Urinary Levels of *N*-Nitroso Compounds in Relation to Risk of Gastric Cancer: Findings from the Shanghai Cohort Study

By

Ling Xu

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N-nitroso compounds (NOCs) formed during food processing and the *in vivo* nitrosation of secondary amines or amides in the presence of nitrites are believed to play a significant role in the development of gastric cancer. Epidemiological data examining the associations between biomarkers of exposure to NOCs and the risk of developing gastric cancer are sparse. A nested case-control study was conducted within the Shanghai Cohort Study, a prospective cohort of 18,244 middle-aged and older men. Urinary levels of N-nitroso-2-methylthiazolidine-4-carboxylic acid (NMTCA), N-nitrosoproline (NPRO), N-nitrososarcosine (NSAR), N-nitrosothiazolidine-4-carboxylic acid (NTCA), nitrate and nitrite as well as serum *H. pylori* antibodies were quantified in samples from 191 incident gastric cancer cases and 569 individually matched control subjects. Odds ratios and 95% confidence intervals for gastric cancer associated with elevated levels of urinary NOCs were calculated from logistic regression models for all subjects as well as for subgroups stratified by *H. pylori* status. Urinary NMTCA level was significantly higher in alcohol drinkers than nondrinkers. Compared with all control subjects, cancer patients overall had comparable levels of urinary nitrate, nitrite, and NOCs. Among individuals with negative *H. pylori* antibodies, elevated urinary nitrate level was associated with increased risk of gastric cancer. The multivariate-adjusted ORs (95%) for gastric cancer in the second and third tertiles of nitrate were 3.39 (0.82~13.96) and 4.18 (0.96~18.13), respectively, compared with the lowest tertile of nitrate (P for trend = 0.057). The present study did not support a direct relationship between urinary biomarkers of *N*-nitroso-compounds and their precursors and the risk of developing gastric cancer in a high-risk population.

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Chapter One: Introduction

1.1 Over view:

Despite the decline in its incidence and mortality over past several decades, gastric cancer is still the fourth most commonly diagnosed cancer and the second most common cause of cancer death in the world [1]. A distinguishing feature of gastric cancer is the remarkable geographic variability in incidence and mortality rates worldwide with especially high incidence in China. Numerous studies suggest that environmental factors play a significant role in the development of gastric cancer. Hence, identification of modifiable environmental risk factors, especially dietary factors, for gastric cancer would inform strategies for primary prevention against the development of this malignancy.

N-Nitroso compounds (NOCs) have shown carcinogenic effects in experimental animal models and certain NOCs have been classified as "probably carcinogenic to humans" by the International Agency for Research on Cancer (IARC) [2, 3]. Take gastric cancer for instance, adenocarcinomas in the glandular stomachs of rats were found after treatment with N-methyl-N-nitro-Nnitrosoguanidine (MNNG) [4]. Administration of MNNG to dogs in the drinking water was also found to result in a high incidence of tumors in the glandular stomach [5]. Furthermore, a Mongolian gerbil (MG) model of stomach carcinogenesis was established using MNU and MNNG as carcinogens [6]. Humans are exposed to NOCs from exogenous sources and through endogenous synthesis. Exogenous NOCs are directly derived from certain types of food, such as processed meat, salted or smoked fish, pickled and dried vegetables, while endogenous NOCs are formed through nitrosation of secondary amines or amides by nitrite, derived either directly from food or water, or indirectly from reduction of nitrate by oral and enteric bacteria [7-13]. Approximately 45%-75% of the total NOC exposure in humans is derived from endogenous synthesis [14]. In humans, nitrosation occurs in low pH environments such as in the normal stomach, especially when inhibitor levels, such

as antioxidant levels, are low. Also, nitrosation reactions can be catalyzed by certain bacteria, especially denitrifying bacteria, as well as under certain inflammatory conditions such as oxidative burst [15]. Individuals with high exposure to NOCs are hypothesized to be at increased risk of developing gastric cancer.

However, epidemiological studies examining the association between NOCs or NOC-containing food and gastric cancer risk in humans provide inconsistent results [16]. These inconsistencies are likely due to measurement error in the assessment of exposure to NOCs in most, if not all, epidemiological studies. A biomarker approach that assesses total NOCs, derived from both exogenous and endogenous sources, would overcome this limitation and increase the validity of results.

Thus, using resources of an established prospective cohort study in a Chinese population, I explored the association between urinary levels of NOCs and the risk of gastric cancer, and to investigate possible interactions between NOCs and cigarette smoking, alcohol drinking, H. pylori infection or dietary nutrient antioxidants. Our findings might provide strategy guidelines for appropriate health policies, especially in developing countries with a high incidence of gastric cancer and limited access to therapeutica choices.

1.2 Study aims:

We plan to test our central hypothesis and accomplish the overall objective of this work by pursuing the following specific aims:

1.2.1 Primary specific aim:

The goals of this study are to use quantified urinary levels of N-nitroso-2-methylthiazolidine-4-carboxylic acid (NMTCA), N-nitrosoproline (NPRO), N-nitrososarcosine (NSAR), N-nitrosothiazolidine-4-carboxylic acid (NTCA), and NOCs-precursor nitrate and nitrite to evaluate the association between urinary levels of these NOCs and the risk of gastric cancer in the Shanghai Cohort Study, a prospective cohort of

18,244 middle-aged and older man in Shanghai, China, with up to 12 years of follow-up.

1.2.2 Secondary aims:

1) Evaluate the difference in the urinary yields of NOCs between subgroups by status of cigarette smoking (ever or never), alcohol consumption (ever or never), H. pylori infection (negative or positive), serum antioxidants levels (high or low) and urinary epigallocatechin level (high or low).

Certain dietary factors might either have higher amounts of NOCs or in vivo inhibit the endogenous synthesis of NOCs[13]. Since we hypothesized that certain NOCs could be potential carcinogens for the stomach, it merits exploring whether these established risk factors or protective factors for gastric cancer would have influence on the urinary yields of NOCs in vivo.

2) Explore the interactive effects between cigarette smoking or alcohol consumption and N-nitroso compounds on the risk of gastric cancer.

Both cigarette smoking and alcohol consumption are well established risks for gastric cancer [17]. Some studies related cigarette smoking to levels of NOCs exposure and some investigated the effect of alcohol consumption on the bioactivation of NOCs [18-23]. Herein, we will to explore the potential that there might be some interactive effects between cigarette smoking or alcohol consumption and NOCs on the development of gastric cancer.

3) Explore the interactive effect between Helicobacter pylori infection and N-nitroso compounds on the risk of gastric cancer.

H. pylori infection is a well established risk factor for gastric cancer[24]. However, H. pylori infection is very prevalent, and only a small proportion of infected people developed gastric cancer. Other environmental factors are suspected to play a modifying

role in the initiation of developing gastric cancer. We hypothesize that there might be some synergistic effect between H pylori infection and NOCs on the development of gastric cancer.

4) Explore the modifying roles of vitamin C, EGC, and β-carotene and other antioxidants in the association between urinary NOCs and the risk of gastric cancer.

Diets rich in these phytochemicals may associate with protection of the gastric corpus from atrophy and a reduction in the incidence of gastric cancer possibly through the ability of reducing oxidative damage to the gastric mucosa by scavenging carcinogenic N-nitroso compounds and free radicals and attenuating the bacteria-induced inflammatory cascade[15, 25]. Herein, we explore the potential modifying effects of antioxidant on the association between NOCs and the risk of gastric cancer.

1.3 Organization of thesis

This thesis includes a detailed review of the literature in chapter two. The third chapter includes the methods and results of the study and is presented as a stand-alone manuscript to be submitted for publication. The final chapter includes conclusions and implications for future studies.

Chapter 2 Literature review

2.1 Source of exogenous N-nitroso compounds

In general, NOCs are a group of compounds which contain a specific chemical structure characterized by a nitroso group (-N=O) attached to a nitrogen atom from a peptide, to form an –N-N=O structure [26]. Based on their chemical structures, NOC are mainly divided into two major groups: N-nitrosamines and N-nitrosamides. N-nitrosamines can be further divided into two subdivisions: volatile (VNA) and non-volatile (NVNA) N-nitrosamines. In general, N-nitrosamines are derived from nitrosation of secondary amines, while N-nitrosamides are derived from nitrosation of N-alkylamides [15]. NOCs are known to be potent carcinogens and numbers of NOCs compounds have been tested in animal models and in vitro for their carcinogenic potentials. Human exposure to NOCs and their precursors (nitrate and nitrite) can occur through exogenous source, such as diet, drinking water, occupation, tobacco or cosmetics products. NOCs also can be formed endogenously.

NOCs have been identified in cured meats, bacon, preserved fish and beer [13]. Some food processing methods can promote formation of NOCs, including pickling or storing food under humid conditions, smoking food in air saturated with nitrogen, drying food at high temperatures (i.e. protein-containing foods as beer ingredients, nonfat dry milk, cooked bacon, or dried meat), and curing food with nitrate or nitrite [27]. Collectively, a wide range of foods have been tested and shown to contain NOCs compounds or its precursor nitrate and nitrite, especially for preserved food products. A most recent NOCs-specific database shows that N-nitrosamines mainly come from meat products with the range from 0-0.45 μg/serving; nitrite mainly comes from meat and bean products with the range from 0-1.84 mg/serving; nitrate mainly comes from fresh vegetable products with the range from 0-189.00 mg/serving [28]. Estimates of the daily median intake of N-nitrosamines, nitrate, nitrite in western diets were 0.472 ug, 40.48 mg and 1.53 mg, respectively [28]. However, these estimations were

mainly based on the food frequency questionnaires of the western diet. There are no available data on dietary exposure of NOCs, nitrate or nitrite for Chinese population. According to the report of surveillance data from 21 provinces in China from year 1990 to 2003, among 13 013 samples tested, 7702 samples (59.2%) contained nitrite and 3792 samples (29.1%) were higher than the national standard [29]. Because of the potential carcinogenic roles of NOCs in animal models, several governmental regulations have been put in place to curb the use of preservative in food processing. The Food and Agriculture Organization of the United Nations, World Health Organization, and the United Nations Food Additive Regulation Commissionrecommended that at present the amount of nitrite additive should be controlled to a minimum level before there is an ideal substitute, and regulate that nitrite maximal residue level (MRL) in general food products should be below 0.2g/kg [30]. Concerning nitrite use, the European Commission has established several MRLs for dry cured meat products as bacon (0.17g/kg), dry non-heated meat products (0.05g/kg), and other meat products (0.1g/kg) [31, 32]. Chinese standards for maximum level of nitrite used in canned meat products is 0.15 g / kg, and the limit for the maximal residue levels of nitrite in canned meat products are 0.05g/kg , 0.07 g/kg for cured meat products and 0.03 g/kg for general meat products [33]. Although efforts to reduce the amount of nitrite used to cure meat and other products have been instituted in the past two decades, naturally occurring precursors of NOCs could be converted to NOCs under appropriate conditions even with low residual levels of nitrite. For instance, a research group found that 2-26ng NMU/10g of meat could be formed in meat samples after two hours incubation at room temperature in an acidified solution [34].

As mentioned in the aforementioned part, they are very often used as food additives to stabilize meat products. In addition, nitrate is used in agriculture as a fertilizer. Thus nitrate could present in all vegetables or in nature water as results of nitrate-containing fertilizer contamination. Nitrate levels in vegetables vary depending on the type of vegetable and how they are grown, stored and cooked. The mean values of NO_3^- levels in fresh vegetables ranged from 26 to 281 mg/kg [34]. Considering cooking methods, boiling could reduce

nitrate contents by 65% to 25%, frying in soy bean oil elevate nitrate contents from 87% to 355%, but baking has no impact on nitrate content[35]. It has been estimated that vegetables can provide up to 85% nitrate of the average daily human diet [36]. However, the anti-oxidant phytochemicals contained in fresh vegetables could compensate the deleterious health effects with regarding to NOCs endogenesis [36, 37]. The increasing use of nitrate containing fertilizer and nitrate of drinking water also contributes a great proportion of total nitrate exposure to humans [38]. In US, public water utilities are required to maintain nitrate levels below a Maximum Contaminant Level (MCL) of 10 mg/L nitrate-N [37]. A nation wide survey in US showed that 9% samples exceeded the regulatory limit and 22% private wells exceed the MCL in agricultural areas [37]. 2 to 3% of the population in the EU are potentially exposed to drinking water exceeding the present WHO standard for nitrate residue in drinking water [39]. In China, the nitrate pollution in public water is more concerning. According to an investigation conducted in the countryside in North China, nitrate contents in ground water and drinking water exceeded 50 mg/L in over half of the 69 investigated locations. In some locations the nitrate content in ground water and drinking water reached 300 mg/L [40].

Other exogenous sources of NOCs include usage of tobacoo, which contains carcinogenic nicotine derived nitrosamines such as NNN (N-nitrosonornicotine) and NNK ((4-methylnitrosamino)-1-(3-pyridyl)-1-butanone) [41]. Also, NOCs could be derived from industrial pollution. For instance, in a study of two industrial rubber plants, considerable concentrations of atmospheric NDMA were detected, and the DNA adducts in workers' peripheral blood were positively associated with exposure of category levels of NDMA exposure[42]. In addition, many drugs contain secondary or teritary amines which could be nitrosated in the body to yield NOCs. Piperazine is a cyclic secondary diamine for the treatment of intestine worms. Its nitrosation could produce mono- and di-nitrosopiperazine (MNP and DNP) [43]. Lung adenomas in mice were attributed to the formation of these nitrosamines in vivo after administration of piperazine and nitrite in diet [44]. A study on

arylamine drugs, which have been tested for the formation of N-nitroso compounds (NOC) by reacting with nitrite, and results showed that 105 of 109 (96.3 %) arylamine drugs were found to form NOCs [45]. Studies also demonstrated that NOCs could come from cosmetics products. A survey in cosmetics from the Dutch market showed that a content of N-nitrosoethanolmaine (NDELA) above the limit of quantification of 5.3 ug/kg was found in 7 out of the 25 cosmetics tested [46].

2.2 Endogenous synthesis of N-nitroso compounds

Human exposure to endogenously formed N-nitroso compounds has frequently been suggested as a causative factor in carcinogenesis. Three ways of endogenous formation have been identified. The first, a direct chemical reaction between secondary or tertiary amino compounds and nitrite, is strongly pH dependent and does not occur rapidly at neutral pH even in the presence of chemical catalysts [47]. The second pathway of NOCs formation depends on the direct bacterial catalysis of N-nitrosation. Studies demonstrated that the bacterial mediated reaction is catalysed by bacterial enzyme systems including nitrate reductase and nitrite reductase, and proceeds much more rapidly at neutral pH than the chemical reaction[14]. Different bacterial species and different isolates of the same species show considerable variation in their abilities to catalyse N-nitrosation reactions under different situations (normal phisical condition vs immunostimulated condition) [47]. The most rapid catalysis is associated with those bacteria capable of reducing nitrate and nitrite by the process of denitrification [47]. The third way depends on inflammatory induced nitrosation through nitric oxide formation by macrophage and neutrophils oxidative burst [15]. It has been estimated that approximately 45%-75% of the total NOCs in humans are derived from endogenous synthesis [14].

2.2.1 Acid-catalyzed Nitrosation

Endogenous NOCs could be formed through nitrosation of amines or amides by nitrite

in the acidic stomach. Nitrite could either directly come from food and drinking water or from ingested nitrate reduction by saliva or enteric enzymes [7-11]. Formation of endogenous N-nitrosamines is thought to be proportional to the amines concentration, which is in excess, and to the square of the nitrite concentration (please see the equation below) [26, 48, 49]. For equation 4, k_1 depends on pH [50]. k_1 and the reaction rate show maximum values at pH = 3.4 corresponding to the concentration of nitrous acid (pKa = 3.37). The reaction rate decreases ten fold for each 1-unit increase in pH when pH is above 3.4 [51]. The main effect of a further reduction in pH will result in a continuous drop of nonionized amine concentrations, causing a decrease in the reaction rate. This reaction also could happen slowly at higher pH values, such as a pH of 5 or even 6, as observed for diethylnitrosamine (DMA) [51]. The reaction rate of N-nitrosamides is proportional to the concentrations of amides and nitracidium ions. This reaction increases about ten fold when pH drops each 1-unit from 3 to 1[43,51, 52].

Equation 1: Formation of the nitrosating species

$NO_2^- + H^+ \rightleftharpoons HNO_2$

$2\ HNO_2 \rightleftharpoons N_2O_3 + H_2O$

$HNO_2 + H^+ \rightleftharpoons (H_2NO_2)^+$

Equation 2: Formation of N-nitrosamines

$RR'NH + N_2O_3 \rightarrow RR'NNO + HNO_2$

Equation 3: Formation of N-nitrosamides

$RNHCOR' + (H_2NO_2)^+ \rightarrow RN(NO)COR' + H_2O + H^+$

Equation 4: general simplified equation of formation of NOCs

$Rate = k_1\ [total\ RR'NH]\ [NO_2^-]^2$

$Rate = k_2\ [total\ RNHCOR']\ [HNO2]\ [H+]$

It has also been suggested that approximately 25% of ingested nitrate is actively secreted into saliva by an anion transport mechanism and that 20% of salivary nitrate is reduced to nitrite by oral bacteria, yielding a conversion of 5% of exogenous nitrate to

endogenous nitrite [53-55]. The majority nitrate exposure is from vegetables. Actually, because fresh vegetables are full of anti-oxidant nutrients, such as vitamin C or vitamin E, in vivo nitrosation could be inhibited by fresh vegetables intake via reducing nitrite to nitric oxide (NO) by vitamins C and E, and NO is not a directly nitrosation agent [43, 56]. On the other hand, the nitrosation of amines could be accelerated by thiocyanates while the nitrosation of amides could be catalyzed by citrate and other organic acids [57]. Thus most studies, when using dietary records to estimate the total NOCs exposure, will adjust fresh vegtales intake as confounding factors to minimize the bias evaluation. However, as inherent of this method, bias on exposure estimation can't be avoided.

2.2.2 Bacterial induced nitrosation

Bacterial nitrosation of carcinogenic NOCs may play a critic role in the etiology of human cancer. Studies found that several bacterial strains, both denitrifying and nondenitrifying, could catalyze the nitrosation of secondary amines [58, 59]. Two enzymes have been found to be related to bacterial induced nitrosation. One is nitrate reductase which contributes to endogenous nitrosation through the reduction of nitrate to nitrite, resulting in an increase in the concentrations of nitrosating species [7-9, 11]. The other is nitrite reductase which facilitates the nitrosation by producting nitric oxide or NO(+)-like species at neutral pH level, which implied that that enhanced endogenous nitrosation might happen in subjects suffering from an achlorhydric stomach [60, 61]. Previous investigation on the bacteria-catalysed formation of NOC revealed that nitrate reductase was mainly associated with the nitrosation in non-denitrifying bacterial strains like E. coli, while nitrite reductase was mainly linked to the nitrosation in denitrifying bacterial such as Neisseria and Pseudomonas [62]. Beside bacterial strains, nitrosation also depends on the physiological state of the bacteria. For example, E. coli could catalyse nitrosation of various secondary amines in the resting phase, while some enteric bacteria only catalyse nitrosation in their growth phase [14, 58, 63].

Presumably, the nitrosation agent formed by nitrite reductase in denitrifying bacteria occurs through the following steps: 1) NO is producted by nitrite reductase; 2) NO is then reacted with dissolved oxygen to form nitrogen dioxide (NO_2), 3）NO_2 is could be either dimerized to N_2O_4 or reacted with NO to form N_2O_3, 4) both N_2O_3 and N_2O_4 are nitrosating agents and could further nitrosate amines or amides in vivo (please see the equations below) [15]. For bacterial nitrosation, studies also found that the specific reaction rate for certain bactrial is also inversely related to the pKa of the amine substrate, while high nitrite concentrations and decreasing pH inhibit bacteria induced nitrosation process [47, 60, 64].

Equation 1: Formation of the nitrosating species

$$2NO + O_2 \rightarrow 2NO_2$$

$$NO + NO_2 \rightleftharpoons N_2O_3$$

$$2NO_2 \rightleftharpoons N_2O_4$$

Equation 2: Formation of N-nitrosamines

$$RR'NH + N_2O_3 \rightarrow RR'NNO + HNO_2$$

$$RR'NH + N_2O_4 \rightarrow RR'NNO + HNO_3$$

$$2RR'NH + N_2O_4 \rightarrow RR'NNO + RR'NNO_2$$

2.2.3 Inflammatory induced nitrosation

Many different mammalian cells produce NO via a common biochemical pathway involving the oxidation of the terminal guanido-nitrogen of L-arginine to citrulline by NO synthase (NOS) [65]. NO could act as a vasodilator in arterioles. The amounts of NO produced in the normal physiological conditions are far less then those produced in the flammatory conditoin during macrophage oxidative burst. Studies found that total endogenous cell-mediated mammalian nitric oxide production under normal physiological conditions is about 1 mM/day or 1 mg/kg body weight/day in human, while in the case of immunostimulated NO synthesis, the rate of NO production could raise up to 1 mM/min for

several hours by macrophages during oxidative burst in response to immune stimulation [14, 66, 67]. Similiar to bacterial induced nitrosating agents, NO produced by macrophages could form N_2O_3 or N_2O_4 to further nitrosate amines in the body to form NOCs in vivo [15]. These results suggest that certain inflammatory conditions which lead to cancer, such as ulcerative colitis for colon cancer, might partly due to NOC formation [68]. However, this deduction needs further validation.

2.3 The role of N-nitroso compounds in cancer development.

NOCs play important roles in the pathogenesis of various cancers in animal models. The hypothesis for the involvement in gastric cancer was first demonstrated in studies that showed that in some gastric precancerous conditions (such as pernicious aneamia, chronic atrophic gastritis and the post-gastrectomy situation), an overgrowth of nitrate-reducing bacteria occured and gastric juice nitrite were elevated above normal levels [8, 14, 69, 70]. Several early population based studies on NPRO yield in urine in relation to the incidence of gastric cancer also confirmed this positive association [71-74]. These findings led to further investigations in the role of NOCs in etiology of cancer development. Up to now, several biologically plausible mechanisms of carcinogenic potential of NOCs have been proposed and demonstrated in both vitro and animal studies.

2.3.1 Formation of N-nitroso-DNA adducts:

Alkylating agents formed by NOCs metabolism could induce DNA adducts which represent the most direct biomarker of the biological effect as potential carcinogens. These primary DNA lesions can either cause certain DNA structure alterations and consequently lead to DNA damages such as strand breakage, mutations, chromosomal rearrangements and deletions, or cause DNA function alteration such as GC island methylation which involve altering the gene expression pattern. If these DNA structure damages or changes were not

effectively repaired, these damages may result in the alternation of an incorrect base during DNA replication, followed by transcription and translation of mutated templates, ultimately resulting in the synthesis of altered protein. The N-nitrosamines require metabolic activation by certain cytochrome P-450 enzymes (e.g. P450 2E1 in a rat model) in the endoplasmic reticulum and successively decompose to yield strong alkylating electrophiles such as diazonium ions that react with DNA at multiple nuleophilic sites [75, 76]. These processes have been identified in cultured human tissue samples as well as in experimental animals [75, 77]. N-nitrosamines are highly organotropic and animal species specific [26]. They generally induce tumors at specific sites independent of the route of administration [75]. In rodents, nitrosamines mainly induce tumors in liver, esophagus, nasal and oral mucosa, kidney, pancreas, urinary bladder, lung and thyroid [78-80]. Different from nitrosamines, nitrosamides could yield similar alkylating agents in a chemical non-enzymatic manner [81]. Hence, nitrosamides mainly induce tumors locally, such as stomach and small intestine and lymphatic systems [78-81]. The most commonly investigated consequences of alkylating agents by NOCs are mutagenic DNA damag such as adducts on N^7, O^6-guanine and O^4 of thymine which might result in base mispairings [15]. For instance, O^6-alkylguanines pair with thymine rather than cytosine and this produces G:C→A:T transition mutation that are thought to be involved in initiating carcinogenesis [15]. In addition, NOCs induced DNA adduct may cause deamination of amino groups of DNA bases via the tautomerization of triazene, which possible yeilds C:G→T:A mutation [15, 82] . Other investigations on the potential carcinogenic role of DNA adduct induced by some cyclic N-nitrosamines provide some new DNA adduct patterns like N2-(tetrahydrofuran-2-yl)-dG from alpha-acetoxy NPYR and N2-(3,4,5,6-tetrahydro-2H-pyran-2-yl)dG (THP-dG) from alpha-acetoxy NPIP [83, 84]. However, the potential carcinogenic mechanism still needs further investigation. In addition, formaldehyde, formed through bioactivation of some NOCs, such as NDMA, has been demonstrated to be mutagenic to cultured human cells and a respiratory carcinogen to rodents, whose potential mechanism might involve the formation of formaldehyde induced

DNA adduct which cause DNA cross link damage [85-90]. Evidence also showed that formaldehyde may synergisticly increase the mutagenicity of N-methyl-N-nitrosurea (MNU) by inhibiting O6-methylguanine repair [90].

2.3.2 Specific gene mutations induced by N-nitroso compounds

The ras family of oncogenes has three primary members: H-ras, K-ras and N-ras [91-93]. All ras protein family members belong to a class of protein called small GTPase, and are involved in transducing growth-promoting and survival signals from membrane-bound receptor tyrosine kinases [94]. The hydrolysis of the γ phosphate of guanosine triphosphate (GTP) into guanosine diphosphate (GDP) abrogates Ras signaling, but oncogenic mutations in ras impair GTP hydrolysis, thus, causing persistent signaling and dysregulated cell signal transduction [95]. Dysregulation were found in various human tumours with up to 90% in certain types of cancer, due to mutations either in the ras genes themselves or in its upstream or downstream signalling sequences, and most common mutations are found frequently in codon 12 [96, 97]. Studies found that a high prevalence of point mutations G:C→A:T in codon 12 of H-ras oncogenes in N-nitrosomethylbenzylamine (NMBA)-induced esophageal preneoplastic lesions and tumors in a rat model [98, 99]. NNK, a tobacco specific N-nitrosamine, is known to be activated in the lung. Activated NNK produces methylating agents, which subsequently induce methylated DNA adducts, such as O^6-methylguanine and N^7-methylguanine, in the bronchial and bronchiolar epithelium [100-103]. Studies in animal models also confirmed that a high frequency of G:C→A:T mutations in condon 12 of the K-ras oncogene could be induced by NNK treatment in rat lung tissues [97, 104-106]. The G:C→A:T mutations in codon 12 or 13 of the K-ras oncogene is a common mutation in colorectal cancer as well [107]. In a human study, volunteers were fed on a diet rich in red meat（420g beef/day）and a vegetarian control diet [108]. Exfoliated colonocytes were isolated and analysed for the DNA adducts

O^6-carboxymethyl-deoxyguanosine (O^6CmeG) [108]. The results showed that the fraction of exfoliated colonocytes with O^6CmeG DNA adducts was significantly increased in the group of high red meat diet, compared with control diet, which suggested that high red meat diet induced endogenous NOCs might involve in K-ras oncogene point mutation in colon cancer development [108].

P53 gene encodes a tumor suppressor protein regulating the cell cycle and functions as a tumor suppressor that is involved in preventing cancer, and mutations in p53 gene might cause gene disfunction thus initiating carcinogenesis [109-112]. One of the most frequently encountered genetic events in human malignancy is alteration of the p53 gene and its encoded protein [113]. Some N-nitroso compounds can be metabolically activated to produce diazoacetate, which can result in the carboxymethylation of DNA. Potassium diazoacetate (KDA) is a stable form of nitrosated glycine and its ability to induce mutations in the p53 gene in a functional yeast assay was studied [114]. Results showed that KDA could induce equal amounts of transitions (gastric cancer/AT) and transversions (gastric cancer/TA and AT/TA) in the p53 gene [114]. The pattern of mutations induced by KDA was very similar to the patterns observed in mutated p53 in human gastrointestinal tract tumours [114]. These results are consistent with the hypothesis that nitrosation of glycine (or glycine derivatives) may contribute to human carcinogenesis in the gastrointestinal system. Another study investigated MNU induced gastic tumors in a genetically modified mouse model: p53 nullizygote (−/−), heterozygote (+/−) and wild-type (+/+). They found in a 15-week experiment, adenomas and a well-differentiated adenocarcinoma were only observed in p53 (−/−) animals after 15 weeks MNU treatment. However, after 40 weeks, there were no significant difference in the incidences of stomach tumors between p53 (+/+) and (+/−) mice which suggests p53 may play an important role as a gatekeeper in rodent stomach carcinogenesis [115]

Normal rat glandular stomach possesses pepsinogen isozymes: Pg1, Pg2, Pg3 and Pg4. However, after treatment of N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG), decreased secretion or disappearance of Pg1 were observed in the early stages of rat glandular stomach

carcinogenesis and in stomach tumors [116]. Significantly increased methylation of the Pg1 gene in both CCGG and GCGC sites were also observed in rat during MNNG induced early carcinogenesis process [117]. The results suggest that the altered methylation of the Pg1 gene observed in stomach cancers might be elicited by MNNG induced DNA methylation and act as an initiating agent in stomach cancer.

β-Catenin codes proteins constituting adherens junctions (AJs). AJs are necessary for the creation and maintenance of epithelial cell layers by regulating cell growth and adhesion between cells, which plays an important role in the Wnt signaling pathway [118]. Aberrent Wnt/β-catenin signaling caused by mutiations in β-catenin has been observed in a number of human malignancies, such as stomach cancer, breast cancer, and intestine cancer [118-120]. Both MNNG and MNU induced stomach adenocarcinomas in animal models showed nuclear β-Catenin localization featuring mutations in exons of β-Catenin gene [121, 122].

2.3.3 N-nitroso compounds induced Change in Genomic response

A recently study analyzed the genome-wide gene expression in the human colon adenocarcinoma cell line Caco-2 after treatment with 2 nitrosamide (N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and N-methyl-N-nitrosurea (MNU)) and 4 nitrosamines (N-nitrosodiethylamine (NDEA), N-nitrosodimethylamine (NDMA), N-nitrosopiperidine (NPIP), and N-nitrosopyrrolidine (NPYR) [10]. Changes in gene ontology gene group, consensus motif gene group and biological pathway were analysed after 24 hour of NOCs exposure by the methods of flow cytometry, comet assay and microarray assay [10]. Results showed that most significant results were induced by nitrosamines but not nitrosamides. Gene group and pathway analyses indicated an increase in apoptosis and inhibition of cell cycle progression following all nitrosamines exposure [10]. Furthermore, NDEA, NPIP, and NPYP strongly affected cellular immune response pathways,

involved in the stimulation of proinflammatory cytokines [10]. In addition, NDEA, NPIP and NPYP strongly affected several developmental motif gene and pathway, which are implicated in essential developmental processes, including embryogenesis [10]. All these data suggested the nitrosamine exposure may be associated with cancer development in human colon cancer. This study provides a new view on potential role of NOCs involved in human cancer development.

2.4 Gastric cancer and potential carcinogenic role of NOCs

Despite a major decline in incidence and mortality over several decades, gastric cancer is still the fourth most common cancer and the second most common cause of cancer death in the world [1]. There is a about 10-fold variation in incidence between populations at the highest and lowest risk. The incidence is particularly high in East Asia, Eastern Europe, and parts of Central and South America [123]. The highest incidence were seen in East Asia with rate over 60 per 10,000 person years [123]. Almost two thirds of the cases of gastric cancer occurred in developing countries and 42% in China alone [124]. Of note, the decline of incidence rate of gastric cancer in China was less dramatic than other countries. Furthermore, the age of onset of developing gastric cancer in Chinese population is younger than that in the West [125]. Despite an overall global decrease in gastric cancer incidence, an increase has been observed in the oldest and the youngest group, and a less remarkable decline has been observed among women than in men [125].

In general, gastric cancer could be classified into two by cell types: the intestinal or the diffuse type, based on histopathological characteristics. With regarding to the epidemioloigic differences, the diffuse type is featured by early onset, equal sex distribution, and similar prevalence by regions while the intestinal type tends to occur in older people, more common in men than women and the incidence rate varies by geographic regions [126]. A human model of gastric carcinogenesis has been proposed, based on a multi-stage model in which gastric mucosa progresses from superficial gastritis, chronic atrophic gastritis, chronic

atrophic gastritis with intestinal metaplasia, dysplastic to final malignant stage [127].

Nutritional factors had long been hypothesized as primary risk factors for gastric cancer. Epidemiological studies have been conducted to identify dietary risk factors for gastric cancer. Some dietary factors, such as high red meat intake, high salty food intake, high dietary nitrate, nitrite and NOCs intake or low intake of fresh fruits and vegetables have been associated with the increased risk of gastric cancer [126]. For instance, the intake of meat and processed meat were both associated with on increased gastric cancer risk in the EPIC study [128]. This increase was related to non-cardia cancer and affected especially H. pylori infected subjects [128]. NOCs exposure is considered as an important risk factor for gastric cancer. So far, this hypothesis is based on circumstantial evidence: 1) Higher nitrite levels in were found in the gastric juice of high-risk population, compared to low-risk population. 2) Experimental carcinogenesis was indentified after administration of NOCs in the diet in animal models. 3) Higher levels of endogenous nitrosation after feeding of proline to high-risk populations were observed, compared to low-risk population. 4) Evidence from epidemiological studies demonstrated that high NOCs, nitrate or nitrite exposure is related to the increased risk of gastric cancer.

According to Correa's gastric cancer model, the development of gastric cancer is a long process, usually starting from superficial gastritis to final carcinoma stage [127]. Which stages are NOCs most likely to act at? Hypothesis has been proposed by several studies that NOCs, together with other deleterious factors such as H. pylori infection, malnutrition and decreased fresh vegetables and fruit intake, might act as irritants and thus induce predisposing pathological changes in the gastric mucosa in first several steps [14, 126, 127]. During the early stages of inflammation and atrophy in stomach tissues, the normal phenotype of the gastric epithelial cells dose not change yet and only cell loss and cell regeneration are involved. Cell loss and regeneration are normal in the gastrointestinal epithelium but become exaggerated when the forces responsible for the atrophic gastritis set in [127]. Investigations have focused on salty food, dried fish and pickled fruits in promoting

the progress of this stage [129, 130]. When processing into chronic atrophic gastritis, cellular atrophy, loss of gastric glands and their acid-secreting parietal cells gradually replaced by intestinal-type glands (intestinal metaplasia) happens[14]. These histopathological changes causes multifocal lesion which rapidly spreads to cover much of the gastric mucosa, thus resulting in an increase in gastric pH [14]. At this moment, nitrite is abundant in the gastric cavity of subjects with atrophic gastritis, probably as a result of the production of nitric oxide or NO(+)-like species by some denitrifying bacteria at neutral pH level[127]. In addition, nitrate and nitrite may be produced by macrophages, which are also present in chronic gastritis [131]. Nitrosation within the achlorhydric region increases the availability of NOCs which might act as initiating carcinogenic irritants by inducing some predisposing pathological changes in the gastric mucosa. The synthesis of nitroso compounds and the cellular damage are probably modulated by other compounds present which may act as facilitators of carcinogenesis [132, 133].

Gastric cancer development is a complex process, possibly influenced by a combination of factors, such as deleterious dietary exposure, smoking and drinking status, H. pylori infection status, chronic inflammation responses and genetic alteration. Different combinations and times of involvement of these elements might lead to different clinical manifestations and pathological changes in stomach tissue. This complexity might explain why numerous results from different studies provide conflicting data. Exploring the real causative factors for gastric cancer may include different environmental factors, clinicopathological characteristics, genotyping methods and so on.

2.5 Epidemiologic Studies on the role of NOCs in gastric cancer

Early attempts in 1980s or early 90s on the association between NOCs and gastric cancer focused on the NOCs yields, especially NPRO endogenous synthesis after L-proline load in human studies, of which, most studies have confirmed this association[72-74, 134]. Later investigations focused more on the dietary exposure of NOCs, nitrate and nitrite in the

relationship to gastric cancer development. A recent Meta analysis of the epidemiological evidence, including over thirty human association studies on the relationship between NOCs and gastric cancer showed mixed results (16). With regard to the characteristic of these studies, they could be roughly divided into 4 different categories: 1) Cohort studies with a focus on the exposure of individual NOCs, nitrate and nitrite, with estimations of exposure mostly from the record of food frequency questionnaires; 2) case control studies with similar scenario as mentioned above; 3) Cohort studies with focus on the exposure of NOCs, nitrate or nitrite containing food, such as hot dogs, red meat products, preserved food and so on; 4) Or case control studies with similar scenario as mentioned above. In general, cohort studies provide very limited and inconsistent results on the relationship between NOCs exposure and the risk of gastric cancer, while most case-control studies provide more consistent results on this positive relationship. The risk ratios of gastric cancer varied across different studies with positive results ranging from 1.10 to 5.51 [16].

However, the evidence from epidemiological studies is limited and weak in supporting the role of NOCs in the development of gastric cancer. First of all, very few studies are prospective studies. However, case-control studies had inherent drawbacks such as recall bias which resulted in biased risk estimation. Secondly, most studies relied on food frequency questionnaires to assess NOCs exposure. Besides the uncomparable food frequency questionnaire among different studies, the results may not reflect the true association because NOCs containing food may contain other cancer-causitive agents including heterocyclic amines, high salt, bacterial or fungus byproducts, which could confound the observed association between NOCs and gastric cancer. Furthermore, as described above, 45%-75% NOCs may come from endogenous synthesis [14]. Thus using a food frequency questionnaire as an estimation method may underestimate the real exposure of NOCs in vivo. In addition, to my best knowledge, no existing studies explored the interactive effect between NOCs and H pylori infection after years of establishment of H pylori infection as a high risk for gastric cancer.

Chapter 3: A Nested Case-Control Study of Gastric Cancer in Shanghai, China

3.1 Chapter Summary

N-Nitroso compounds (NOCs), formed *ex vivo* during food processing and *in vivo* via nitrosation of secondary amines or amides in the presence of nitrites, are believed to play a significant role in the development of gastric cancer. However, epidemiological data examining the associations between biomarkers of NOC exposure and the risk of developing gastric cancer are sparse. We have conducted a nested case-control study within the Shanghai Cohort Study, a prospective cohort of 18,244 middle-aged and older men, to test the hypothesis that NOC exposure increases risk of gastric cancer. Urinary levels of N-nitroso-2-methylthiazolidine-4-carboxylic acid (NMTCA), N-nitrosoproline (NPRO), N-nitrososarcosine (NSAR), N-nitrosothiazolidine-4-carboxylic acid (NTCA), nitrate and nitrite as well as serum *H. pylori* antibodies were quantified in samples from 191 incident gastric cancer cases and 569 individually matched control subjects. Urinary NMTCA level was significantly higher in alcohol drinkers than nondrinkers. Compared with control subjects, gastric cancer cases had comparable levels of urinary nitrate, nitrite, and NOCs. Among individuals seronegative for *H. pylori*, elevated urinary nitrate level was associated with increased risk of gastric cancer. The multivariate-adjusted ORs (95%) for gastric cancer in the second and third tertiles of nitrate were 3.39 (0.82~13.96) and 4.18 (0.96~18.13), respectively, compared with the lowest tertile of nitrate (*P* for trend = 0.057). The present study does not support a direct relationship between urinary biomarkers of other *N*-nitroso-compounds and their precursors and the risk of developing gastric cancer in a high-risk population.

3.2 Introduction

Despite the decline in its incidence and mortality over the past several decades,

gastric cancer is still the fourth most commonly diagnosed cancer and the second most common cause of cancer death in the world [1]. Even if the current decline in incidence continues, this malignancy will remain as one of the most common cancers worldwide given the current population trends in high-risk regions[135]. A distinguishing feature of gastric cancer is the remarkable geographic variability in incidence and mortality rates worldwide. Eastern Asia has the highest incidence rate of gastric cancer with more than 60 per 100,000 person-years, significantly higher than the rates in North America and Africa which are below 9 per 100,000 [123]. However, gastric cancer rates among Japanese immigrants to the United States in the past several decades were significantly decreased relative to their counterparts in Japan [136]. These data suggest that environmental factors play a significant role in the development of gastric cancer. Hence, identification of modifiable environmental risk factors, especially dietary factors, for gastric cancer would inform strategies for primary prevention against this malignancy.

N-Nitroso compounds (NOCs) have shown carcinogenic effects in experimental studies. Approximately 300 NOCs have been tested for carcinogenicity in laboratory experiments, with 90% of them demonstrating carcinogenic affects across organ sites and animal species, including higher primates [137, 138]. NOCs may also be etiologic in human cancers [10, 15, 139, 140], and certain NOCs have been classified as "probably carcinogenic to humans" by the International Agency for Research on Cancer (IARC) [2, 3]. Humans are exposed to NOCs from exogenous sources and through endogenous synthesis. Exogenous NOCs are directly derived from certain types of food, such as processed meat, salted or smoked fish, pickled and dried vegetables [12]. Available data suggest that NOCs in food are found more frequently, and at higher concentration, in Asia than Western countries [141]. Endogenous NOCs are formed through nitrosation of secondary amines or amides by nitrite, derived either directly

from food or water, or indirectly from reduction of nitrate by oral and enteric bacteria [7-11]. Approximately 45%-75% of the total NOC exposure in humans is derived from endogenous synthesis [14]. In humans, nitrosation takes place in low acidity environments such as the stomach, especially when antioxidant levels are low. Nitrosation reactions can be enhanced by certain bacteria as well as under certain inflammatory conditions such as oxidative burst [15]. Individuals with high exposure to NOCs are hypothesized to be at increased risk of developing gastric cancer.

Epidemiological studies examining the association between NOCs or NOC-containing food and gastric cancer risk in humans provide inconsistent results [16]. These inconsistencies are likely due to measurement error in the assessment of exposure to NOCs in most, if not all, epidemiological studies. A biomarker approach that assesses total NOCs, derived from both exogenous and endogenous sources, would overcome this limitation and increase the validity of results. In the present study, we quantified levels of nitrate and nitrite, two precursors of NOCs, as well as *N*-nitroso-2-methylthiazolidine-4-carboxylic acid (NMTCA), *N*-nitrosoproline (NPRO), *N*-nitrososarcosine (NSAR), and *N*-nitrosothiazolidine-4-carboxylic acid (NTCA) in urine samples collected more than 10 years before subjects developed gastric cancer. This study was nested within the Shanghai Cohort Study, a prospective cohort of 18,244 middle-aged and older men, in Shanghai, China with up to 12 years follow-up. The primary aim of the present study was to evaluate the association between urinary levels of nitrate, nitrite, or NOCs and risk of developing gastric cancer. The present study also investigated the potential modifying effect of infection with *H. pylori* bacteria, alcohol consumption, cigarette smoking, serum antioxidants and urinary epigallocatechin (EGC) on the associations between urinary nitrate, nitrite or NOCs and risk of gastric cancer.

3.3 Material and Methods

3.3.1 Study Population

The design of the Shanghai Cohort Study has been described in detail elsewhere [142, 143]. Briefly, four small, geographically defined communities over a wide area of the city of Shanghai were selected for a prospective, epidemiologic study of diet and cancer. Complete rosters of all residents in these selected communities for identification of eligible study subjects were obtained from local police stations. The eligibility criteria were men aged 45-64 years who had no history of cancer. Between January 1986 and September 1989, 18,244 men (~80% of eligible subjects) participated in the study. Each participant was interviewed in person using a structured questionnaire to obtain demographic information, use of tobacco and alcohol, usual adult diet, and medical history. At the completion of the interview, a 10-mL nonfasting blood sample and a single-void (i.e., spot) urine sample were collected from each participant. Blood and urine samples were collected usually between 5 pm and 9 pm and placed in an icebox (~4 ℃) immediately after collection. Multiple aliquots of serum and urine from each subject were prepared and stored at ultra-low temperature until analysis. From each subject one 25 ml vial of urine was mixed with 100 mg sodium hydroxide (NaOH) before freeing for long-term storage. The study was approved by the Institutional Review Boards at the University of Pittsburgh, University of Minnesota and the Shanghai Cancer Institute.

Current diet was assessed through a food frequency questionnaire that included 45 food items that represented commonly consumed local foods in early 1980's. Annual follow-up for incident cancers and deaths has been carried out since 1986. All surviving participants were contacted in-person annually and we also performed record linkage analysis with databases of the Shanghai Cancer Registry and Shanghai Municipal Vital Statistics. By the end of 2008, only 550 (3.0%) original cohort participants were lost to our anuual follow-up interview. In addition, 492 subjects refused our request for annual follow-up interviews, although their

cancer and vital status were confirmed through record linkage analysis. Thus the follow-up of the cohort for incidence of cancer and death was almost complete.

3.3.2 Case patients

By March 1998, 197 cohort participants had developed gastric cancer and were eligible for the present study. Diagnoses of 179 (91%) cancers were based on histopathologic evidence. The remaining 18 (9%) cases were diagnosed based solely on clinical evidence (n = 14) or death certificate (n = 4).

3.3.3 Control Subjects

For each case, three control subjects were chosen among all eligible participants of the cohort study. All three control subjects were matched to index cases by age (±2 years), month and year of biospecimen collection, and neighborhood of residence at recruitment.

3.3.4 Laboratory assays

NSAR, NPRO, NTCA and NMTCA were analyzed by gas chromatography coupled with Gas Chromatography-Thermal Energy Analysis (GC-TEA) according to a previously reported method [144]. From each subject a 7.5 mL aliquot of NaOH-treated urine was extracted 3 times with 20 mL of methanol-dichloromethane (1:9, v/v) after addition of 75 ng N-nitrosopipecolic acid (NPIC) as internal standard, 2.0 g NaCl, and 1.5 mL 20% ammonium sulfamate solution in 1.8 M H_2SO4. The combined extracts were dried over anhydrous sodium sulfate and concentrated to dryness by rotary evaporator at 30 ℃, and derivatized in 2 ml ether with excess diazomethane (prepared with 2 g N-Methyl-N-Nitroso-p-toluenesulfonamide, 60 ml ether, 12 ml of 60 % potassium hydroxide and 12ml of methanol).The methyl ester of the five N-nitrosamino acids in ethereal solution were concentrated to 0.1 ml and quantified with a 10 ul aliquot by GC-TEA. For GC, a glass column (2 m x 3 mm i.d.) packed with 5 % FFAP on

Chromosorb WHP (80-100 mesh) was used at a temperature of 180 ℃. The temperature of the injection port of the GC was 200 ℃, and the flow rate of the nitrogen carrier gas was 50 ml/min. For the TEA, the temperature of the pyrolyzer was 500 ℃, interface was 200 ℃, and vacuum to 0.9 mm Hg. The recoveries of NSAR, NPRO, NMTCA, and NTCA added at 30 ug/L each were 75 %, 79 %, 91 % and 96 % respectively. The detection limits ranged from 0.1 - 0.5 ug/L, depending on the compound.

Nitrate and nitrite were analyzed according to the method described previously with some modification [145]. Cadmium was prepared by the reaction of zinc with 20% cadmium sulphate. Ammonium chloride buffer solution was prepared by diluting 20 ml HCl to 500 ml, then mixing HCl solution with 50 ml NH_4OH, and finally diluting the mix solution to 1L with water. Adjust ammonium chloride buffer solution pH to 9.6-9.7. 0.5% Sulfanilamide solution was prepared by dissolving 1.25g sulfanilamide with 6N HCl to 250 ml. Standard solutions of nitrate and nitrite were prepared by dissolving 0.724 g of KNO_3 and 0.493 g of $NaNO_2$ with water to 1000 ml, respectively. Urine samples were deproteined before measurement of nitrite and nitrate was performed. After adding 15 ml of water and 2.4 ml of 0.5 N NaOH to 2 ml urine sample to adjust pH to 8-9, the mixture was incubated at 50-60 ^{o}C for 10 min, then 2 ml 12% $ZnSO_4$ solution was added and incubated at 50-60^{o}C for another 10 min, and additional 1 ml 0.5 N NaOH was added before the contents cooled to room temperature. After adding 17.6 ml of water, the sample was passed through filter paper and the filtrate was collected after discarding the first 10 ml filtrate. For measurement of nitrite, 5 ml 0.5% sulfanilamide and 2 ml 0.5% N- (1-naphthyl) ethylenediamine dihydrochloride was added to the 10 ml aliquot of filtrate described above before the absorbance of the final solution was read at 540 nm. For determination of nitrate, 5 ml NH_4OH buffer solution and 18 ml water was added to 2 ml filtrate before it passed through a Cd column at 3-5 ml/min. The column was washed with 15 ml water and the combined effluent collected. After adding 5 ml 0.5% sulfanilamide and 2 ml 0.5% N-(1-naphthyl) ethylenediamine dihydrochloride, the absorbance of the final solution was read at 540 nm after standing for 20 min. The final concentrations of nitrite and nitrate

were calculated using the standard curve.

Untreated serum samples were used for measurements of serum antioxidants including α-carotene, β-carotene, β-cryptoxanthin, lycopene, lutein/zeaxanthin, retinol, α-tocopherol, γ-tocopherol, and vitamin C. The serum concentrations of all these micronutrients, except vitamin C, were determined by high performance liquid chromatography using methods described previously [146, 147]. The serum concentration of vitamin C was determined by a method developed previously with some modifications[148, 149]. The urinary concentration of epigallocatedchin (EGC) was measured according to a validated method described previously [150]. A history of infection with *H. pylori* was determined by detection of serum immunoglobulin G antibodies to *H. pylori* using an enzyme-linked immunosorbent assay (ELISA) described previously [17]. This ELISA was developed and validated using *H. pylori* strains that were prevalent in the study population [17].

For all laboratory measurements, samples within a given matched set were assayed in the same run. Six cases and three control subjects who were not matched to these six cases were missing at least one NOC measurement and were excluded from the study. Also, an additional control who was not eligible for this study was also excluded. Thus, the present study included 191 cases and 569 control subjects.

3.3.5 Statistical Analysis

Nitrate, nitrite and NOCs levels were expressed in units of urinary creatinine (mol/g Cr) to correct varying water contents of individual spot urine samples. The distributions of urinary NOCs levels were markedly skewed with few subjects possessing high values, which were corrected to a large extent by transformation to logarithmic values. Therefore, formal statistical tests on continuous values of NOCs, nitrate and nitrite were performed on logarithmically transformed values. Spearman rank correlation analysis was performed to assess the relationships between individual urinary NOCs, serum antioxidants and urinary EGC. The χ^2 test was used to compare the distributions of selected demographics, cigarette

smoking, alcohol consumption and seropositivity to *H. pylori* between cases and controls. The analysis of covariance (ANCOVA) method was used to examine differences in the geometric mean of urinary NOCs among different levels of alcohol consumption and selected food in controls and Wilcoxon test for the difference in the center of the distributions of analytes between cases and controls.

We used conditional logistic regression models to examine associations between urinary NOCs, nitrate and nitrite levels and gastric cancer risk [151]. The associations were measured by odds ratios (OR) and their corresponding 95% confidence interval (CI) and *Ps* for trends. Study subjects were grouped into three levels (low, intermediate, and high) based on the tertile or high/low distributions of detectable urinary NOCs among all control subjects. The linear trend tests for exposure-disease associations were based on ordinal values (1-3) for the three exposure categories. We also examined the NOCs-gastric cancer risk associations in subgroups stratified by cigarette smoking (ever or never), alcohol consumption (ever or never), seropositivity to *H. pylori* (negative or possitive), serum antioxidant levels (high or low) and urinary EGC level (high or low). For the subgroup analyses, matched sets were broken and unconditional logistic regression was used to maximize the sample size in each subgroup analysis. The matching factors (age, year of biospecimen collection, and neighborhood of residence at recruitment) were included in all unconditional logistic regression models as covariates. The presence of *H. pylori* antibodies in serum, cigarette smoking, and heavy alcohol consumption were identified risk factors for gastric cancer in this study population[17, 24]. The distribution of level of education showed a significant difference between case and control in this population [152]. These potential confounders were included in the multivariate logistic regression models of NOCs, nitrate and nitrite and gastric cancer risk. We also examined the NOCs-gastric cancer association with further adjustment for epigallocatedchin (EGC), β-carotene and vitamin C that were previously demonstrated to be inversely related to the risk of developing gastric cancer in this study population [150, 152].

Statistical computing was conducted using the using the SAS version 9.2 (SAS Institute

Inc., Cary, NC). All p values reported are two-sided, and those that were less than 0.05 were considered to be statistically significant.

3.4 Results

The present study included 191 case patients with gastric cancer and 570 matched controls. The mean age (SD) of case patients at cancer diagnosis was 63.4 (5.6) years. The average time interval between biospecimen collection and cancer diagnosis was 5.1 (3.0) years, ranging from 1 month to 12 years. There was no significant difference in body mass index (kg/m^2) or level of education between cases and controls. Individuals who developed gastric cancer consumed more cigarettes and alcohol, and were more likely to be seropositive for *H. pylori* than their matched controls (Table 1). Compared to never smokers, ever smokers had a statistically significant 60% increased risk of gastric cancer (OR=1.60, 95% CI=1.11–2.29), and heavy drinkers of alcoholic beverages (4 or more drinks/day) had a statistically significant 141% increased risk of gastric cancer (OR=2.41, 95% CI=1.41–4.11) compared to nondrinkers. Of the 182 cases with known serologic status, 162 (89%) were positive for antibodies to *H. pylori* whereas 447 (82.6%) of the 541 controls with known *H. pylori* serology had detectable antibodies to *H. pylori* (OR=1.70, 95% CI = 1.02-2.85).

The median, 5^{th} and 95^{th} percentiles of urinary NOCs, nitrate and nitrite in gastric cancer cases and control subjects are presented in Table 2. Overall, there were no significant differences in urinary concentrations of NMTCA, NPRO, NSAR, NTCA, nitrate and nitrite between case patients and control subjects. The correlation coefficients between individual NOCs ranged from 0.16 to 0.70 (Table 3). The highest correlation coefficient was seen between NPRO and NTCA (r = 0.70), followed by NPRO and nitrate (r = 0.51), NTCA and nitrate (r=0.51). A moderate correlation with r less than 0.5 was seen for NMTCA and NTCA (r = 0.48), nitrite and nitrate (r = 0.31) and NPRO and NMTCA (r = 0.29). The associations between serum carotenoids, vitamin C and vitamin E and urinary NOCs were much weaker. All Spearman correlation coefficients were in the range of -0.16 to 0.13 (Table 4). Urinary

EGC levels were positively associated with urinary nitrite (r = 0.23, p= 0.0001).

Next, we examed urinary levels of NOCs by the status of consumption frequency of red meat (pork), and eight kinds of preserved food, including furu, bean paste, salted vegetables, pickled vegetables, salted fish, salted pork, sausage&ham, and salted egg (significnat results were shown in table 5). Total salted animal protein consumption was significantly and dose-dependently associated with increased urinary levels of NMTCA (*P*=0.03). Salted vegetables and total preserved vegetables consumption were significantly and dose-dependently associated with decreased urinary levels of nitrate and statistical significance remains after the adjustment by fresh vegetables intake ($P < 0.003$ and $P < 0.002$, respectively). Other preserved food consumption has no effect on urinary levels of NOCs.

We also investigated the association of NOC biomarkers and disease risk factors among control subjects (Table 6). Alcohol drinking was significantly associated with increased urinary levels of NMTCA (*P*<0.0001). The association was dose-dependent; NMTCA was 3.75 (ug/g Cr) for drinkers consuming less than 2 drinks/day and 4.21 (ug/g Cr) for drinkers with 2 or more drinks/day (*P* for trend <0.0001). Smokers showed higher levels of urinary NSAR and nitrite than nonsmokers, but their differences were not statistically significant. Control subjects seropositive to *H. pylori* had statistically significantly higher urinary levels of nitrite than those without detectable *H. pylori* antibodies (P =0.048). There were no significant differences in NPRO, NTCA and nitrate by smoking, drinking or *H. pylori* status.

Relative risk of gastric cancer in relation to tertile levels of urinary NOCs, nitrate and nitrite was investigated in all study subjects (Table 7). Overall, there was no statistically significant association between levels of NOCs, nitrate, or nitrite and risk of gastric cancer before or after adjustment for alcohol use, cigarette smoking, and seropositivity to *H. pylori*. Further adjustment for EGC, β-carotene and vitamin C did not materially alter these null associations.

We also examined whether *H. pylori* status modified the association between urinary levels of NOCs and gastric cancer risk using stratified models (Table 8). Among individuals

seronegative to *H. pylori*, elevated urinary nitrate was associated with a borderline statistically significant increase in gastric cancer risk; the multivariate-adjusted ORs (95%) for gastric cancer in the second and third tertiles of nitrate were 3.39 (0.82~13.96) and 4.18 (0.96~18.13), respectively, compared with the lowest tertile of nitrate (P for trend = 0.057). There was no evidence for effect modification of the NOCs-gastric cancer association for smoking, alcohol consumption, serum antioxidants, and urinary EGC (data not shown).

3.5 Discussion

To the best of our knowledge, the present study was the first to use pre-diagnostic urinary biomarkers of NOCs and their precursors (i.e., nitrate and nitrite) to investigate the association between NOCs and the risk of developing gastric cancer. We observed a marginally significant positive association between urinary nitrate and gastric cancer risk among *H. pylori* negative individuals. In addition, among control subjects, elevated levels of urinary nitrite were associated with seropositivity to *H. pylori*, suggesting that infection with *H. pylori* may enhance the reduction of nitrate to nitrite *in vivo*. Further, there was a strong dose-response association between alcohol consumption and urinary levels of NMTCA, indicating that NMTCA could be a biomarker for alcohol intake.

Infection with *H. pylori* has been found to be associated with risk of developing gastric ulcer and gastric cancer[26]. The presence of *H. pylori* in the stomach causes inflammatory damage to the mucosa of the stomach, which may enhance the endogenous formation of NOCs in the stomach [15, 153, 154]. An elevated level of urinary nitrite in individuals seropositive to *H. pylori* supports this hypothesis of *H. pylori* contribution to nitrosation in the stomach. The interactive role of *H. pylori* and *N*-nitroso compounds in the development of gastric cancer requires further studies.

Among individuals seronegative to *H. pylori*, urinary levels of nitrate, a precursor of *N*-nitroso compounds, were positively associated with risk of gastric cancer. Chronic infection with *H. pylori* is an established risk factor for gastric cancer. It is not surprising that

the association between nitrate and gastric cancer risk was more apparent among individuals without *H. pylori* infection, a relatively low risk population. The findings of the present study suggest that the effect of nitrate through the nitrosation pathway could be masked by *H. pylori* infection, especially in the present study population where *H. pylori* infection is highly prevalent.

Certain dietary factors might have higher amounts of NOCs or favoring conditions which benefit the endogenous synthesis of NOCs[13]. We found that total salted animal protein consumption was significantly and dose-dependently associated with increased urinary levels of NMTCA. Salted vegetables and total preserved vegetables consumption were significantly and dose-dependently associated with decreased urinary levels of nitrate (P<0.003 and P<0.002). These results are consistent with the findings from the investigation of western diet that processed meat products have high level of NMTCA and nitrate exposure mainly come from fresh vegetable comsumption [27, 28].

High levels of NMTCA in the urine of alcohol drinkers suggest that NMTCA could be a metabolic biomarker of alcohol intake. Acetaldehyde, a metabolite of ethanol [155], participates in the synthesis of NMTCA through the reaction with L-cysteine [156]. Administration of L-cysteine and acetaldehyde together with nitrate significantly increased urinary excretion of NMTCA in an animal model [157]. The present study observed a similar effect of alcohol intake on NMTCA formation in humans. These findings may shed some light on the mechanism of alcohol intake in relation to risk of gastric cancer. Additional studies are warranted to confirm our findings.

In the present study, we found that several NOCs levels in urine were correlated with each other. NTCA and NPRO had the highest correlation (r=0.70) and both were significantly correlated with nitrate (r=0.51). Due to the complexities of the assays for multiple NOCs in a single batch run, there are very limited prior data on the relationships among NOCs on an individual basis. According to a recent survey on the food sources of NOCs，NTCA and NPRO are two of the most frequently assayed NOCs and share similar food sources such as

processed meat products [27]. In regard to endogenous formation of these two NOCs, the *in vivo* administration of nitrate and their respective secondary amine precursor has shown high efficiency in the production of NTCA and NPRO [144, 158]. Therefore, it is not surprising to see a high correlation for NTCA and NPRO within individuals given their similar exogenous sources and endogenous synthesis.

A positive association between dietary intake of nitrites and nitrosamines and gastric cancer risk has been observed in several case-control studies [159-162]. The consumption of meat, processed meat, preserved fish and preserved vegetables increase the risk of gastric cancer from 1.10 to 5.51 fold [16]. However, several cohort studies showed inconsistent results. For instance, a large multicenter European cohort study reported that individuals with the highest propensity for endogenous NOCsformation had an elevated risk for gastric cancer [128]. In contrast, a large Finnish cohort study found no association between estimated intake of nitrites, nitrates and NMDA and risk of gastric cancer [163]. Using selected urinary biomarkers of NOCs and their precursors, we found a marginally positive association between prediagnostic urinary nitrate level and subsequent development of gastric cancer among *H. pylori*-negative individuals only. The lack of an overall positive association between urinary levels of selected NOCs and their precursors and risk of gastric cancer could be that the selected few biomarkers tested in the present study might not be representative of total NOCs. Further investigation using a more complete profile of NOCs in urine might be necessary to more completely understand the role of NOCs in the development of gastric cancer.

Studies have demonstrated that vitamin C, vitamin E and polyphenols in green tea extract indirectly inhibit the nitrosation of amino compounds by scavenging nitrosating agents [15, 25]. In the present study, serum measurements of antioxidants were not inversely associated with urinary levels of NOCs. The null association between urinary NOC and the risk of developing gastric cancer remained regardless of serum levels of antioxidants measured.

Our study had several strengths. A prospective study design and the availability of prediagnostic urinary specimens minimized the possible influence of disease symptoms on dietary intake and other lifestyle factors and also ruled out the possibility of recall bias on exposure. Considering the limitation of food frequency questionnaire in the estimation of NOCs exposure and considerable endogenous NOCs production [14], urinary levels of NOCs as exposure biomarkers may overcome such limitations and capture both exogenous and endogenous NOCs. The simultaneous adjustment for cigarette smoking, alcohol drinking, *H. pylori* infection, urinary level of EGC, serum levels of β-carotene and vitamin C could reduce their potential confounding effect on the NOCs-gastric cancer associations. The almost complete follow-up for incident cancer and death minimized the potential bias on results due to the loss to follow-up.

There are also several potential limitations to our study. One cannot presume that NOCs levels in a randomly timed, single void urine sample represented the long-term exposure to both exogenous and endogenous NOCs. It would be ideal, but rarely feasible to assess NOC exposure at multiple time points prior to disease occurrence, especially using a biomarker approach as this study, due to the high cost and logistical complexity involved in collecting biospecimens from large numbers of study participants. Another major limitation of the present study was the relatively small sample size given the labor intensive assays of urinary NOCs, which prohibited us from conducting more detailed statistical analyses.

In summary, the present study showed a borderline statistically significant positive association between prediagnosic urinary levels of nitrate and gastric cancer development in *H. pylori* negative people. The positive nitrate-gastric cancer association was independent of cigarette smoking, alcohol consumption and exposure to dietary intake of EGC, β-carotene and vitamin C. The present study showed a statistically significant dose-response relationship between alcohol intake and urinary levels of NMTCA. The elevated urinary nitrite level in *H. pylori* positive subjects suggests that *H. pylori* at the stomach may enhance the endogenous formation of NOCs in the presence of amines or amides.

Part 4 Summary of Findings and Future Research Recommendations

Gastric cancer is the fourth most common cancer in the world, but incidence rates vary between countries with profound high incidence in Asian countries like China and Japan. N-nitroso compounds (NOCs) are potential carcinogens synthesized during food processing or by nitrosation of secondary amines or amides in vivo, and have been considered as one of dietary deleterious factor determining gastric cancer risk. This study used quantified urinary levels of N-nitroso-2-methylthiazolidine-4-carboxylic acid (NMTCA), N-nitrosoproline (NPRO), N-nitrososarcosine (NSAR), N-nitrosothiazolidine-4-carboxylic acid (NTCA), and NOCs-precursor nitrate and nitrite as exposure proxy to evaluate the association between prediagnostic urinary levels of these NOCs and risk of gastric cancer in the Shanghai Cohort Study, a prospective cohort of 18,244 middle-aged and older man with up to 12 years of follow-up. In the present study, we found a borderline significant positive association between urinary level of nitrate and gastric cancer development in H. pylori negative subgroup by logistic regression models. The multivariate-adjusted ORs (95%) for gastric cancer in the second and third tertiles of nitrate were 3.39 (0.82~13.96) and 4.18 (0.96~18.13), respectively, compared with the lowest tertile of nitrate (*P* for trend = 0.057). Our study also found that urinary NMTCA level was significantly higher in drinkers than nondrinkers (P<0.0001) and showed a dose-dependent effect (p<0.0001). Significant linear relationships amongst urinary level of NTCA, NPRO and nitrate were also found in present study with r^2 ranging from 0.51 to 0.71.

With regarding to future research, it might be more ideal to assess exposure at multiple time points prior to disease occurrence. And instead of collecting one spot urine, 24 hour urine samples might provide more accurate estimation of NOCs in vivo exposure. Different subdivisions of gastric cancer defined either by carcinoma's location or histopathology might

to have different pathogenesis [124, 127]. Analyses on the NOCs-GC association stratified by those factors are also suggested to be performed in future studies. In fact, up to date, more than hundreds NOCs have been reported and tested for carcinogenicity and epidemiological data from showed that urinary concentrations of these four NOCs are highly variable in urine for Chinese population and these four NAAs investigated in the present study may only reflect a partial fraction of total NOCs in urine [140]. Hence, further investigation on a more completed profile of NOCs, derivatives or total NOCs in the urine sample might provide more information. Also, due to most NOCs will metabolized in the body after exposure, it may be difficult to detect its original form in urinary samples. Thus directly detecting them from gastric fluid samples might provide more accurate results. Recently, more studies focus on the roles of gene polymorphisms on the development and progress of gastric cancer. A numbers of single nucleotide polymorphisms of candidate genes, including inflammation-related genes, gastric acid inhibition-related genes, and immune response-related genes have been studied and showed the potential involvement in the development of gastric cancer [164]. Thus for future studies, interaction roles between NOCs exposure and gastric candidate genes' polymorphisms might provide a more panoramic idea of the etiology of gastric cancer. In addition, most previous studies related NOCs' carcinogenic properties to its potential DNA alkylating ability. Thus, determination of alkylated nucleic acid bases in stomach tissue may provide more clues of the roles NOCs play in the development of gastric cancer [165].

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Appendix

Table 1. Demographic characteristics of patients with gastric cancer (Cases) and their control subjects (Controls), The Shanghai Cohort Study.

	Cases	Controls	P value*
Number	191	569	
Age at interview (year), mean (SD)	58.3 (5.3)	58.3 (5.1)	0.98
Body mass index (kg/m2), mean (SD)	22.4 (3.4)	22.2 (3.1)	0.36
Level of education (%)			
No formal schooling or primary school	71 (37.1)	188 (33.0)	0.28
Jr. middle school	64 (33.5)	175 (30.8)	
Senior high school	22 (11.5)	95 (16.7)	
College graduates or above	34 (17.8)	111 (19.5)	
Alcohol drinking on a weekly basis (%)			
Nondrinkers	97 (50.8)	325 (57.1)	0.13
Ever Drinkers	94 (49.2)	244 (42.9)	
No. of alcoholic drinks per day (%)			
Nondrinkers	97 (50.8)	325 (57.1)	0.021
<1	27 (14.1)	87 (15.3)	
1 to <2	21 (11.0)	56 (9.8)	
2 to <4	18 (9.4)	62 (10.9)	
4+	28 (14.7)	39 (6.9)	
No. of years of alcohol drinking (%)			
Nondrinkers	97 (50.8)	325 (57.1)	0.16
<20	22 (11.5)	68 (12.0)	
20 to <40	54 (28.3)	116 (20.4)	
40+	18 (9.4)	60 (10.5)	
Cigarette smoking (%)			
Never smokers	59 (30.9)	238 (41.8)	0.027
Former smokers	22 (11.5)	53 (9.3)	
Current smokers	110 (57.6)	278 (48.9)	
No. of years of smoking (%)			
Never smokers	59 (30.1)	238 (41.8)	0.011
<30	52 (27.2)	109 (19.2)	
30+	80 (41.9)	222 (39.0)	
No. of cigarettes per day (%)			
Never smokers	59 (30.9)	238 (41.8)	0.014
<20	69 (36.1)	154 (27.1)	
20+	63 (33.0)	177 (31.1)	
H. pylori antibody serologic status (%)			
Negative	20 (11.0)	94 (17.4)	0.041†
Positive	162 (89.0)	447 (82.6)	

*P-values (2-sided) were derived from t-test (for means) or Chi-Square (for frequencies) statistic.

†Nine cases and twenty eight controls with missing H. pylori status were excluded from these data analysis.

Table 2. Median (range) levels of urinary N-nitroso compounds in patients with gastric cancer (cases) and controls subjects (controls), The Shanghai Cohort Study

N-nitroso compounds	Cases (5th-95th percentile)	Controls (5th-95th percentile)	2-sided P Value*
NMTCA (ug/g Cr)	2.22 (0～39.87)	2.11 (0～36.52)	0.69
NPRO (ug/g Cr)	4.52 (0.59～20.29)	4.30 (0～42.94)	0.89
NSAR (ug/g Cr)	0 (0～2.67)	0 (0～4.65)	0.87
NTCA (ug/g Cr)	5.63 (0～48.88)	7.3 (0～72.78)	0.20
Nitrate (mg/g Cr)	169.13 (49.41～684.67)	190.03 (40.91～1189.32)	0.56
Nitrite (mg/g Cr) †	7.41(2.15～71.49)	8.08 (1.95～72.85)	0.34

* 2-sided P values were derived from Wilcoxon test.
†Eighty seven cases and two hundred and sixty one controls with missing values of Nitrite were excluded from these data analysis.

Table 3. Spearman correlation coefficients between urinary N-nitroso compounds among control subjects only, Shanghai Cohort Study

	NPRO	NSAR	NTCA	Nitrate	Nitrite†
NMTCA	0.29*	-0.066	0.48*	0.20*	-0.070
NPRO		0.067	0.70*	0.51*	0.20*
NSAR			-0.029	-0.077	0.027
NTCA				0.51*	0.16*
Nitrate					0.31*

*P<0.05

†Two hundred and sixty one controls with missing values of Nitrite were excluded from these data analysis

Table 4. Spearman correlation coefficients between micronutrients and urinary N-nitroso compounds among control subjects only, Shanghai Cohort Study

	NMTCA	NPRO	NSAR	NTC	Nitrat	Nitrite†
EGC(umol/g Cr)※	-0.051	0.087*	-0.0025	0.0023	0.094*	0.23*
Vitamin C(mg/100ml)	0.022	0.090*	0.081	0.087*	0.061	-0.030
Alpha-carotene(mcg/dl)☆	-0.091*	-0.093*	-0.088*	-0.12*	-0.052	-0.110
Beta-carotene(mcg/dl)☆	0.046	0.099*	-0.015	0.093*	0.058	0.033
Total carotenes(mcg/dl)☆	0.031	0.084*	-0.018	0.073	0.051	0.017
Alpha-tocopherol(mg/l)☆	0.040	-0.009	0.098*	-0.030	-0.067	-0.056
Gamma-tocopherol(mg/l)☆	0.065	0.048	0.003	0.050	0.024	-0.042
Total tocopherol(mg/l)☆	0.047	0.009	0.083*	-0.007	-0.047	-0.076
Cryptoxanthin(mcg/dl)☆	0.13*	0.080	0.014	0.095*	-0.053	0.018
Lycopene(mcg/dl)☆	-0.025	-0.060	-0.056	-0.071	-0.060	-0.16*
Lutein+Zeaxanthin(mcg/dl))☆	0.047	0.014	-0.046	-0.006	-0.049	-0.002
Retinol (mcg/dl)☆	0.12*	-0.035	0.057	0.0051	-0.025	0.022

*P<0.05
†Two hundred and sixty one controls with missing values of Nitrite were excluded from these data analysis.
※Twenty controls with missing values of EGC were excluded from these data analysis.
☆Three controls with missing values of Alpha-caroten, Beta-caroten, total Carotenes, Alpha-tocopherol, Gamma-tocopherol, total Tocopherol, Cryptoxanthin, Lycopene , Lutein+Zeaxanthin and Retinol were excluded from these data analysis, respectively.

Table 5. Urinary levels of NOCs (Geometric means with 95% CI) by the status of consumption frequency of preserved food in control subjects only, Shanghai cohort study.

		N[&]	NMTCA (ug/g Cr)	NPRO (ug/g Cr)	NSAR (ug/g Cr)	NTCA (ug/g Cr)	Nitrate (mg/g Cr)	Nitrite† (mg/g Cr)
Salted vegetables	None	129	2.29(1.44~3.44)	5.11(3.85~6.77)	0.43(0.25~0.67)	7.33(5.42~9.91)	224.88(176.68~289.03)	8.87(6.17~12.6)
	< Monthly	74	3.1(1.77~5.05)	6.32(4.37~8.97)	0.43(0.19~0.73)	7.41(4.93~10.94)	205.44(148.9~283.29)	8.3(5.3~12.74)
	< Weekly	237	2.46(1.77~3.31)	5.3(4.31~6.46)	0.35(0.21~0.49)	7.67(6.1~9.49)	199.34(166.34~238.85)	8.78(6.77~11.18)
	Weekly+	129	2.53(1.61~3.71)	4.42(3.26~5.82)	0.3(0.13~0.49)	6.77(4.99~9.18)	144.47(112.3~183.93)	10.7(7.58~14.8)
	P* for trend		0.87	0.30	0.15	0.76	0.003	0.37
Total preserved vegetable (Salted & pickled)	None	121	2.42(1.53~3.66)	5.3(3.9~7)	0.46(0.26~0.68)	7.5(5.49~10.13)	231.76(180.27~300.87)	8.87(6.03~12.87)
	< Monthly	76	3.01(1.72~4.87)	5.82(4.05~8.3)	0.42(0.19~0.7)	7.08(4.7~10.36)	199.34(144.47~274.89)	8.12(5.23~12.46)
	< Weekly	237	2.39(1.75~3.22)	5.3(4.31~6.54)	0.35(0.21~0.49)	7.58(6.1~9.49)	197.34(164.67~236.46)	8.78(6.85~11.18)
	Weekly+	135	2.49(1.61~3.66)	4.47(3.35~5.89)	0.3(0.13~0.49)	6.92(5.11~9.28)	145.94(115.75~185.79)	10.82(7.76~14.8)
	P* for trend		0.84	0.29	0.12	0.77	0.002	0.31
Total salted animal protein	< Monthly	44	1.8(0.7~3.66)	4.47(2.67~7.25)	0.32(0.04~0.68)	6.1(3.48~10.13)	203.38(133.29~310.06)	10.82(6.03~18.69)
	< Weekly	241	2.29(1.64~3.06)	5.42(4.42~6.69)	0.35(0.22~0.49)	7.76(6.24~9.59)	185.79(155.02~222.63)	8.58(6.69~11.06)
	1-2 /Week	220	2.63(1.89~3.53)	5.17(4.16~6.39)	0.46(0.31~0.62)	7.25(5.75~9.18)	205.44(169.72~248.64)	9.28(7.08~12.07)
	3+ /Week	64	3.71(2.1~6.17)	4.58(3.01~6.85)	0.17(-0.04~0.43)	7.17(4.58~10.82)	159.77(112.3~227.15)	9.49(5.89~15.12)
	P* for trend		0.03	0.74	0.77	0.93	0.66	0.96

N[&]: Subjects in different subgroups.
P* for trend are derived from glm model; Values of NOCs are log-transferred; Freqency of Food were divided to 4 groups, and treated as continuous value.
†Two hundred and sixty one controls with missing values of nitrite were excluded from these data analysis.

Table 6. Geometric mean levels of Urinary N-nitroso compounds by status of drinking, smoking and H.Pylori infection among control subjects only, Shanghai Cohort Study.

		Subjects (n)	NMTCA (ug/g Cr)	NPRO (ug/g Cr)	NSAR (ug/g Cr)	NTCA (ug/g Cr)	Nitrate (mg/g Cr)	Nitrite† (mg/g Cr)
Alcohol drinking	No	325	1.70 (1.34~2.12)	5.38 (4.68~6.18)	0.38 (0.28~0.48)	7.74 (6.68~8.96)	190.40 (168.50~215.14)	8.50 (7.20~10.04)
	Yes	244	3.92 (3.18~4.82)	4.88 (4.14~5.72)	0.36 (0.26~0.48)	6.86 (5.78~8.14)	191.74 (166.52~220.76)	10.08 (8.30~12.20)
	P		<0.0001	0.36	0.82	0.30	0.94	0.19
Cigarette smoking	No	238	2.22 (1.72~2.82)	4.98 (4.22~5.86)	0.3 (0.20~0.40)	7.42 (6.22~8.80)	192.84 (167.18~222.40)	7.94 (6.54~9.60)
	Yes	331	2.72 (2.22~3.30)	5.30 (4.60~6.06)	0.42 (0.32~0.52)	7.32 (6.30~8.46)	189.66 (168.02~214.06)	10.2 (8.62~12.04)
	P		0.2	0.58	0.080	0.90	0.86	0.053
H. Pylori Status*	Negative	94	2.66 (1.80~3.80)	4.92 (3.82~6.28)	0.34 (0.16~0.52)	6.82 (5.18~8.92)	166.46 (133.66~207.24)	7.00 (5.20~9.30)
	Positive	447	2.50 (2.08~2.96)	5.08 (4.54~5.70)	0.38 (0.30~0.48)	7.48 (6.60~8.46)	191.38 (173.08~211.60)	9.64 (8.40~11.04)
	P		0.75	0.82	0.59	0.55	0.26	0.048

*Seropositiveity of H. pylori antiboedies. Twenty eight controls with missing H. pylori status were excluded from these data analysis.
†Two hundred and sixty one controls with missing values of nitrite were excluded from these data analysis.

Table 7. Levels of urinary N-nitroso compounds in relation to the risk ofgastric cancer, the Shanghai

	Low	Intermediate	High	P for trend
NMTCA (ug/g Cr)	0	≤6.79	> 6.80	
Case/Control*	79 / 233	56 / 158	47 / 150	
OR (95%CI)※	1.0	1.11 (0.73~1.70)	0.85 (0.55~1.32)	0.55
NPRO (ug/g Cr)	≤2.60	2.61～7.51	>7.51	
Case/Control*	54 / 181	70 / 186	58 / 174	
OR (95%CI)※	1.0	1.38 (0.90~2.12)	1.11 (0.71~1.73)	0.67
NSAR (ug/g Cr)	0	≤0.77	> 0.78	
Case/Control*	122 / 350	24 / 94	36 / 97	
OR (95%CI)※	1.0	0.69 (0.40~1.2)	1.00 (0.61~1.64)	0.80
NTCA (ug/g Cr)	≤3.63	3.64～12.16	>12.16	
Case/Control*	63 / 177	65 / 191	54 / 173	
OR (95%CI)※	1.0	0.96 (0.63~1.46)	0.83 (0.54~1.28)	0.40
Nitrate (mg/g Cr)	≤114.9	115.0～285.46	>285.46	
Case/Control*	50 / 179	81 / 190	51 / 172	
OR (95%CI)※	1.0	1.41 (0.92~2.17)	1.01 (0.64~1.61)	0.99
Nitrite (mg/g Cr) §	≤5.30	5.31～11.87	>11.87	
Case/Control*	39 / 100	23 / 103	40 / 99	
OR (95%CI)※	1.0	0.61 (0.32~1.15)	1.04 (0.54~2.03)	0.92

* Number of cases/number of controls.

※Odds ratios (ORs) were derived from conditional logistic regression models. This model also included the following covariates as

covariates: level of education (No formal schooling or primary school, junior middle school, senior high school, and college or above), number of alcoholic drinks per day (non, 1, 1 to <2. 2 to <4, and 4 or more), cigarette smoking (never, ever), and seropositivity of antibodies to H. Pylori (negative, positive). Nine cases and twenty eight controls with missing H. pylori status were excluded from these data analysis.

§Eighty seven cases and two hundred and sixty one controls with missing values of Nitrite were excluded from these data analysis

Table 8. Odds Ratios for Gastric Cancer in Relation to Urinary N-nitroso Compounds by H.pylori serologic status, the Shanghai Cohort Study

	Subjects with negative H. pylori serology					Subjects with positive H. pylori serology			
	Low	Intermediate	High	P for trend		Low	Intermediate	High	P for trend
NMTCA									
Cases/Controls*	7/37	9/31	4/26			72/196	47/127	43/124	
OR (95% CI)†	1.0	1.66(0.52~5.36)	0.73(0.18~2.96)	0.75		1.0	1.08 (0.69~1.68)	0.88 (0.56~1.39)	0.65
NPRO									
Cases/Controls*	6/29	10/36	4/29			48/152	60/150	54/145	
OR (95% CI)†	1.0	1.22(0.36~4.09)	0.57(0.13~2.44)	0.45		1.0	1.27 (0.81~2.00)	1.16 (0.73~1.84)	0.53
NSAR									
Cases/Controls*	17/58	1/19	2/17			105/292	23/75	34/80	
OR (95% CI)†	1.0	0.19(0.02~1.60)	0.34(0.07~1.68)	0.091		1.0	0.89 (0.52~1.51)	1.2 (0.75~1.93)	0.55
NTCA									
Cases/Controls*	6/33	10/38	4/23			57/144	55/153	50/150	
OR (95% CI)†	1.0	1.25(0.39~3.95)	0.79(0.18~3.39)	0.80		1.0	0.89 (0.57~1.39)	0.84 (0.53~1.31)	0.44
Nitrate									
Cases/Controls*	3/35	9/34	8/25			47/144	72/156	43/147	
OR (95% CI)†	1.0	3.39(0.82~13.96)	4.18(0.96~18.13)	0.057		1.0	1.35 (0.87~2.10)	0.87 (0.54~1.41)	0.58
Nitrite §									
Cases/Controls*	3/24	3/23	3/12			36/76	20/80	37/87	
OR (95% CI)†	1.0	1.33(0.20~8.97)	1.10(0.14~8.77)	0.90		1.0	0.58 (0.30~1.10)	0.94 (0.51~1.74)	0.83

* Number of cases/number of controls.

† Odds ratios were derived from unconditional logistic regression models that included matching variables (age, neighborhood and duration of sample storage), and level of education (No formal schooling or primary school, junior middle school, senior high school, and college or above), number of alcoholic drinks per day (non, 1,1 to <2. 2 to <4, and 4 or more) and cigarette smoking (never, ever).

§Eighty seven cases and two hundred and sixty one controls with missing values of Nitrite were excluded from these data analysis.