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Serum ghrelin is inversely associated with risk of subsequent oesophageal squamous cell carcinoma

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Abstract

Background—Oesophageal cancers rank as the eighth most common cancer and the sixth most common cause of cancer death, worldwide. Gastric atrophy, as determined by a low serum pepsinogen I/II ratio, may be associated with an increased risk of oesophageal squamous cell carcinoma (OSCC). Ghrelin, a hormone which, like pepsinogen, is produced in the fundic glands of the stomach, may be a sensitive and specific marker of gastric atrophy, but its association with OSCC is not known.

Methods—To examine the relationship between baseline serum ghrelin concentration and subsequent risk of OSCC, we conducted a nested case-control study within the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study. 82 cases of OSCC were matched (1:1) by age and date of blood draw to controls from the ATBC study. Serum ghrelin was measured by radioimmunoassay. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated using conditional logistic regression with adjustment for potential confounders.

Results—For those individuals in the lowest quartile of serum ghrelin, compared to those in the highest, the multivariate odds ratio of subsequent OSCC was 6.83 (95% CI: 1.46, 31.84). These associations were dose dependent (*P* for trend = 0.005 for both), and independent of the effects of low pepsinogen I/II ratio (a marker of gastric fundic atrophy) and *Helicobacter pylori* infection. The significance of these associations remained even for individuals developing OSCC up to 10 years after baseline ghrelin measurement, though they become attenuated after 10 years.

Conclusion—Lower baseline concentrations of serum ghrelin were associated with an increase in risk of OSCC. Further studies are needed to confirm this finding in other populations and to explore the role of ghrelin in the aetiology of OSCC.

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Keywords

ghrelin; oesophageal squamous cell carcinoma; atrophy

Introduction

In 2008 there were an estimated 482,000 new cases of oesophageal cancer globally and 407,000 oesophageal cancer deaths, so that it ranks as the eighth most common incident cancer and the sixth most common cause of cancer-related death worldwide [1]. Oesophageal cancer is comprised of two histologic subtypes, adenocarcinoma and squamous cell carcinoma, which show striking variation by both geography and aetiologic factors [2]. Though smoking and alcohol intake have been identified as strong risk factors for oesophageal squamous cell carcinoma (OSCC) in the West [3, 4], these risk factors alone do not explain the aetiology of the disease [5].

Ghrelin, a hormone produced in the fundic glands of the stomach, is known to have a variety of metabolic functions which range from stimulation of gastric acid and regulation of gastrointestinal tract motility to regulation of energy balance and control of appetite [6]. Pepsinogen I is another hormone secreted from the gastric fundic mucosa and low levels of serum pepsinogen I and/or a low serum pepsinogen I/II ratio are commonly used as serologic markers of gastric fundic atrophy. Our group has recently reported an increase in risk of OSCC for individuals with a low serum pepsinogen I/II ratio [7]. Ghrelin, like pepsinogens I and II, may be altered by gastric fundic atrophy [8], but its association with OSCC has not yet been studied. A previous prospective study of oesophageal adenocarcinoma (OA) reported a five-fold increase in risk of cancer for those in the lowest quartile of serum ghrelin concentration [9]. In another prospective analysis, we have also reported a five-fold increase in risk of both non-cardia gastric adenocarcinoma (GNCA), and oesophagogastric junctional adenocarcinoma (OGJA), among individuals with low serum ghrelin relative to those with higher serum ghrelin, an increase in risk which persisted even for cases diagnosed more than 10 years after ghrelin measurement [10].

The aim of this study was to investigate the association between serum ghrelin concentration and subsequent risk of OSCC in a case-control study nested within the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study cohort.

Methods

Cases and controls in this study were drawn from the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) study, a randomized, double-blind placebo-controlled, primary prevention trial to determine whether daily supplementation with alpha-tocopherol (50mg/day), beta-carotene (20mg/day), or both, would reduce the incidence of lung or other cancers in male smokers [11]. Between 1985 and 1988, 29,133 eligible Finnish male smokers between 50 and 69 years old were recruited to the ATBC study. The trial ended in 1993, however, participants continue to be followed as a cohort. This study was approved by the institutional review boards of both the National Cancer Institute in the United States and the National Public Health Institute (currently the National Institute for Health and Welfare) in Finland. All participants provided written informed consent.

All case subjects had incident OSCC, as defined by the International Classification of Diseases, 9th Revision [12] code 150 and the International Classification of Diseases for Oncology, 2nd Edition [13] code 8070 (8070, 80703, 80706, 80708, 80713), diagnosed through April 30th, 2006. Only squamous cell cancers are included in this analysis. Cancer

cases (n=82) were identified via the Finnish Cancer Registry. Controls were alive and cancer-free at the time of case diagnosis and were matched to cases (1:1) on age at randomization (± 1 year) and date of blood draw (± 30 days).

Data Collection

Participants completed questionnaires at baseline (1985–1988) regarding general risk factors, medical history, and dietary intake and provided a serum sample which was stored at −70°C. All blood samples were drawn in the morning, following an overnight fast. Dietary intake was assessed using a food frequency questionnaire, assessing usual food consumption over the previous year, including 276 common foods and mixed dishes, using a picture booklet to aid estimation of portion size[14]. The food frequency questionnaire was satisfactorily completed by 27,111 participants (93%) at study entry. The weight and height of all participants was measured by trained study staff.

Laboratory Analysis

Total ghrelin was measured by radioimmunoassay using reagents obtained from Millipore Linco Research (St. Charles, MO). This assay utilizes an antibody that is specific for total ghrelin, and requires 0.1 ml of serum in a 2-day disequilibrium assay. Using a 10% blinded quality control sample (n=16) from a single serum pool from the ATBC Study, randomly interspersed among study samples, the coefficient of variation for these assays was calculated as 11.6%.

Serum pepsinogen I and II concentrations were measured in duplicate using ELISA (Biohit, Plc, Helsinki, Finland[7]). The mean of the duplicate readings was used for analysis. Using 15 blinded quality control samples (~10%) from a single serum pool from the ATBC Study, the coefficients of variation for these assays were calculated as 6.0% and 8.7% for pepsinogen I and II, respectively.

Helicobacter pylori (*H. pylori*) sero-status was determined using IgG antibodies against whole cell *H. pylori* and CagA antigens by ELISA, as previously described [15, 16]. Whole cell *H. pylori* seropositivity was defined as an absorbance ratio ≥1 and CagA seropositivity as an absorbance ratio ≥0.35. Samples were assayed in duplicate and 15 blinded quality control samples from a single serum pool for the ATBC Study were inserted into the batches. Using these blinded quality control samples the coefficients of variation for these assays were calculated as 7.8% for whole cell *H. pylori* and 11.8% for the CagA antigen.

Statistical Analysis

Statistical analyses were performed using STATA version 10.1 (Stata Corp. LP) and all *p*-values were two-sided. The distribution of baseline characteristics between OSCC and controls were compared using Student's *t* test for continuous variables and Pearson's chi squared test for categorical variables. The associations between baseline characteristics and serum ghrelin quartiles were determined using the Jonckheere-Terpstra test for trend for continuous variables and the Mantel-Haenszel trend test for categorical variables (SAS, version 9.1.3; SAS Institute Inc., Cary, NC). Correlations between pepsinogen I and II levels and ghrelin were determined using Pearson's correlation (*r*) among the 82 controls.

Odds ratios (OR) and 95% confidence intervals (95% CI) for the association between serum ghrelin concentration and risk of OSCC were calculated using conditional logistic regression models. For continuous estimates of ghrelin, the ORs are scaled to 181 pg/mL (½ the interquartile range observed in ATBC controls: (Q3–Q1)/2) and represent the increase in OSCC risk for each unit (181 pg/mL) decrease in serum ghrelin. Preliminary multivariate

models of risk were adjusted for: age at randomization, total years of smoking, total cigarettes/day, alcohol (g/day), BMI (kg/m^2), fruit intake (g/day), vegetable intake (g/day), education (post-primary school), *H. pylori* (either whole cell or CagA antigen positivity), low pepsinogen I/II ratio (<5), and alpha-tocopherol or beta-carotene treatment. Final fully-adjusted models included age and known risk factors for OSCC (smoking, alcohol and education) as well as any variable which altered the β-estimate by more than 5% in a univariate model (CagA antigen positivity and alcohol). Lag analysis was performed by excluding cases diagnosed less than, or more than, 10 years after baseline.

Results

Baseline characteristics of the cases and controls are shown in Table 1. Controls were similar to cases with regard to baseline characteristics, with the following exceptions: alcohol consumption (g/day) was higher in cases than controls, while fruit and vegetable consumption (g/day) was lower; OSCC cases were also more likely to have low levels of pepsinogen I (≤ 25μg/L), a low pepsinogen I/II ratio (<5) and lower serum ghrelin concentrations. However, serum ghrelin was not strongly correlated with either pepsinogen I or pepsinogen II levels (r=0.16 ghrelin *vs.* pepsinogen I; r=−0.11 for ghrelin *vs.* pepsinogen II; r=0.39 ghrelin *vs.* pepsinogen I/II).

Using conditional logistic regression models, baseline serum ghrelin (as a continuous variable, scaled to ½ the inter quartile range of serum ghrelin in controls: 181 pg/ml) was significantly inversely associated with risk of OSCC (OR: 1.49, 95% CI: 1.15, 1.92) in an age-adjusted model (Table 2), so that for every unit (181 pg/ml) decrease in serum ghrelin, risk of OSCC increased by 49%. This association did not change following adjustment for age, total years of smoking, total cigarettes/day, alcohol intake, education, and *H. pylori* CagA antigen positivity (OR: 1.49, 95% CI: 1.09, 2.04).

In quartile analyses using an age-adjusted model, the risk of developing OSCC increased significantly from the highest (referent) to the lowest quartile of baseline serum ghrelin (OR: 6.02, 95% CI: 1.84, 19.66; Table 2). This association was dose-dependent (P_{trend}< 0.001). Following full adjustment, the risk estimate for OSCC for those in the lowest quartile of serum ghrelin, relative to those in the highest quartile was 6.83 (95% CI: 1.46, 31.84; P_{trend} = 0.005).

Lag analysis was performed using fully adjusted models: OSCC cases were classified according to whether they occurred within 10 years of baseline or more than 10 years after baseline (Table 2). The inverse association noted for ghrelin and OSCC risk was stronger within the first 10 years of baseline and became attenuated, and no longer significant, for those cases diagnosed 10 years after ghrelin measurement.

Discussion

This is the first investigation of an association between serum ghrelin concentration and future risk of oesophageal squamous cell carcinoma. In this nested case-control study, performed in the prospective ATBC study cohort, we found a nearly seven-fold increase in risk of developing OSCC among subjects in the lowest quartile of baseline serum ghrelin, compared to those subjects in the highest quartile of serum ghrelin. This association was independent of *H. pylori* seropositivity or serum pepsinogen concentrations, and it remained in cases diagnosed up to 10 years after baseline ghrelin measurement, though the association is attenuated after 10 years of follow-up.

Few previous studies have prospectively investigated the association between serum ghrelin and gastrointestinal cancer. We have recently reported, in the ATBC study, a five-fold

increase in risk of gastric non-cardia adenocarcinoma (GNCA) and oesophagogastric junction adenocarcinoma (OGJA) among individuals in the lowest quartile of serum ghrelin, compared to those in the highest[10]. This significant increase in risk of GNCA was independent of *H. pylori* infection and pepsinogen I levels and it was observed even for cases developing more than 10 years after baseline. The only previous prospective study of ghrelin and upper gastrointestinal cancer was an evaluation of 31 individuals who developed OA, and 79 controls in the multiphasic health check-up cohort in the Kaiser Permanente Medical Care Program (California)[9]. This study, also reported a five-fold increase in risk of OA for those in the lowest, relative to the highest, quartile of serum ghrelin concentration. Thus low serum ghrelin levels have now been associated with all four major upper gastrointestinal cancers: GNCA, OGJA, OA and OSCC, despite differing etiologic and risk factor profiles across these sites, suggesting that low levels of ghrelin may be broadly carcinogenic within the upper gastrointestinal tract. Nonetheless, these single reports could, conceivably, be the result of chance and, as such, all require replication in additional studies.

A number of previous reports have noted associations between gastric fundic atrophy (as measured by serum pepsinogens) and an increased risk of OSCC [7, 17, 18]. There is also some evidence to suggest an association between gastric fundic atrophy, as demonstrated by low pepsinogen I/II ratio, and an increased risk of oesophageal squamous dysplasia, the precursor lesion for OSCC [19]. One proposed mechanism by which atrophy may induce or promote oesophageal squamous cell carcinogenesis is bacterial overgrowth in the stomach, with associated production of nitrosamine compounds or other carcinogenic products [20, 21]. However, data from a large longitudinal study challenge this interpretation. Although gastric atrophy was associated with a significant increase in risk of developing OSCC (Relative Risk (RR): 2.2; 95% CI: 1.8, 2.6) in their study, this risk did not increase with histological severity of disease[22]. The same study also noted a significant association between gastric atrophy and small cell lung carcinoma (RR: 1.8; 95% CI: 1.6, 2.1) suggesting that confounding, most probably by smoking, may explain both this and the association noted for OSCC. In our analysis, however, smoking was not associated with ghrelin levels and adjustment for smoking did not alter risk estimates for ghrelin and OSCC.

Serum ghrelin is altered by gastric fundic atrophy [8], however in both this analysis, and our previous investigation of gastric and oesophagogastric junctional adenocarcinoma, it was not strongly correlated with either pepsinogen I or II. That ghrelin levels did not correlate with pepsinogen levels is not surprising. These agents are produced by different gastric cell types: pepsinogen by the chief cells and ghrelin by the P/D1 cells [23, 24, 25], which may react differently to severity and chronicity of inflammation. Such a differential response might mean that one cell type could be irreparably harmed by a specific quality or grade of inflammation, atrophy (or loss of normal cells) may also occur differentially under different conditions of stress. Pepsinogens are polypeptide pro-enzymes, while ghrelin is a peptide hormone, and their metabolism and functions may be altered differently in different microenvironments. Finally, and perhaps most importantly, ghrelin is part of a complex hormonal milieu affecting the gastrointestinal tract - alterations in this hormonal context before or after gastric atrophy may induce or promote carcinogenesis in the oesophagus, the stomach and/or elsewhere in the gastrointestinal tract.

In this, as in our previous analysis of GNCA and OGJA, the association between low serum ghrelin and risk of OSCC was not significantly influenced by whole cell *H. pylori* status. However we, and others, have demonstrated that serum ghrelin levels are lower in *H. pylori* positive subjects[10, 26]. We also note that associations between *H. pylori* and OSCC were null in our cohort [7] and inconsistent in previous studies [27]. In our analysis, adjustment for whole cell *H. pylori* had no significant effect, and adjustment for CagA only marginally affected risk estimates.

We measured total ghrelin, however, there are two isoforms of ghrelin: acylated ghrelin, which binds the growth hormone secretagogue receptor 1a (GHS-R1a), and des-acylated ghrelin which lacks acylation at the third serine residue. In the stomach, des-acylated ghrelin is present at levels which are three times higher than acylated ghrelin. Des-acyl ghrelin was initially thought to be biologically inactive, recent evidence suggests this is not the case[28]. While acylated ghrelin has been investigated in the context of *H. pylori* infection[29], further studies are needed to determine the significance of acylated and des-acylated ghrelin in the context of upper gastrointestinal disease.

Ghrelin's role in inflammation has been widely studied, with the majority of studies reporting that ghrelin suppresses the expression and/or production of cytokines both *in vivo* and *in vitro*[28], with further evidence to suggest that ghrelin treatment increased the level of anti-inflammatory cytokines *in vitro*[30]. In fact, ghrelin-mediated suppression of cytokine expression correlated with better outcomes for a number of diseases, and survival, in a number of studies[31, 32, 33]. Taken together this data supports a possible role for ghrelin in initial host reponse to *H. pylori* infection and suggests that decreased ghrelin levels in the wake of gastric atrophy (of ghrelin producing cells) could allow bacterial overgrowth and drive gastric inflammation.

The potential role of ghrelin in carcinogenesis is not yet understood. In addition to gastric fundic glands, ghrelin is secreted by colorectal carcinoma cells and appears to act in both an autocrine and paracrine manner to promote the proliferative and invasive nature of these cells[34, 35]. Other studies, however, have demonstrated that ghrelin can have both growth-promoting and -inhibiting, as well as pro- and anti-inflammatory effects, often dependent on the histological cell type and the dose and timing of ghrelin administration[36, 37, 38, 39].

The prospective design of our study is one of its major strengths, allowing the use of baseline, pre- diagnostic, serum for analysis of serum ghrelin, *H. pylori* infection and pepsinogens I and II. The ATBC study also includes a wealth of covariate information allowing adjustment for potential confounders. In contrast, the relatively small number of cases included in our analysis is a limitation which means that sub-group analyses (such as the lag analysis) are under-powered and should be interpreted with caution. The serum ghrelin measurement used in this analysis was from a single time point and it is possible that ghrelin levels may change with changes in *H. pylori* status and BMI. As The results of our study may not be generalizable in that the incidence of OSCC varies markedly between different geographic regions and ethnic differences in ghrelin expression levels have not been studied. Our study was conducted in a population of smokers, so that we are unable to assess associations between serum ghrelin and OSCC in never smokers. Neither smoking duration nor intensity were associated with serum ghrelin levels and adjustment for these factors had little effect on observed associations. Nevertheless, future studies which include never smokers are needed to replicate our results.

In this prospective investigation, we found that individuals with lower baseline serum ghrelin concentrations had a significant increase in risk of developing OSCC subsequently and that this effect appears independent of both *H. pylori* infection and low pepsinogen I/II ratio. Further work is needed to confirm these findings and, if replicated, to determine the etiologic role of ghrelin in the development of OSCC and the significance of the hormonal context within which ghrelin is acting.

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Abbreviations

CI	confidence interval
GNCA	non-cardia gastric adenocarcinoma
OA	esophageal adenocarcinoma
OGJA	oesophagogastric junctional adenocarcinoma
OSCC	oesophageal squamous cell carcinoma
OR	odds ratio

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Summary Box

What is already known about this subject?

- Ghrelin is a hormone produced in the stomach and known to have a variety of metabolic functions which range from stimulation of gastric acid to regulation of energy balance and control of appetite.
- Like pepsinogen, ghrelin may be a marker of gastric fundic atrophy.
- Recent reports have indicated low serum ghrelin is associated with an increased risk of gastric cancer and oesophageal adenocarcinoma.

What are the new findings?

- In this nested case-control study, we found a nearly seven-fold increase in risk of developing OSCC among subjects in the lowest quartile of baseline serum ghrelin, compared to those subjects in the highest quartile of serum ghrelin.
- This association appeared independent of *Helicobacter pylori* seropositivity or serum pepsinogen concentrations, and it remained in cases diagnosed up to 10 years after baseline ghrelin measurement.

How might it impact on clinical practice in the foreseeable future?

- Low serum ghrelin levels have now been associated with non-cardia gastric adenocarcinoma, oesophagogastric junctional adenocarcinoma, oesophageal adenocarcinoma and oesophageal squamous cell carcinomas, despite differing etiologic and risk factor profiles across these sites, suggesting that low levels of ghrelin may be broadly carcinogenic within the upper gastrointestinal tract.
- Ghrelin may be useful as a biomarker for upper gastrointestinal cancers; it may also have an etiologic role in the development of these cancers.

Table 1

Descriptive characteristics of OSSC cases and controls from the ATBC cohort.

Variable	Controls	Oesophageal Squamous Cell Carcinoma	*P value*
Total N	**82**	**82**	
Age at baseline	57.9 (4.7)	57.9 (4.7)	*0.99*
Years of smoking	36.5 (8.5)	37.4 (8.4)	*0.47*
Cigarettes/day	20.7 (10.5)	21.2 (7.3)	*0.72*
Alcohol, g/day	15.9 (14.0)	30.3 (29.3)	*<0.001*
Education: post-elementary school n (%)	16 (19.5)	12 (14.6)	*0.40*
BMI (kg/m^2)	26.6 (4.0)	25.5 (4.1)	*0.10*
Fruit g/day	276.4 (258.0)	182.9 (181.8)	*0.01*
Vegetables g/day	324.0 (124.2)	279.0 (104.3)	*0.02*
Helicobacter pylori positive (%)	63 (80)	64 (82.1)	*0.71*
Helicobacter pylori: CagA positive (%)	36 (46.0)	35 (45.0)	*0.93*
Low Pepsinogen I (≤ 25 μg/L) n (%)	4 (5.0)	7 (13.2)	*0.001*
Low Pepsinogen I:II ratio (<5) n (%)	23 (28.4)	42 (51.2)	*0.003*
Serum ghrelin concentration	802 (297)	647 (300)	*0.001*

Data presented as mean (SD), unless otherwise stated.

*P values from Student's t test and Pearson's χ^2 test, as appropriate. % may not sum to 100 because of missing data.

Table 2

Odds ratios (ORs) and 95% confidence intervals (CIs) for the association between serum ghrelin concentration and risk of OSCC in the ATBC cohort.

	Continuous‡			Quartiles							P_{trend}
				Q1		Q2		Q3		Q4	
	OR	95% CI	P_{value}	OR	95% CI	OR	95% CI	OR	95% CI		
Oesophageal Squamous Cell Carcinoma											
Age-adjusted	1.49	1.15, 1.92	*0.003*	6.02	1.84, 19.66	2.30	0.67, 7.90	1.26	0.42, 3.77	*1.00 (ref)*	*0.001*
Fully-adjusted*	1.49	1.09, 2.04	*0.014*	6.83	1.46, 31.84	3.68	0.79, 17.15	0.65	0.16, 2.68	*1.00 (ref)*	*0.005*
Time to cancer diagnosis											
≤10 years* (n=41)	2.13	1.10, 4.00	*0.025*								
>10 years* (n=41)	1.12	0.73, 1.72	*0.606*								

*Fully-adjusted: Adjusted ORs and 95% CIs were calculated using models which included: age at randomization, total years of smoking and total cigarettes/day, alcohol (g/day), education (post-primary school), CagA +/−.

‡Continuous ORs are scaled to 181 pg/mL, which is one half of the inter-quartile range in the ATBC controls (75^{th} percentile − 25^{th} percentile % 2) and represent the increase in OSCC risk with every unit (181 pg/mL) decrease in serum ghrelin concentration.