

Swedish moist snuff accelerates gastric cancer development in *Helicobacter pylori*-infected wild-type and gastrin transgenic mice

Björn Stenström¹, Chun-Mei Zhao¹, Arlin B. Rogers², Hans-Olof Nilsson³, Erik Sturegård^{3,4}, Steinar Lundgren^{1,5}, James G. Fox², Timothy C. Wang⁶, Torkel M. Wadström³ and Duan Chen^{1,*}

¹Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, NO-7006 Norway,

²Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, MA 01451, USA, ³Department of Medical Microbiology, Lund University, Lund SE-22362, Sweden, ⁴Department of Clinical Microbiology, Malmö University Hospital, Malmö SE-20502, Sweden, ⁵Department of Oncology, St Olav's Hospital, Trondheim NO-7006, Norway and ⁶Division of Digestive and Liver Diseases, Columbia University Medical Center and Irving Cancer Research Center, NY 10032, USA

*To whom correspondence should be addressed. Tel: +47 725 73320;
Fax: +47 725 76412;
Email: duan.chen@ntnu.no

The Swedish variant of moist oral smokeless tobacco (snus) is popular in Sweden and Norway, banned from sale within the European Union and is currently being introduced in USA. The aim of the present study was to determine if snus is carcinogenic to the stomach, particularly in *Helicobacter pylori* (*H.P.*)-infected hosts at increased risk for gastric cancer development. Snus (GeneralTM; Swedish Match, Sweden) was mixed with powdered standard mouse chow at a concentration of 5–9% (wt/wt) and given to wild-type (WT, FVB) and gastrin transgenic (INS-GAS, FVB) mice for 6 months with or without *H.P.* (strain 67:21, CagA⁺, VacA⁺) infection. At necropsy, pathological evaluation of stomachs from uninfected snus-treated WT mice showed mild morphological changes, whereas 50% snus-treated INS-GAS mice developed carcinoma *in situ* (CIS), compared with 25% not exposed to snus. When snus was given to *H.P.*-infected mice, 9 of 17 WT mice developed CIS with intramucosal invasion, and the remaining 8 of 17 WT mice developed high-grade dysplasia (score >1.5) that was associated with increased gastritis, epithelial defects, oxyntic atrophy, hyperplasia and intestinal metaplasia. Twelve of 12 *H.P.*-infected INS-GAS mice developed CIS with intramucosal invasion and submucosal herniation. We suggest that snus is a potential gastric carcinogen in mice. The development of CIS was associated with increased rates of the epithelial cell proliferation and apoptosis, common features of gastric carcinogenesis.

Introduction

Use of smokeless tobacco increased in USA during the 1970s and 1980s, and fuelled the tobacco-related public health problem by serving as an entry for youth to develop nicotine dependence and eventually switch to cigarette smoking (1,2). The main types of smokeless tobacco in Western countries are chewing tobacco, which is predominantly used in USA, and oral snuff, i.e. snus, in Sweden. Snus is currently banned from sale within the European Union, but being introduced in USA. During the last decade, the number of users of snus has dramatically increased in Sweden and Norway, especially among teenagers; 21% of boys and 8% of girls, 15 years of age, use snus on a regular basis in Sweden (3). The International Agency for Research on Cancer, a branch of the World Health Organization, has recognized the evidence of a relationship between smokeless tobacco and cancer (in 1985 and 2004), largely based on regional epidemio-

logical data, concluding that use of oral smokeless tobacco is carcinogenic (group IIB) (4,5). The Swedish National Institute of Public Health published a report on snus in 2005, concluding that the overall assessment of the experimental and epidemiological evidence indicates that Swedish snus is a carcinogen, containing ingredients that are more toxic than would be permitted in foods or medicines (3). The tobacco-specific *N*-nitrosamines, e.g. *N'*-nitrososonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are well-documented carcinogens (6–10). The primary risk of smokeless tobacco use (possibly including snus) is cancer of the mouth, nose, throat and pancreas (6,10).

Gastric cancer is the 4th most frequent cancer worldwide and 2nd only to lung carcinoma in global cancer mortality (11). This disease has also been recognized as a tobacco-related cancer based on epidemiological studies (12,13). Cigarette smoking was confirmed to increase the incidence and mortality of gastric cancer in a dose- and time-dependent manner (12,13), whereas snus did not seem to be associated with gastric cancer (13). However, due to the small number of exposed cases of snus ever-users, the precision of this estimate was poor (13). Snus is popular in Sweden and Norway where chronic *Helicobacter pylori* (*H.P.*) infection rate is ~40% in the young adult population. Based on sufficient evidence for its carcinogenicity among humans, *H.P.* has been classified as a group I definite carcinogen by the International Agency for Research on Cancer (14). One would expect that the risk of gastric cancer will be significantly increased if these two carcinogens co-exist. Large prospective epidemiological studies of snus and *H.P.* infection status are limited, and there is no report yet about the possible carcinogenic synergism with the combination of snus and *H.P.* infection in animal models.

In several long-term (15 months to 2 years) studies of various wild-type (WT) mouse strains, *H.P.* infection was found to induce gastric inflammation, gastric ulcer and gastric intestinal metaplasia/dysplasia, but not invasive adenocarcinoma (15–19). Moreover, gastric cancer has not been observed in experimental animals when snuff (not snus) was given at a 5% concentration in the diet for 18 months to rats, at a 25% concentration for 15 months to mice or at a 20% concentration for 2 years to hamsters (20,21). Nevertheless, it seems likely that development of gastric cancer depends upon the interaction of multiple factors, including the specific carcinogen used [e.g. tobacco-specific *N*-nitrosamines (TSNAs)], the *H.P.* strain employed and the genetic constitution of the host (22,23). The peptide hormone gastrin, which is produced and released from the antral G-cells in the gastric mucosa, has long been recognized not only as a gastric acid secretagogue (e.g. amidated gastrin-17) but also as a trophic hormone and a tumor-promoting factor for the oxyntic mucosa of the stomach (23,24). Accordingly, a mouse model of gastric cancer (the so-called INS-GAS mouse) has been created by insertion of a human gastrin gene, under insulin promoter control, in pancreatic beta-cells. INS-GAS mice exhibit elevated levels of circulating amidated gastrin, and develop spontaneous intestinal metaplasia, dysplasia and carcinoma *in situ* (CIS); by 20 months of age, the majority of INS-GAS mice develop spontaneous cancer in the stomach (25). In these animals, *H.P.* or *Helicobacter felis* infection accelerates carcinoma development, such that the majority of infected mice show gastric cancer by 7 months (25,26). Similar to humans, *H.P.*-infected INS-GAS mice develop prodromal atrophic gastritis, as defined by loss of parietal and chief cells and replacement by pseudopyloric metaplasia (25,26).

The aim of the present work was to study if snus is potentially carcinogenic in the stomach; especially in such hosts with a high risk for gastric cancer development and a concurrent *H.P.* infection. Snus was given to WT and INS-GAS mice with or without *H.P.* infection in the diet for 6 months.

Abbreviations: CIS, carcinoma *in situ*; ECL, enterochromaffin-like; *H.P.*, *Helicobacter pylori*; TSNA, tobacco-specific *N*-nitrosamines; WT, wild-type.

Materials and methods

Animals and experimental design

Male WT mice (FVB/N, Taconic, Germantown, NY) and gastrin transgenic (INS-GAS) FVB mice (TC Wang's laboratory, Columbia University, NY), on average 6 months old, were used. Males were used because gastric cancer development has been shown to be sex dimorphic in INS-GAS mice (26). The mice were housed in individually ventilated Makrolon type-III cages, on a wood chip bedding, at regulated room temperature and humidity ($22 \pm 1^\circ\text{C}$; 45–55%), with fluorescent lighting on a 12 h light/dark cycle. They had free access to standard powdered mouse food [RM1 (E) FG SQC maintenance diet; SDS, Witham, Essex, UK] with or without snus, and tap water *ad libitum*. The animals were divided into six groups: (i) WT, (ii) WT + snus, (iii) INS-GAS, (iv) INS-GAS + snus, (v) WT + snus + *H.P.* and (vi) INS-GAS + snus + *H.P.* (Table I).

The experiments were conducted both in Trondheim, Norway, and in Lund, Sweden, after approval by the Norwegian Animal Welfare Committee (Forsøksdyrutvalget, FDU), and by the local animal ethical committee in Sweden (Centrala Forsöksdjursnämnden Malmö/Lund).

Snus ingestion

Snus with the brand name General™, one of the most popular brands in Sweden and Norway, was bought over the counter. The snus was mixed with powdered rodent feed using an ordinary food processor. Larger batches of the mix were kept at -20°C . A week's portion was kept at ambient temperature before use. The mixture was loaded into the so-called powder hoppers (Techniplast, Buguggiate, Italy), by which the mice had unlimited access to feed. In a pilot experiment, we started to feed mice at a snus concentration of 20% wet weight (wt/wt). Since many animals suffered weight loss, the feed protocol was adjusted to a gradual increase over 2 months, starting with snus content from 5% and increasing to a final concentration of 9%. This was well tolerated. Body weight gain was similar to non-exposed mice (Table I). The mice were fed with the snus containing diet for 6 months, starting 6 weeks after *H.P.* infection (see below). The age of the mice at the start of the experiment as well as the duration of the experiment was chosen based on the observation that the majority of INS-GAS mice (30 of 36 mice) 16 months or older developed gastric oxyntic mucosal hyperplasia/dysplasia (C.M.Zhao, B.Stenström and D.Chen, unpublished observation).

Snus contains nicotine in the range of 11.3–18.1 mg/g (27). Nicotine has a short half-life, but the metabolite cotinine is well correlated to nicotine exposure, and can be detected in plasma and other tissues (including kidney). In this study, the level of cotinine accumulated in the kidney was measured as an indicator of snus intake. Kidneys from each mouse were collected and stored at -20°C . Kidneys were homogenized and analyzed by gas chromatography (method NM-018-9; Bioanalytical laboratory, Analytical laboratories, Pfizer Consumer Health AB, PCH, R&D, Helsingborg, Sweden).

H.P. infection

Prior to the experimental challenge, feces were collected from all cages, and DNA was extracted and analyzed for the presence of *Helicobacter* species using a semi-nested polymerase chain reaction–denaturing gradient gel electrophoresis assay, specific for the genus *Helicobacter*, as described previously (28). Before the start of the experiment, all fecal samples from INS-GAS mice were positive for *Helicobacter hepaticus*, whereas all samples from FVB/N mice were *Helicobacter* genus negative. A non-mouse-adapted clone of *H.P.* strain 67:21, originally isolated from an antral biopsy obtained from a Swedish female with gastric ulcer, was used for inoculation. It is VacA⁺ and contains the entire Cag pathogenicity island (PAI) with genetic stability in the Cag PAI, demonstrated by microarray analysis, as well as three other genes over a 10-month course of mice inoculation (29). The mice were inoculated on three separate days in a 5-day period.

At sacrifice, samples composed of a quarter of the ventral stomach wall, including the antrum and fundus (oxyntic part) but without the squamous forestomach, were obtained from all mice for culture of *H.P.* The samples were carefully rinsed in phosphate-buffered saline before the mucosa was gently streaked onto *H.P.*-selective agar and incubated under microaerobic conditions (30). The bacteriologist handling all cultures of stomach samples and determination of *H.P.* status was blinded to the sample source. Mice were considered *H.P.* positive as determined by typical colony morphology, Gram staining and catalase, oxidase and urease activity (30).

Radioimmunoassay of serum gastrin level

Serum samples from each animal were collected for measurement of gastrin levels by a radioimmunoassay using synthetic human gastrin-17 as a standard (Phoenix Pharmaceuticals, Burlingame, CA).

Histopathological analysis

The samples for histology comprised multiple linear strips along the greater curvature of the stomach wall, extending from the squamocolumnar junction through the antrum. They were briefly rinsed in saline, fixed in 4% formaldehyde (Chemicon, Malmö, Sweden) for 8–12 h at 4°C and embedded in paraffin. Sections (4 μm thick) were stained with hematoxylin and eosin, diastase-resistant periodic acid-Schiff, or immunohistochemistry was performed by a DA-KO AutoStainer (Universal Staining System with DAKO EnVision® System, code no. K4011 and K3954, Dako, Glostrup, Denmark). Primary antibodies for staining of proliferating cell nuclear antigen (at a working dilution of 1:100), caspase-3 (1:20), pancreastatin (1:300) and ghrelin (1:7000) were used.

The evaluation was performed by one comparative pathologist and one histologist who were blinded to the sample source. The gastric lesions were scored on an ascending scale from 0 to 4 using the criteria adopted from a previous report (18). Defining characteristics for dysplasia and CIS were adopted from consensus guidelines on mouse models of intestinal cancer (31). The positive-stained cells with nuclei were counted, and the results were expressed as volume density (%) or numerical densities (number of cells per gland or number of cells per millimeter length of mucosa, as measured along the submucosa).

Data analysis

The values are expressed as % or as means \pm standard error of the mean. Statistical analyses were performed using Fisher's exact test, Student's *t*-test or two-way analysis of variance followed by Tukey's or Bonferroni test, as appropriate. A *P*-value < 0.05 was considered statistically significant.

Results

Snus intake, rate of *H.P.* infection and serum gastrin concentration

The levels of cotinine accumulated in the kidneys, an indicator of snus intake, were similar in both the WT and INS-GAS mice with or without *H.P.* infection (Table I). The *H.P.* infection rates were 17 of 20 (85%) in WT mice and 12 of 22 (55%) in INS-GAS mice. No control animal became *H.P.* positive during the experiment. For further analysis, only animals with the intended *H.P.* status were included. The serum gastrin-17 concentration was not elevated by snus ingestion with or without *H.P.* infection (data not shown).

Pathological alterations and incidence of CIS

Mild morphological changes without statistical difference were seen in the stomachs of WT mice exposed to snus alone. As expected, CIS was seen in two of eight INS-GAS mice not exposed to snus at 12

Table I. Snus intake (cotinine concentration in the kidney), body weight, *H.P.* infection rate and diagnosis of gastric CIS in WT and transgenic (INS-GAS) mice that were untreated or fed with a snus-containing diet for 6 months, starting after inoculation or sham inoculation with a non-mouse-adapted, CagA⁺ and VacA⁺, *H.P.* strain

Group (number of mice at start)	Snus intake (cotinine $\mu\text{g}/\text{ml}$) (n)	Body weight (g)	<i>H.P.</i> infection (rate %)	Number of mice at end for pathology	Diagnosis CIS
WT (11)	0	31 \pm 3	0	11	0/11
WT + snus (8)	724.6 \pm 88.0 (8)	27 \pm 5	0 (0%)	8	0/8
WT + snus + <i>H.P.</i> (20)	665.7 \pm 60.3 (20)	30 \pm 2	17/20 (85%)	17	9/17
INS-GAS (8)	0	24 \pm 2	0	8	2/8
INS-GAS + snus (8)	836.0 \pm 90.3 (8)	29 \pm 4	0/8 (0%)	8	4/8
INS-GAS + snus + <i>H.P.</i> (22)	838.8 \pm 75.8 (22)	26 \pm 3	12/22 (55%)	12	12/12

months of age. When exposed to snus, four of eight (50%) INS-GAS mice developed CIS. When snus was given to *H.P.*-infected WT mice, CIS with intramucosal invasion was observed in 9 of 17 (53%) animals, high-grade dysplasia (score >1.5) was seen in the remaining eight mice (Figure 1A and B) and increases in scores for gastritis, epithelial defects, oxyntic mucosal atrophy, hyperplasia and intestinal metaplasia were observed in all 17 mice (Table II). Moreover, when snus was given to *H.P.*-infected INS-GAS mice, high-grade dysplasia and CIS with intramucosal invasion and submucosal herniation were found in 12 of 12 (100%) animals (Table I and Figures 1 and 2). Interestingly, *H.P.* infection in INS-GAS mice significantly increased the incidence of CIS without significant exacerbation of inflammation

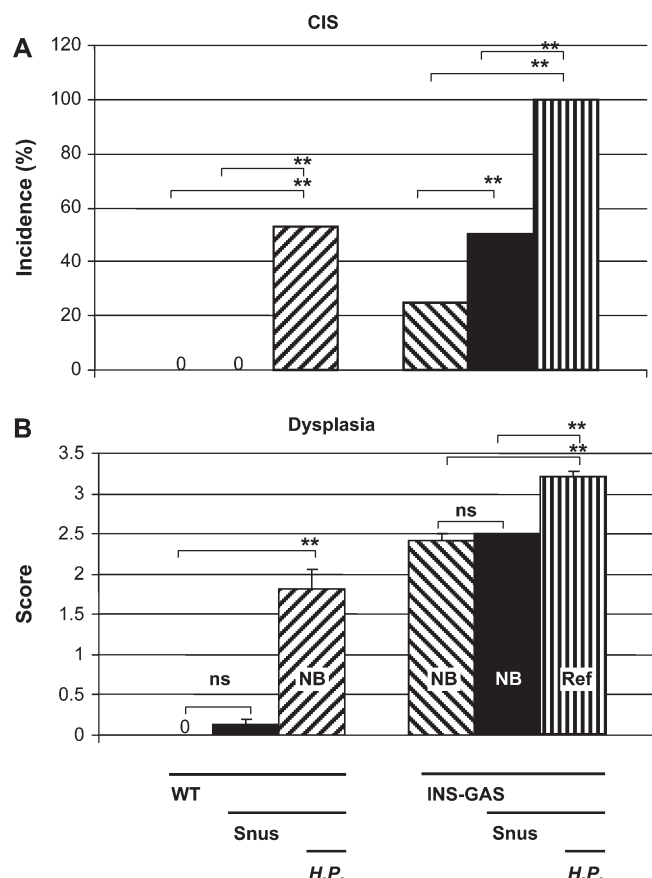


Fig. 1. WT and transgenic (INS-GAS) mice were fed a snus-containing diet for 6 months, starting 6 weeks after *H.P.* inoculation. (A) Frequency of CIS (dysplasia grade > 3) across the treatment groups. (B) Dysplasia scores across the treatment groups. NB in the columns indicates that the mice have high-grade dysplasia just falling short of reaching score for CIS diagnosis. Ref. in the column indicates all mice are CIS (grade > 3) as reference. ***P* < 0.01, ns, not significant.

and other mucosal lesions (Table II and Figure 1B). It should be noted that the mice developed oxyntic atrophy prior to dysplasia/neoplasia (Table II).

Proliferation, apoptosis and endocrine cells

An increase in proliferation (indicated by numbers of proliferating cell nuclear antigen-positive cells) was associated with an increased incidence of CIS (Figure 3A) in both WT and INS-GAS mice. The number of apoptotic cells (numbers of caspase-3-positive cells) was elevated by snus alone and even more so by a combination of snus and *H.P.* infection in WT mice. Apoptotic cell numbers were higher in INS-GAS mice compared with WT mice. The apoptotic rate was not affected by snus alone but reduced by snus + *H.P.* infection in INS-GAS mice (Figure 3B). The volume density of mucous cells (number of PAS-stained cells) was unchanged in both mouse strains when exposed to snus alone or to the combination snus and *H.P.* infection (Table III). The enterochromaffin-like (ECL) cell density was reduced in both strains of mice exposed to snus alone and to the combination snus and *H.P.* infection (Table III). The A-like cells were unaffected in all groups (Table III).

Discussion

The results of the present study support the hypothesis that snus exposure accelerates gastric cancer development in the setting of hypergastrinemia and/or *H.P.* infection. Six months of snus exposure increased the rate of carcinoma (i.e. CIS) in uninfected INS-GAS mice from 25 to 50%. When snus was given to *H.P.*-infected INS-GAS mice, CIS was found in all animals. Intramucosal invasion and submucosal herniation were frequent, but CIS invasion into muscle tissue was not observed, possibly due to the relative short observation period of this study. The mechanism of gastric cancer development in INS-GAS mice has not been fully defined, but likely involves gastrin-dependent increases in apoptosis and proliferation leading to progressive parietal cell loss and achlorhydria (for review, see ref. 23).

Gastric neoplasia following *H.P.* infection alone is uncommon in WT mice (19), although CIS has been observed in WT (C57BL/6 × 129S6/SvEv) mice infected with *H.P.* at 2 months of age and carried out to 13 months (18). However, in the present study, CIS developed in 9 of 17 WT mice infected with *H.P.* at 6 months of age and then exposed to snus for 6 months. This suggests that snus may act synergistically with another carcinogenic factor, i.e. *H.P.* infection, leading to neoplastic development. Indeed, there were non-significant low-grade lesions and mild pathological alterations present in snus-treated animals, whereas there were no pathological lesions in the untreated animals. The mechanisms underlying CIS development induced by the combination of snus and *H.P.* infection are presently unknown. In general, our current model for gastric carcinogenesis involves a sequence: chronic inflammation, resulting in increased rates of apoptosis and cell proliferation, the development of atrophy and perhaps achlorhydria, followed by progression to metaplasia and dysplasia and the accumulation of genetic alterations (32). The present study has demonstrated similar alterations, including increased rates of apoptosis and proliferation, in mice exposed to snus with or without *H.P.*

Table II. Pathological grades in the stomach in WT and transgenic (INS-GAS) mice that were untreated or fed with a snus-containing diet for 6 months, starting after inoculation or sham-inoculation with a non-mouse-adapted, CagA⁺ and VacA⁺, *H.P.* strain

Groups (n)	Intestinal metaplasia	Foveolar hyperplasia	Oxyntic gland atrophy	Epithelial defects	Inflammation
WT (11)	0	0	0	0	0
WT + snus (8)	0.13 ± 0.08 ^{NS}	0.06 ± 0.06 ^{NS}	0.25 ± 0.16 ^{NS}	0.19 ± 0.13 ^{NS}	0.25 ± 0.13 ^{NS}
WT + snus + <i>H.P.</i> (17)	2.29 ± 0.19*	2.33 ± 0.18*	2.32 ± 0.19*	2.03 ± 0.14*	1.88 ± 0.13*
INS-GAS (8)	2.88 ± 0.13*	3.00 ± 0.13*	3.00 ± 0.09*	1.81 ± 0.13*	1.63 ± 0.08*
INS-GAS + snus (8)	3.06 ± 0.15*	3.31 ± 0.16*	3.13 ± 0.08*	2.50 ± 0*	2.13 ± 0.08*
INS-GAS + snus + <i>H.P.</i> (12)	3.04 ± 0.09*	3.41 ± 0.10*	3.17 ± 0.07*	2.42 ± 0.06*	2.54 ± 0.07*(^{NS})

Means ± standard error of the mean. **P* < 0.05 compared with WT; NS, not significant between WT + snus and WT; (NS), not significant between INS-GAS + snus + *H.P.* and INS-GAS + snus.

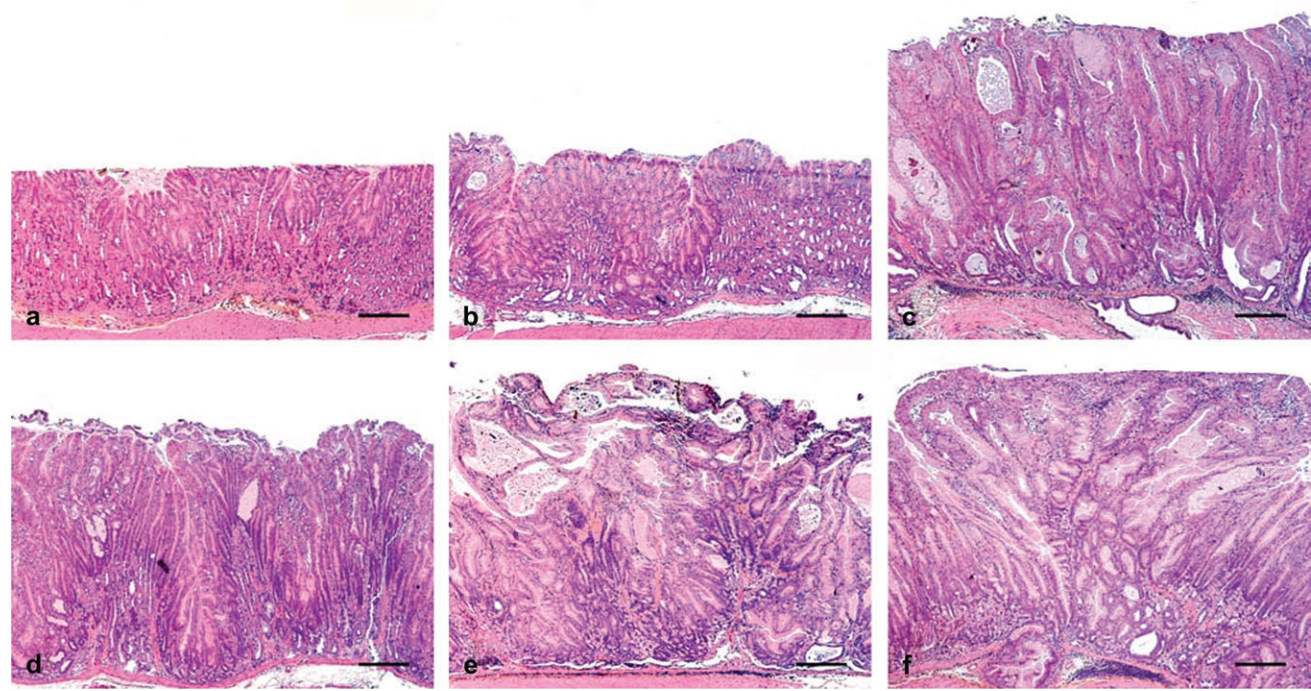


Fig. 2. Mouse gastric histopathology of WT (a), WT + snus (b), WT + snus + *H.P.* (c), transgenic INS-GAS (d), INS-GAS + snus (e) and INS-GAS + snus + *H.P.* (f). Note: in (b) minimal gastric lesions; in (c) gastritis and marked hyperplasia, intestinal metaplasia and dysplasia consistent with CIS and intramucosal invasion; in (d) hyperplasia; in (e) marked dysplasia and in (f) uniform high-grade gastric dysplasia and CIS, often with intramucosal invasion and submucosal herniation.

infection. An exception was the INS-GAS mice exposed to both snus and *H.P.*, which showed the highest proliferation rates but reduced apoptosis. In the current study, the ghrelin-producing A-like cells in the stomach did not differ between WT and INS-GAS mice, and were unaffected by snus intake with or without *H.P.* infection, suggesting little if any role for ghrelin (33). In addition, CIS development was accompanied by a decreased ECL cell density, probably suggesting little role for ECL cells in this process (25), although it has been claimed that gastric carcinomas may develop through dedifferentiation of the ECL cells (34,35).

The carcinogenic effects of snus are probably dependent on the amount of absorbed TSNAs. An average snus user consuming ~20 g snus per day is estimated to absorb ~0.042 µg/kg/day of NNK. For comparison, smoking 20 cigarettes per day yields an NNK dose of 0.05 µg/kg/day (36). In the current model, the snus exposure was probably higher than the amounts a daily snus user would typically experience (20,36). In addition, the effect of snus on gastric carcinogenesis probably reflects the combination of direct effects along with secondary effects resulting from absorbed and circulating TSNAs. There have been no reports comparing TSNAs' absorption rate between humans and mice, but the rate of elimination in humans is generally slower than in rodents, so that accumulation of TSNAs may occur more readily in humans than in mice. Smoking has been reported as a risk factor for gastric cancer, as well as for intestinal metaplasia and gastric dysplasia arising from chronic atrophic gastritis (37). However, in this report, there was no comparison of the effect of cigarette smoking in subjects between normal stomachs versus subjects with chronic atrophic gastritis, most probably because no adult in the population studied had a normal stomach (38). In the present study, snus intake caused the high incidence of CIS with intramucosal invasion from the high-grade dysplasia and atrophic gastritis in all WT animals that were *H.P.* infected.

Swedish epidemiological data suggest that snus use does not lead to an observable risk increase of cancers of any type, including gastric cancer (7, 13, 38), with an exception of pancreatic cancer (7). However, the results of the present study illustrate the potential co-carcinogenic

Table III. Quantification of immunohistochemical staining with antisera against pancreastatin (ECL cells predominately) or ghrelin (A-like cells) and of diastase-resistant periodic acid-schiff staining for mucous cells in the oxyntic mucosa of the stomach in WT and transgenic (INS-GAS) mice that were untreated or fed with a snus-containing diet for 6 months, starting after inoculation or sham inoculation with a non-mouse-adapted, CagA⁺ and VacA⁺, *H.P.* strain

Mice (n)	ECL cells (no./mm)	A-like cells (no./mm)	Mucous cells (volume density %)
WT (11)	104.8 ± 5.2	29.1 ± 1.3	20.5 ± 2.3
WT + snus (8)	75.0 ± 4.2 ^a	21.6 ± 2.3	18.8 ± 1.8
WT + snus + <i>H.P.</i> (17)	69.0 ± 2.6 ^a	27.3 ± 2.8	27.9 ± 1.8
INS-GAS (8)	92.4 ± 5.1	26.9 ± 2.0	29.9 ± 2.5
INS-GAS + snus (8)	44.7 ± 2.5 ^b	28.6 ± 1.5	32.1 ± 2.6
INS-GAS + snus + <i>H.P.</i> (12)	42.6 ± 2.3 ^b	27.7 ± 1.3	29.6 ± 2.6

The positive-stained cells were expressed as numerical densities (number of cells per millimeter length of mucosa, as measured along the submucosa) or volume density (%). Means ± standard error of the mean.

^a*P* < 0.01 compared with WT.

^b*P* < 0.01 compared with INS-GAS.

nogenic effect of snus in animal models, which may be relevant for a subset of patients. Furthermore, there are other regions of the world, such as Sudan or India, where current use of smokeless tobacco involves products with decidedly higher concentrations of TSNAs and other harmful substances (20), which also have populations with much higher *H.P.* prevalence rate than Sweden and Norway. Moreover, large populations of foreign immigrants in Europe use such smokeless tobacco products, from their countries of origin. In a global perspective, it is also possible that snus could become popular in countries like China and Japan where the risk of gastric cancer development (most probably due to *H.P.* infection) is already very high. The present study also serves as a model for further investigation on

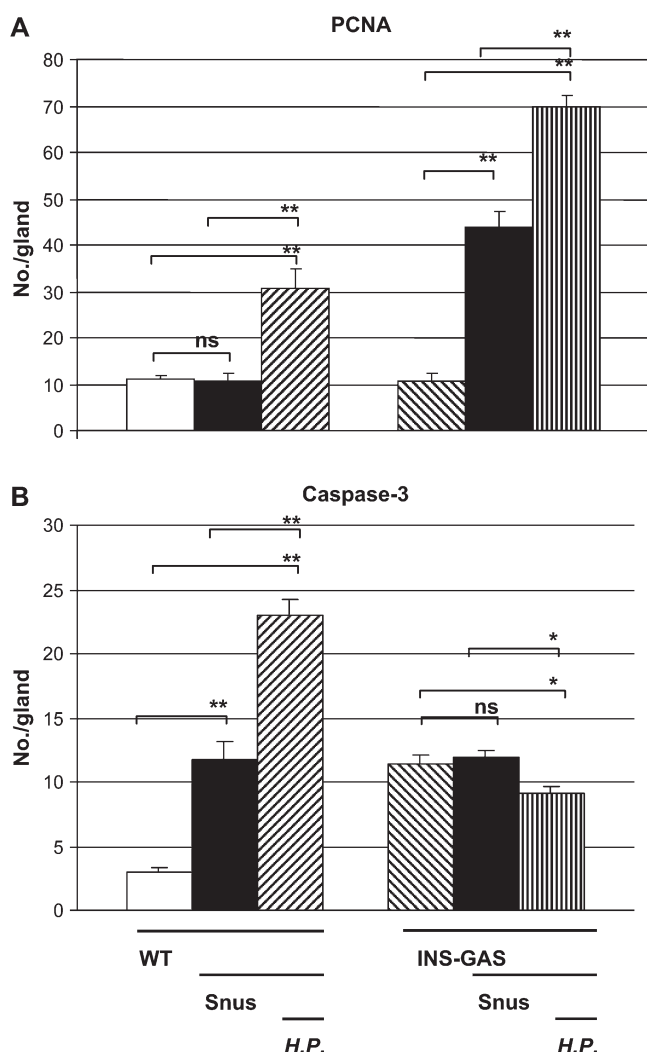


Fig. 3. WT and transgenic (INS-GAS) mice were fed a snus-containing diet for 6 months, starting 6 weeks after *H.P.* inoculation. (A) Proliferating cell nuclear antigen (PCNA) immunoreactive cells across the treatment groups. (B) Caspase-3 immunoreactive cells across the treatment groups. * $P < 0.05$, ** $P < 0.01$ and ns, not significant.

the carcinogenic effects of snus combined with other risk factors in other organs, such as the liver, pancreas, colon, kidneys and urinary bladder.

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