and matched controls with available serum samples**. (Table content was reproduced after modification from a previously-published paper(121) by the author with permission from publisher)

Characteristics	Controls (n=524)	ESCC cases (n=293)	Controls without serum samples (n=47)	p value[1]
Mean age (SD), years	65.5 (10.4)	64.4 (11.1)	62.1 (9.9)	0.07
Sex (%)				
Men	256 (48.8)	147 (50.2)	22 (46.8)	0.8
Women	268 (51.2)	146 (49.8)	25 (53.2)	
Fruit and vegetable consumption (%)				
≤ median	235 (44.8)	139 (47.4)	20 (42.5)	0.76
> median	289 (55.1)	154 (52.6)	27 (57.5)	
DMFT (%)[2]				
≤ median (28)	266 (51.0)	117 (40.3)	28 (59.6)	0.25
> median	256 (49.0)	173 (59.7)	19 (40.4)	
Tooth brushing habit				
Ever	221 (42.4)	59 (20.3)	18 (39.1)	0.66
Never	300 (57.6)	232 (79.7)	28 (60.9)	

DMFT: Sum of decayed, missing, and filled teeth. [1]p value for significance of difference between controls with and without serum samples. [2]Numbers of cases and controls were less than total because of missing data.

Using validated cutoff for serologic diagnosis of gastric atrophy showed that PGI < 55µg/l was associated with a 1.39-fold non-significant elevated risk of ESCC (95% CI: 0.93 - 2.09). Other published cutoff values i.e. PGI < 30 µg/l, PGI/PGII < 3, PGI <70 µg/l and PGI /PGII ratio < 3 did not change the point-estimate materially. Whereby, combination of PGI/PGII ratio <5 and PGI< 55 µg/l, was associated with a significant 1.62-fold increased risk of ESCC (95% CI = 1.01 − 2.65). Excluding those who reported ever use of proton pump inhibitors did not change the estimates. Based on our validation study results, diagnostic ability of PGI alone was as god as its combination with ratio.

Furthermore we observed a group of ESCC cases and controls (13 cases and 7 controls) with extremely high level of PGI (>250 µg/l) or PGII (> 60 µg/l). This group rendered an OR of 3.08 (95% CI = 1.20 – 7.90) for ESCC risk. The best we could conclude of the presence of this group was the possibility of a high inflammation (gastritis) in our study subjects.

We stratified two groups of fundal atrophy (defined by PGI < 55 µg/l) by level of PGII. As we reported earlier, PGII > 11.8 µg/l was a marker of severe pangastritis in the study population. We did not detect any elevated ESCC risk associated with gastric atrophy among those with pangastritis. We combined marker of severe non-atrophic pangastritis with PGI and evaluated association between gastric fundal atrophy with ESCC because we assumed that gastritis might be caused secondary to ESCC. Combination of PGI < 55µg/l and PGII < 11.8µg/l was associated with a 2-fold increased risk for ESCC (Table 6).

Table 6. Serologic gastric fundal atrophy association with ESCC.

	Controls (%)	ESCC cases (%)	Adjusted [1]OR (95% CI)
Serologic fundal atrophy and non-atrophic pangastritis			
PGI ≥ 55 & PGII[2] < 11.8	189 (36.1)	71 (24.2)	Referent
PGI < 55 & PGII < 11.8	57 (10.9)	42 (14.3)	2.01 (1.18 - 3.45)
PGI ≥ 55 & PGII ≥ 11.8	260 (49.6)	167 (57)	Referent
PGI < 55 & PGII ≥ 11.8	18 (3.4)	12 (4.4)	1.20 (0.54 - 2.66)

[1]Adjusted for education, ethnicity, fruit/vegetable use, and tobacco/opium consumption.
[2]For one case PGII was missing.

H. pylori infection serology, measured as antibodies against some *H. pylori* antigens implemented in immunoblot kit was not associated with ESCC risk. In total CagA band was detectable (including faint bands) in 90% of controls and 88% of ESCC cases. We were unable to detect an association between *H. pylori* and ESCC in our study samples by using this particular immunoblot kit (Table 7).

Table 7. Adjusted[1] OR (95%CI) for association of H. pylori and ESCC risk.

	Reference: *H. pylori*-	*H. pylori*+CagA-	*H. pylori*+CagA+
H. pylori status[2]			
H. pylori-	1	1.3 (0.7-2.4)	0.7 (0.4-1.2)
H. pylori+ CagA-	0.7 (0.40-1.37)	1	0.5 (0.3-1.1)
H. pylori+ CagA+	1.3 (0.82-2.12)	1.8 (0.9-3.5)	1

[1] Adjusted for ethnicity (non-Turkmen or Turkmen), alcohol consumption, tobacco or opium use, education level, fruit and vegetable use.
[2]*H. pylori* was considered positive if (1) both 19.5 and 30 KD were present or (2) any of the 35, 37, or 89 KD band was present. CagA was positive if 116 KD band was accompanied by any of the above- mentioned bands.

To test for possible biologic synergism between poor oral health and gastric fundal atrophy, we tested the additive interaction. Due to low power, we broke the matching frame and added the matching factors (age, gender, rural or urban residence) to an unconditional logistic regression model. Adjusted OR for association of DMFT with ESCC was 1.60 (95% CI: 1.14 - 2.24) (Table 8).
Based on small number of cases, presence of poor dental hygiene, indicated as higher than median DMFT, and gastric atrophy rendered a more than 4-fold excess risk of ESCC compared with the reference group. The relative excess risk due to interaction

between low poor dental hygiene and fundal atrophy was 1.47 (95% CI: -1.15 - 4.1), synergy index (95% CI) was 1.90 (0.6 - 6.1), and the attributable proportion (95% CI) was 0.36 (-0.14 - 0.85) (Figure 3).

To test if the finding was dependent on our particular criteria for dental hygiene, we used frequency of tooth brushing as another measure of oral hygiene. Frequency of tooth brushing in combination with fundal atrophy resulted in an 8-fold increase in ESCC risk (OR = 8.65, 95% CI: 3.65 - 20.46).

Table 8. OR (95% CI) for the association between DMFT and ESCC comparing different choices of categories of DMFT

	OR (95% CI)	Adjusted[1] OR (95%CI)
DMFT number		
0-10	Referent	Referent
11-20	1.68 (0.78 - 3.58)	1.89 (0.86 - 4.20)
21-30	2.14 (1.03 - 4.44)	2.07 (0.96 - 4.49)
31-32	3.30 (1.58 - 6.90)	3.16 (1.45 - 6.92)
DMFT median		
< median (28)	Referent	Referent
≥ median	1.77 (1.27 - 2.46)	1.60 (1.14 - 2.24)
DMFT Centiles		
1st (≤ 18)	Referent	Referent
2nd (19-25)	1.73 (1.03 - 2.91)	1.63 (0.95 - 2.79)
3rd (26-31)	2.26 (1.37 - 3.74)	1.90 (1.12 - 3.22)
4th (32)	2.49 (1.54 - 4.04)	2.23 (1.35 - 3.69)

[1] Adjusted for ethnicity (non-Turkmen or Turkmen), alcohol consumption, tobacco or opium use, education level, fruit and vegetable use.

DMFT: Decayed, missing, filled teeth. ESCC: Esophageal squamous cell carcinoma

The relative excess risk due to interaction between habit of never brushing teeth and atrophy was 4.34 (95% CI: -1.07 - 9.76), synergy index 2.24 (95% CI: 0.97 - 5.15), and attributable proportion 0.49 (95% CI: 0.14 - 0.83). Based on fewer sets, conditional logistic regression model showed similar results.

Figure 3. Joint effect of poor dental health and gastric atrophy on ESCC risk.
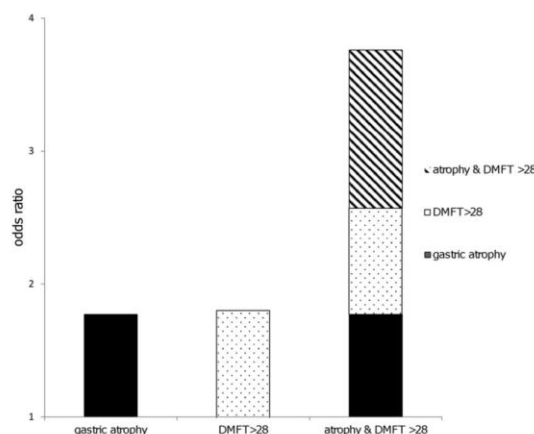
5.4 STUDY III

5.4.1 Subjects

Of 579 non-cancerous subjects who were referred to Atrak clinic, 18 with squamous dysplasia and no evidence of cancer were diagnosed. We found 19 ESCC patients in early clinical stages. For each subject with a diagnosis of dysplasia or early stage cancer, one age- and sex-matched control was randomly selected from subjects with a healthy esophagus from endoscopy clinic controls. We also tried to select subjects with a diagnosis of esophagitis in the mid esophagus as a separate control. During study period, a total of 17 age- and sex-matched esophagitis control subjects could be identified. As our multiplexing method was optimized for 95 samples, we added 2 more subjects with mid-esophagitis and their age- and sex-matched controls. Esophagitis had been considered to be extremely high (80%) in the high risk areas of Linxian and Gonbad in two reports (122, 123). Histology criteria for diagnosis of esophagitis was based on more recent publication (124) which was different from early criteria and estimated prevalence of esophagitis was around 5%. Prevalence of esophagitis and squamous dysplasia in our study sample was in accordance with reports from high risk areas for ESCC (125).

The mean age (SD) of ESCC and squamous dysplasia was 64.5 (11.8), for controls 63.4(14.6) and for mid-esophagitis was 62.1(16.3). Fifty-one percent of ESCC and squamous dysplasia subjects were men.

5.4.2 Sequence data

After combining two lanes of 454 sequencer, 369, 539 sequences with mean length of 419 nt were obtained from 93 gastric tissue samples. Average sequence per sample was 3,004. A total of 25% of sequences were removed as noise or chimera. Two samples had less than 1000 reads (504 and 710 reads) and were excluded. As a result, 36 matched pairs of esophageal cancer/dysplasia-healthy control and 17 pairs of matched mid-esophagitis-healthy control were left. After clustering unique sequences at 3%, 5%, and 7% of dissimilarity rate, 2075, 1636, and 1300 OTUs were shaped. Based on SILVA database (release 111) theses OTUs could be represented by 1283 bacterial taxa. OTUs which could not be assigned, were considered unclassified. Majority of these unclassified OTUs (80%) were singleton (n = 517) and double tone (n = 123). At the sample level, highest numbers of unclassified OTUs were observed in 2 samples with 8.5% and 2.9% of total OTUs. Mean percentage of unknown OTUs per sample after excluding these 2 subjects was 0.5%.

Figure 4. depicts gastric mucosa-associated microbiota representatives at the Phylum level in subjects with mid-esophagitis, ESCC plus squamous dysplasia and normal esophagus (126). Observed cumulative, and mean value of species richness over samples were 1308, 117.6 for the esophageal cancer or dysplasia group, 1154, 100.7 for control group, and 626, 84.5 for mid-esophagitis group respectively. Comparison of OTU richness, measured by mean Chao1 showed similar results for the esophageal cancer or dysplasia group versus control group ($p = 0.4$) and non-significantly lower for mid-esophagitis ($p = 0.06$).

OTUs were assigned to 31 phyla, 53 classes, 90 orders, 168 families, and 336 genera. Five most abundant phyla were *Firmicutes*, *Bacteroidetes Proteobacteria*,

Actinobacteria, and *Fusobacteria*. Phyla composition was consistent across esophageal cancer or dysplasia, control and mid-esophagitis groups.

After ordination of dissimilarity measurements based on Unifrac and weighted-Unifrac distance using PCoA, conditional logistic regression model was applied to compare study groups. A significant difference in Unifrac distance between ESCC or squamous dysplasia group and controls in the three coordinates was observed ($p=0.003$). First coordinate based on weighted-Unifrac dissimilarity index, explained 60% of variance and differs significantly two groups ($p =0.018$). Similar results were obtained when matched analysis was based on multidimensional scaling ordination using Unifrac ($p=0.005$), and weighted-Unifrac ($p=0.016$).

After excluding early cancer patients and restricting conditional logistic regression analysis to squamous dysplasia, results did not change substantially. Results on comparing dissimilarity distances between mid-esophagitis and ESCC or squamous dysplasia subjects were not suggestive of significant difference (PCoA ordination on weighted Unifrac distance: $p=0.463$)

Figure 4. Gastric mucosa-associated microbiota composition at Phylum level with probable phylogenetic match among a pool of subjects with mid-esophagitis, esophageal squamous cell carcinoma and dysplasia, and with normal esophagus.
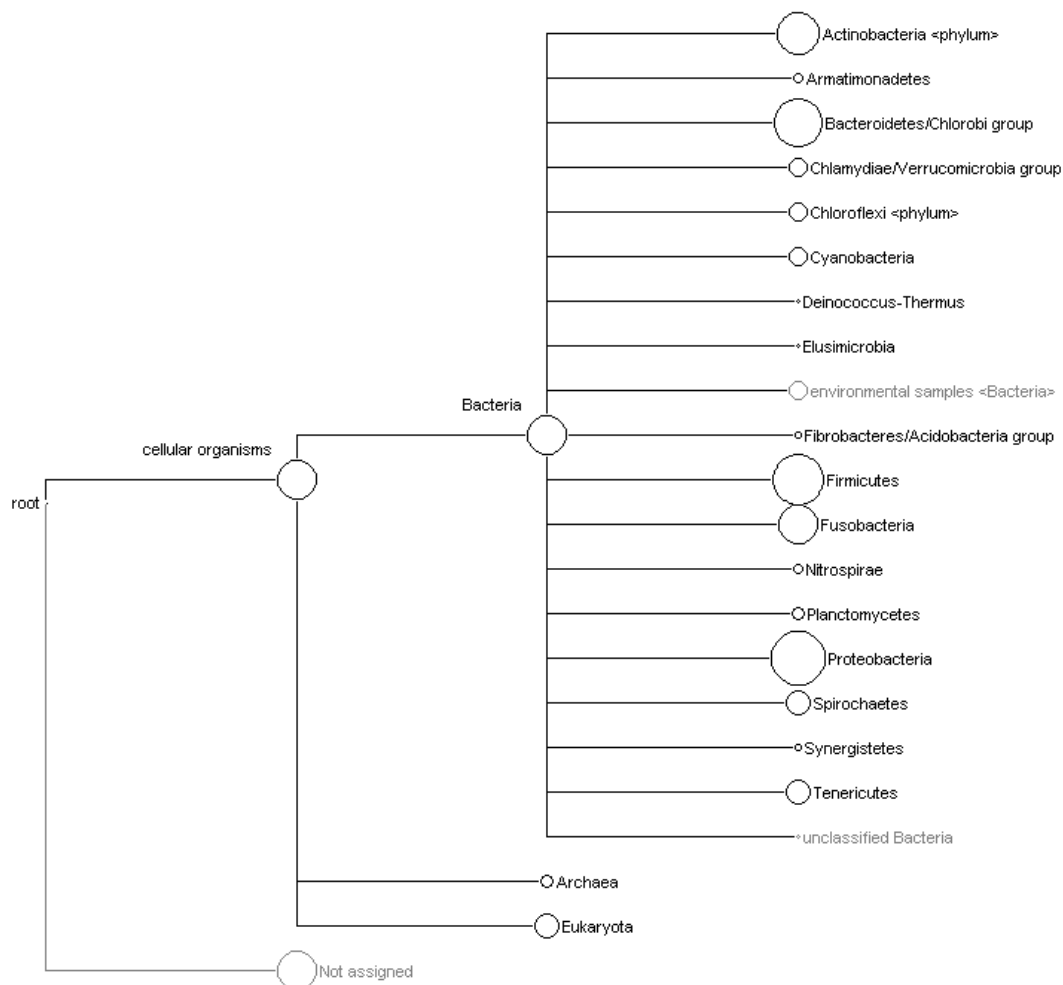
5.5 STUDY IV

The study included 300 incident case patients and 571 matched control subjects from whom animal contact history was available for 287 (96%) cases and 542 (95%) controls. Of the 42 subjects (13 cases, 28 controls) with missing history of animal contact 18 case-control pairs were missing due to lack of information on cases in 13 pairs and lack of exposure data on controls in 5 pairs. For the rest of subjects with missing data, at least one control for each matched-pair existed. A total of 19 subjects (11 controls and 8 ESCC cases) reported to have contact with more than one animal type without further specification; there was no association with risk of ESCC among them (OR = 1.87, 95% CI: 0.62 - 5.69) and they were excluded when association with particular type of animal was investigated. No substantial difference was observed between subjects with available and missing history of exposure to animals considering demographics and confounders.

There was no difference between men and women in terms of contact with four types of animal. Contact with ruminants was associated with an eight fold increased risk of ESCC (95% CI: 4.40 -15.44) (Table 9). We observed a significant excess risk associated with contact with dogs among ESCC patients (OR = 1.92, 95% CI: 1.35 - 2.72). When exposure to ruminants and dogs, alone or in combination was considered, the adjusted OR (95% CI) for dog contact alone was 3.82 (1.00 - 14.63).

Exposure to different animals overlapped among participants. We compared exposure to 2, 3 and 4 different types of animals with exposure to only one type as a reference group. The observed excess risks were constant through different strata without obvious trend.

Table 9. Animal contact and risk of ESCC.

	Controls (%)	Cases (%)	Adjusted[1] OR (95% CI)
Equines			
Never	234 (44.1)	121 (43.1)	Referent
Ever	297 (55.9)	160 (56.9)	0.92 (0.66 - 1.29)
Ruminants			
Never	166 (31.3)	15 (5.3)	Referent
Ever	365 (68.7)	266 (94.7)	8.24 (4.40 - 15.44)
Domestic canines			
Never	251 (47.3)	82 (29.2)	Referent
Ever	280 (52.7)	199 (70.8)	1.92 (1.35 - 2.72)
Poultry			
Never	69 (13.0)	35 (12.5)	Referent
Ever	462 (87.0)	246 (87.5)	1.06 (0.63 - 1.78)

[1]Adjusted for potential confounders including education (as a proxy for socioeconomic status), ethnicity (Turkmen versus non-Turkmen), opium and tobacco use (four categories), fruit and vegetable use, alcohol consumption, source of drinking water (piped water versus otherwise) and use of animal waste as fuel.

6 DISCUSSION

6.1 METHODOLOGICAL CONSIDERATION (design, bias, confounding)

Esophageal cancer is a rare disease and case-control design is an efficient way to study several exposures in a limited period of time for this cancer. Due to sharp geographic boundary of high incident area for esophageal cancer in northern Iran, source population was defined as those who were inhabitants of the eastern Golestan province. Cancer registry data showed that approximately 70% of incident cases in source population were enrolled in this study. For control selection, random sampling from source population was not feasible at the time of study and risk of cancer within catchment area varied by geographical regions (3). One convenient way was to sample controls who were individually matched to cases from the same neighborhood. Although not all incident cases in the source population were enrolled, neighborhood controls still constituted a useful control series (127). As physicians in the study catchment area were asked to refer their patients suspected of having upper gastrointestinal tract cancers to the enrolling clinic, there is no evidence that neighborhood controls may include people who would not have visited the same clinic had they developed the disease under study. Matching might improve efficiency in some degrees (128) but also it has some disadvantages (129).

In study IV, animal contact was the exposure of interest and matching on neighborhood could potentially provide a more efficient stratified analysis (130) when confounders such as source of pasture for grazing or exposure of farm animals to pesticides and herbicides varied regionally and have numerous categories. In study III matching on age and probably sex, improved power of this study with small sample size as age and gastrointestinal microbiota are linked (131). On the other hand, the effort which had been made to find matched controls probably resulted in decreased number of controls per case, thus it might not be a cost efficient method.

In study II, processing time of blood samples from neighborhood controls was longer than cases due to the distance from study center. However efforts were made to transfer whole blood at 4°C, if whole blood storage conditions affect pepsinogen level measurement, it would bias the results. We compared pepsinogen levels in sera of controls from areas close to processing center and areas located in long distance and did not observe significant difference.

Biologic specimen in study II and III were collected at the time of cancer diagnosis. In study III, the observed microbiota pattern in stomach might be secondary to changes in esophageal cancer, although we observed similar pattern in stomach of those with esophageal squamous dysplasia. In study II, majority of subjects were diagnosed at advanced stages of ESCC, though induction period for esophageal cancer is probably shorter than that for gastric atrophy development, reverse causality is unlikely.

In study I, people in Golestan may start using opiates to alleviate pain prior to their cancer diagnosis and therefore reverse causality is a concern. However, excluding the subjects who had recently (one year before diagnosis or enrollment) started using opium from the analysis made no material difference in the study results, and younger age at first use was a strong predictor of cancer risk. Selection bias due to non-participation of controls is ignorable as 77% of the enrolled controls were the first randomly selected individuals and 11 and 3% were the second and third choices (110).

Although diagnosis of esophageal cancer is not difficult and it is estimated that 90% of tumors were of squamous origin in the study area, 100% of the cases in this study had pathological confirmation of ESCC and misclassification of outcome is unlikely.

The possibility of misclassification of exposure in study I was declined by performing validation study (132) which showed high validity and reliability of questionnaire to assess opium consumption. In study II, laboratory experiment was designed in a blind manner to the case/control status and matched sets were analyzed in the same plate. However misclassification of *H. pylori* status would be of concern due to 3 main factors: (1) the immunoblot kit was not validated for the population under study, (2) the immune response in cancer patients to *H. pylori* antigens might have changed due to change in nutrition and malignancy status, (3) precise history of *H. pylori* eradication was not available from study subjects.

Low sensitivity for atrophy detection might lead to loss of precision and power by unnecessary reducing the exposed sample size. However, we chose atrophy cutoff point at high specificity level (97%), false negative probability of 0.4 in addition to low prevalence of gastric atrophy in the population could potentially result in misclassification of exposure among cases and controls which we believe if evolves it would be differential. False negative results are inherent in serologic diagnosis of atrophy due to the low sensitivity of the diagnostic method. It is believed that resulted misclassification will drive the association toward null if non-differentiality assumption is met. However atrophy is an uncommon exposure and its binary value is the result of categorizing continuous PGI variable. Our results of sensitivity analysis confirmed that there is an effect of misclassification due to ignoring non-atrophic pangastritis on association between atrophy and ESCC. We used PGII as a marker of severe inflammation as stratifying factor to decrease this effect.

In study III, contamination of biopsy samples might be of concern, however we made all efforts to avoid it by using Ultraviolet light and filtering the solutions. Sequencing errors and PCR chimera formation were corrected by using noise removal pipelines. Although microbial community assessment based on 16S rRNA gene sequencing is useful in bacterial classification, it has low phylogenetic power at the species level (133). Furthermore, V4 and V5 are less specific for species level (134) and we avoided assigning genus or species nomenclature to the OTUs. Although 16S is a gold standard of bacterial phylogeny, presence of its multiple copies in some bacteria with slight difference in sequence could lead to identifying multiple types of the same bacterium (135, 136) which might have resulted in misclassification of exposure. In our data unassigned OTUs were unevenly distributed among samples, as five samples shared most of those. These rare species could be formed due to intrinsic errors of pyrosequencing (137).

Recall bias, which is common in exposure assessment based on questionnaire, is a source of concern in study I and IV. Neither interviewers nor study subjects were aware of study hypothesis about animal contact and opium consumption as potential risk factors.

Detailed information on potential confounders was collected during this study which let us to adjust for several factors.

In study II, our statistical inference on presence of interaction between poor oral hygiene and gastric atrophy had low precision due to the modest sample size. In study III, accuracy of our estimate for alpha diversity is questionable due to number of denoised sequences (138). Although our studies were among the largest up to date, the

sample size in study III is small. The cost of increasing sample size in 454 sequencing platform would result in lower depth of sequencing. Newer version of next generation sequencing platforms will provide deeper reads with higher number of samples.

6.2 INTERPRETATION OF FINDINGS

6.2.1 Opium consumption and risk of esophageal squamous cell carcinoma

Opium was common enough in study area at the time that most of our study subjects had started consuming opium (about 40 years ago). Long term opium use at low doses was the common feature of its consumption among this population. Opium consumption is associated with a stigma among populations and this might limit the validity of questionnaire responses. For reliability of the questionnaire, a subset of rural and urban residents in the catchment area were interviewed twice about ever use of opium. An agreement between responses to opium questions in our questionnaire for ever use of opium was 0.99 and the corresponding kappa statistic was 0.96 (11).

We tested the validity of self-reported current use of opium by measuring codeine or morphine in the urine. It was found that self-report of current opium use had a sensitivity of 0.93 and specificity of 0.89 (132). However we were not able to test the validity of questionnaire on duration or amount of opium use, we decided to use questionnaires to measure opium use as the exposure. The percentage of opium users among control subjects found in this study is close to the percentage found in the 50,000 subjects enrolled in a cohort study among the same source population (11, 22). The results of our study showed a 2-fold increased risk of ESCC associated with opium use. Similar odds ratio was obtained when we restricted the analysis to tobacco non-users.

The results of this study showed that consumption of both crude and refined opium are associated with increased risk of ESCC. Both routes of administration, smoking and ingesting, were associated with an elevated risk of ESCC. Whereby inhaling opium smoke might be linked to the PAH exposure, similar risk of ESCC for ingesting opium does not fully consistent with this hypothesis. A recent study in the same population has reported that opium might have a role in gastric cancer carcinogenesis as it was associated with more than 3-fold increase in gastric adenocarcinoma risk with a dose-response relationship (139) which is even higher than the risk we observed for ESCC. Esophageal cancer and gastric cancer are the two most common malignancies in the study area and observing an association with opium is plausible. While decline in opium use during recent decades is in line with decline in ESCC incidence, the prevalence of gastric cancer is increasing in the study area.

Limited number of animal studies on carcinogenic effect of opium did not show persistent results. A case-control study with hospital controls in the same area did not detect association between opium and ESCC risk (140). A prospective study on cause of death among 50,000 individual recruited in a cohort study in Golestan province, did not show a significant hazard ratio for ESCC mortality among men who used opium. Although, the hazard for ESCC mortality was more than 2-fold among women who ever used opium (141) . One may raise this possibility that opium per se might not be a carcinogenic agent for ESCC; rather the reason that users consumed opium might be the main risk factor. As in the study area opium has been mostly used as a medication

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for gastrointestinal and upper respiratory infectious diseases, it might be a proxy for lifelong history of certain infectious diseases. Opium was significantly elevated hazard of death due to infectious diseases by 9-fold (141).

Cigarette smoking is a major risk factor for ESCC in low incident areas, we found a weak to moderate association between cigarette smoking and ESCC in eastern Golestan. It is likely that smoking is not a strong risk factor for ESCC in this population because incidence of ESCC is similar among men and women in the study area but smoking prevalence among rural women is 1% and among urban men is 39%. Weak association between smoking and ESCC risk may also be due in part to the relatively low cumulative tobacco exposure among the smokers; as median cumulative use among the smoking controls in this study was 13.5 pack-years.

In our study, although the confidence intervals are wide, the point estimates suggest that smoking hookah and chewing nass are at least as strong risk factors for ESCC as cigarette smoking. The intensity and duration of nass use showed a dose-response association with ESCC risk. We did not detect a trend for duration of hookah use. This might be partly due to some people changing from hookah use to cigarettes.

6.2.2 Gastric fundal atrophy and risk of esophageal squamous cell carcinoma

Serologic gastric atrophy, as indicated by low serum pepsinogen, in the absence of severely inflamed gastric tissue was associated with an increased risk of ESCC. We used serum pepsinogens for measurement of exposure however, pepsinogens are markers of different histologic conditions including gastritis and atrophy (142). Atrophy and inflammation are often mixed and both present in the same stomach. When inflammation burns out, atrophy detection would be more precise. Additionally gastritis could be of distinct etiologies in cancer patients. PGII has been suggested as a marker for all types of gastritis (143, 144). We observed an association between serologic non-atrophic pangastritis (measured by PGII) with ESCC risk. This finding raised the possibility of developing gastritis secondary to esophageal cancer. This could be happened as;

- Cancer patients may develop gastritis due to non-steroidal anti-inflammatory drug (NSAIDs) use, opportunistic infections in the stomach (Cytomegalovirus, Epstein-Barr virus), and malnutrition (145).
- Stage of tumors in most of our study population were III or IV at the time of diagnosis and dysphagia was the presenting symptom which might led to high proportion of NSAID consumption among cases compared to controls.

The best we could conclude was that in studying the serologic relationship between gastric atrophy and ESCC when serum collection period is close to cancer diagnosis, the association might be influenced by severe inflammation in stomach. This condition will elevate secretion of PGII and influences validity of PGI/PGII ratio for detection of atrophy. Which if happens, it decreases PGI/PGII ratio and might inflate the estimate of risk.

Sever inflammation might influence the assessment of PGI accuracy as well. Histology is considered as the gold standard for gastric atrophy diagnosis, however

mucosal inflammation and separation of glands by inflammatory infiltrate would add more diagnostic difficulties in severe gastritis (119). The error probabilities for unexposed cases and controls might not be identical because inflammation among our cancer patients was more probable than healthy controls. To decrease the effect of mixed category, we stratified gastric fundal atrophy by the presence of non-atrophic pangastritis and observed an increased ESCC risk just in strata without severe inflammation.

Furthermore *H. pylori* eradication and use of proton pump inhibitors are both affect pepsinogen level. Studies have shown PGI is a more stable marker after H. pylori eradication (146, 147). We did not collect specific information on treatment for *H. pylori* and used PGI alone as a marker of fundal atrophy. Similar ability of PGI/PGII ratio and PGI has been previously reported (148) but majority of the studies showed superior ability of the joined use of PGI and PGI/PGII ratio (149) or the PGI/PGII ratio alone (150-152). In our study sample, combining PGI and PGI/PGII ratio rendered a risk for association between fundal atrophy and ESCC risk without further stratifying by the presence of pangastritis.

In terms of the magnitude of risk, the result based on our validated threshold (combination of PGI and PGI/PGII ratio) was not different from applying PGI alone with stratification by PGII level. Using both criteria we detected an approximately 2-fold risk for ESCC. Prevalence of serologic atrophy in our study population was similar to the one in the low risk area for ESCC (10%).

In this study, we could not detect any association between infection with CagA positive *H. pylori* and ESCC risk. Spontaneous disappearance of *H. pylori* over time or *H. pylori* eradication might influence our estimate. Immunodeficiency among advanced malnourished cancer patients could be another explanation as their immune response to antigens might be declined. These possibilities could lead to heterogeneity of our reference group as *H. pylori* negative category. It is plausible that in this aged population with high prevalence of CagA+ (approximately 90%) our serologic measurement of previous *H. pylori* infection is not conclusive.

We found suggestive evidence that the estimated joint effect of poor dental hygiene and gastric atrophy together was larger than the sum of their effects alone. This finding could be linked to the joint effect of bacterial overgrowth in both atrophic stomach and low hygiene periodontal mucosa (153). Upstream microbial community members from the oral mucosa have the potential to translocate to the stomach by salivary follow, and they might act as reservoirs for recolonization.

The observed interaction might be due to other mechanisms: Poor orodental health may be an indicator of a less healthy life style. However we did not detect association between education as a socioeconomic status indicator and DMFT. Also low DMFT could be a marker for distinct diet due to reduced masticatory ability. Yet the detected association might be due to residual confounding.

6.2.3 Gastric mucosa-associated microbiota and risk of esophageal squamous dysplasia and squamous cell carcinoma

In this study, we observed a significant difference in microbiota composition of gastric fundal mucosa in subjects with early ESCC and squamous dysplasia compared to those with normal esophagus. This difference remained stable after exclusion of cancer cases.

Similar to other studies on gastric mucosal microbiota (71), the most common phyla in our samples were *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Fusobacteria*.

The relation between *H. pylori* and ESCC risk is controversial. *H. pylori* abundance might be a single most important factor in diversity of gastric microbiota. In our study population, more than 80% were seropositive for *H. pylori* (121). A cluster of subjects with extremely low and zero *Helicobacteraceae* abundance showed low diversity. This group of subjects might be representative of:
- Those with healthy stomach with acidic environment and without *H. pylori* infection.
- Those who have been successfully treated upon *H. pylori* eradication program or *H. pylori* has been self-eradicated.

This pattern influenced structure of sample clusters, with marginal difference in proportion of esophageal cancer/dysplasia subjects and control individuals in the 2 clusters shaped by OTUs.

In this study, 16S rRNA with coverage of 464 nt of region V3-V4 was used to distinguish bacteria. Although it did not cover whole hyper variable V1-V9 regions, the most divergent regions were covered and it was possible to align with SILVA reference with 90.5% coverage.

Most of gut microbiota are anaerobes and difficult to cultivate and some of them could not be assigned to reference databases. Through OTU formation we tried to reduce this effect. Low number of study subjects in the pyrosequencing platform was compensated with higher depth of sequencing. Number of reads per sample in our study was around 3000. While studies have shown that a depth of 100 read is enough to separately cluster microbiota of healthy from inflamed bowel, to separate microbiota clusters of lean from obese 10,000 reads are needed (154). It is noteworthy that the possible difference in microbiota harboring in organs adjacent to cancerous tissue might be more subtle than the cancerous tissue. Like any PCR-based methods, amplification bias is a concern as some less abundant taxa might be underestimated or ignored.

6.2.4 Animal contact and risk of esophageal squamous cell carcinoma

We found an increased risk of ESCC among those who were in contact with domestic animals, particularly with ruminants which showed a more than 8-fold increased risk of ESCC.

Other observations verified our results on high level of contact between human and animals in study area. Golestan province has the highest incidence of animal bite and animal rabies compared to its neighbor provinces (155). Moreover serologic prevalence of human hydatidosis, a common zoonotic disease with canine-cattle-human cycle, is 2.3% in the study area which is higher than its neighbor regions (156). Population of domesticated ruminants in the catchment area during the study period was 1,733,000 with 83% small ruminants (74% sheep and 9% goat) and 17% cattle, which did not differ in total number from its neighbor areas with lower incidence rate of esophageal cancer (157). Even though the ruminant population in the study area is increasing, by improvement in the lifestyle of inhabitants (158) and industrialized animal husbandry, the contact with animals is decreasing in parallel with decline in ESCC incidence.

Contact with canine showed a two-fold increased risk for ESCC. This contact includes domestic and stray dogs. Wild canines are distributed in the study area in large extent and domestic dogs are applied by villagers to guard ruminants which could be one explanation for this association. After combining exposure to both ruminants and dogs, the risk due to only dogs disappeared and no excess risk was attributable to the contact with both animals compared to ruminants alone.

An excess risk of esophageal cancer among poultry workers has been reported (93), although we did not find any association between poultry and ESCC in our study. Possible explanation could be that in the cohort of poultry related industry the type of esophageal cancer has not been stated: whether majority were adenocarcinoma or squamous cell carcinoma. Further lack of adjustment for major confounders i.e. tobacco smoking, alcohol consumption, and contact with other animals could be another concern.

In the majority of subjects, the start of exposure to ruminants was from birth. Based on common lifestyle in the study area we can speculate that subjects were exposed to ruminants' materials prenatally. Median duration of contact with ruminants was 54 years which is approximately a lifelong exposure.

One explanation for our finding could be exposure to ruminants' products without industrial and healthy processing which is more common among villagers who keep animals within household. A study showed that small ruminants fed by a family of *Euphorbiaceae* flower plants extract a group of carcinogens (*diterpene ester*) in their milk (159). This genus is one of the most species-rich in flowering plants taxonomy and several of its species occur in the study area (160). We keep reservation that the association we observed in this study might be due to dietary factors in ruminants.

Another possible explanation for our findings is animal zoonoses i.e. viruses which could cause cancer in ruminants. In addition to the multi-host nature of some zoonoses, the transmission pattern could be complex, i.e. rodents as carriers or wild animals as sources.

7 CONCLUSION

- Opium and tobacco consumption are associated with ESCC risk.

- Gastric fundal atrophy in the absence of severe gastritis is associated with the risk of ESCC. Poor dental health and oral hygiene habit may act synergistically in increasing the risk.

- Applying high-throughput DNA sequencing, gastric microbiota composition differs between subjects with esophageal cancer or dysplasia and those with normal esophagus. Confirmation of this early result is needed.

- Inhabitants of high risk area for esophageal cancer in northern Iran who were in long-term contact with ruminants have an about 8-fold risk for ESCC.

8 FUTURE STUDIES

The underlying mechanism for the observed association between opium consumption and ESCC is not known. Recent studies have shown opium is linked to the risk of gastric adenocarcinoma in the same study area. Further studies are needed to explain the source of this association.

Although serologic marker of gastric atrophy has been linked to ESCC, dose-response relationship between severity of fundal atrophy and ESCC was once studied and failed to show any relation. One of the hypotheses for this association is the effect of atrophy on composition of gastric microbiota. This hypothesis is needed to be tested through next generation sequencing methods of gastric biopsies from atrophic and non-atrophic stomach tissues. The possible synergism with poor orodental hygiene warrants larger sample size and adding microbiological examination of oral mucosa and saliva.

The marginal difference in composition of gastric microbiota among those with normal and pre-neoplastic esophagus is needed to be repeated by other sequencing platform with higher sequencing depth. To verify the validity of phylogenetic candidates, qPCR of tissue samples with specific primers should be performed.

The observed strong association between animal contact and ESCC remains to be explained. It might be due to a shared environment, infectious agent(s), or a chance finding. As the first step, evaluation of serum virome through next generation sequencing might help to evaluate possible zoonotic origin of the observed link.

9 ACKNOWLEDGEMENTS

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