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Plasma and Dietary Vitamin C Levels and risk of Gastric Cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST).

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Abstract

Vitamin C is an antioxidant and inhibitor of carcinogenic N-nitroso compound production in the stomach. Higher dietary vitamin C consumption is associated with decreased risk of gastric cancer (GC) in numerous case-control studies but data from prospective studies is limited, particularly so for blood measures of vitamin C. The objective of this study was to determine the association of plasma and dietary vitamin C levels with the risk of GC in a case-control study nested within the European Prospective Investigation into Cancer and Nutrition (EPIC), a large cohort involving 10 European countries. Using a fluorometric method, vitamin C was measured in pre-diagnostic plasma from 215 GC cases (matched controls=416). Conditional logistic regression models adjusted by body mass index, total energy intake, smoking status/duration/intensity and Helicobacter pylori (Hp) infection status were used to estimate relative cancer risks. No association with GC risk was observed for dietary vitamin C whereas an inverse GC risk was observed in the highest versus lowest quartile of plasma vitamin C (OR=0.55, 95%CI=0.31-0.97, P_{trend}=0.043) which was maintained after exclusion of cases with ≤2yrs follow-up (OR=0.40, 95%CI=0.19-0.83, P_{trend}=0.064). The inverse association was more pronounced in subjects consuming higher levels of red and processed meats, a factor that may increase endogenous N-nitroso compound production. The effect of plasma vitamin C was not different by GC anatomical sub-site (cardia/non-cardia) or histological sub-type (diffuse/intestinal) and there was no significant interaction of effect with Hp. The results of this study show, in a prospective setting, an inverse association of GC risk with high levels of plasma vitamin C and suggest an interaction with the intake of red and processed meats, whose consumption may elevate endogenous N-nitroso compound production.

Introduction

In many Western countries, the relative incidence rates for adenocarcinomas of the gastric cardia have increased [1;2]. Several environmental factors, particularly diet, are thought to be involved in the etiology of gastric cancers (GC). Out of the many dietary components that have been associated with GC risk, antioxidants tend to show the strongest protective effects, and by far the most effective of these is vitamin C [3]. In fact, the consumption of citrus fruits, which are rich dietary sources of vitamin C, has been shown to be inversely associated with GC risk [3-5].

Vitamin C is an important enzyme co-factor [6] and has also been suggested to have anti-proliferative and pro-apoptotic roles *in vitro* [6], to inhibit the growth of Helicobacter pylori (Hp) [7] and to regulate the immune response towards Hp infection [8]. But perhaps most importantly, vitamin C can quench reactive oxygen species produced in the gastric environment, thus limiting free radical-mediated damage in the gastric epithelium [9], and it can scavenge nitrite, inhibiting *in vivo* nitrosation and the production of carcinogenic N-nitroso compounds [10]. Normally, gastric mucosa and juice contain high levels of vitamin C, perhaps even higher levels than in plasma [11]. But in the presence of gastric pathology, the vitamin C secretion into the gastric juice is affected, causing lower vitamin C concentrations [12]. GC patients have

been shown to have decreased levels of blood vitamin C, increased ph and nitrite in their gastric juice [13] and higher overall levels of oxidative stress [14]. In fact, low dietary vitamin C intake can enhance the progression of gastric dysplasia to GC [15], whereas vitamin C supplementation can slow the progression of gastric mucosal atrophy [16] and increase the regression rate of gastric pre-cancerous lesions [17].

Many case-control studies show an inverse association between dietary vitamin C intake and GC risk [3;4;18;19]. Although the effect of diet on GC risk may differ based on the sub-site localization within the stomach or histological subtype of the cancer [20;21], few previous studies have considered these factors. In recent case-control studies, dietary vitamin C has been shown to have either no association with cardia GC [22-24], or to be inversely associated with both GC sub-sites [25;26] and sub-types [25;27]. Much of this disparity in results may be due to the inherent measurement errors in dietary recall instruments and biases associated with case-control study designs. In the few existing data from prospective studies, both increased dietary [28-30] and blood [15;31;32] vitamin C levels have been associated with a decreased GC risk.

Thus, the aim of this case-control study, nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort, was to determine the association of plasma and dietary vitamin C levels with risk of GC (as well as its sub-sites and sub-types), taking into account Hp infection status.

Materials and Methods

Study Population and Collection of Blood Samples. The rationale and methods of the EPIC study have been previously discussed in detail [33;34]. Briefly, EPIC consists of 23 centers in 10 European countries (Denmark, France, Greece, Germany, Italy, Netherlands, Norway, Spain, Sweden and United Kingdom). Between 1992 and 1998, standardized lifestyle and personal history questionnaires, anthropometric data and blood samples were collected from most participants. In addition, diet over the previous 12 months was measured at recruitment by validated country-specific questionnaires designed to ensure high compliance and better measures of local dietary habits [33;34]. Values for total energy and dietary vitamin C were computed using country-specific food composition tables. Data on the intake of vitamin C from dietary supplements are not available and were not assessed here.

As has been previously documented [33;34], in each of the 23 centers, blood samples of at least 20mL were drawn from all participants and stored at 5-10°C protected from light and transported to local laboratories for processing and aliquoting. The only exceptions were the EPIC-Oxford centre (UK) where blood samples were collected from a network of general practitioners and transported to a central laboratory in Norfolk via mail, and centers in Sweden where blood was aliquoted within one hour of drawing.

In all countries, except Sweden, blood was separated into 0.5mL fractions (serum, plasma, red cells and buffy coat for DNA extraction) and stored in heat-sealed straws at ultra-low temperatures (-196°C) under

liquid nitrogen. One half of all aliquots were stored at the local study centre and the other half in the central EPIC biorepository at the International Agency for Research on Cancer (IARC; Lyon, France). In Sweden, samples were stored in -80°C freezers.

Short term losses of blood Vitamin C from handling, transport and storage prior to long term freezing have previously been shown to be minimal [35]. Prior to long term freezing, blood samples were not treated with compounds, such as meta-phosphoric acid, for the specific stabilization of vitamin C. Regardless, a recent study specifically on EPIC plasma samples shows that after freezing at -196°C for up to 11 years, vitamin C can still be measured with reasonable reliability as a biomarker [36].

Follow-up for Cancer Incidence and Vital Status. Follow-up is based on population cancer registries (Denmark, Italy, Netherlands, Norway, Spain, Sweden and the United Kingdom) and other methods, such as health insurance records, pathology registries, and active contact of study subjects or next of kin (France, Germany and Greece). The follow-up period for the present study was for data reports received at IARC to the end of October 2002, representing complete follow-ups until either December 2000 or December 2001 for all centers using cancer registry data and until 2002 for France, Germany and Greece. Cancers of the stomach included cancers coded as C16 (10^{th} Revision of the International Statistical Classification of Diseases, Injury and Causes of Death). The diagnosis, tumor site classification and morphology (according to ICDO2 and Lauren classifications) of each identified cancer was confirmed and validated by an independent panel of pathologists with a representative from each EPIC country and a coordinator. The pathologist panel reviewed histological slides and/or re-cuts from the paraffin blocks and original histo-pathology reports provided by each EPIC centre.

Nested Case-Control Study Design and Selection of Study Subjects. The present study includes all GC incident cases (excluding gastric lymphomas, gastric stump cancers, other gastric non-adenocarcinoma, esophageal non-adenocarcinomas and otherwise unspecified malignant neoplasms) with available blood samples that were diagnosed after recruitment from all EPIC countries except Norway (blood samples only recently collected). Out of a total of 230 gastric adenocarcinomas and 18 adenocarcinomas of the gastro-esophageal junction (GEJ) cases identified, plasma samples for 33 were of insufficient volume/quality for vitamin C analysis. Thus, the present study includes a total of 199 gastric adenocarcinomas and 16 GEJ, which are grouped together (n matched controls= 416) and referred to as GC. GC were also divided into three groups by anatomical sub-site: (i) cardia tumors (n cases=59, n matched controls=113), combining tumors that reached the gastro-esophageal junction, either crossing it from below (all 16 GEJ adenocarcinomas) or not, (ii) non-cardia tumors (n cases=113, n matched controls=223) grouping cases from other sites in the stomach and (iii) tumors from unknown or mixed sites (n cases=43, n matched controls=80). When divided by histological sub-type, of the 215 GC cases, 86 were classified as diffuse (n matched controls = 166) and 83 as intestinal (n matched controls = 163) histological subtypes according to

the Lauren classification. The remainder (n cases=46, n matched controls=87) were of unknown or mixed histological types. For each identified GC case, control subjects with available blood samples were selected from all cohort members who were alive and free of cancer (except non-melanoma skin cancer) at the time of diagnosis of the case patient. Controls were matched (1:2) by gender, age group (± 2.5 years), study centre and date of blood sample collection (±45 days; to account for follow-up time and seasonality). Plasma vitamin C measurements could not be obtained for 14 control subjects and so they were excluded.

Laboratory Assay - Helicobacter pylori Infection Status. The methodology for the determination of Hp infection status is detailed elsewhere [5;37]. Briefly, quantification of anti-Hp antibodies in plasma of all cases and controls was done by ELISA using the lysate of the Hp CCUG strain. Briefly, various dilutions of plasma samples (starting dilution 1:200) were incubated with the Hp lysate in solid phase (1 ug/ml). After one hour and extensive washings, plates were incubated with an alkaline phosphatase-conjugated polyclonal affinity purified goat anti-human IgG (Sigma chemical Co, St Louis, MO). After three hours incubation and further washings, the enzymatic reaction was revealed by addition of p-nitrophenylphosphate as a substrate. Hp-specific IgG antibody titres were expressed as ELISA Units (EU), and were determined by interpolation relative to a standard curve constructed by a serial dilution of a standard positive control. A cut-off value of 100 EU was defined using serum samples from individuals negative for H. pylori infection as determined by clinical, microbiological and serological assays (Western blotting). Serum samples with EU values above 100 were considered as positive for anti-H. pylori IgG antibodies. In previous experiments this assay exhibited specificity and sensitivity higher than 90%.

Laboratory Assay – Vitamin C. Vitamin C measurements were performed at Addenbrookes Hospital (Cambridge, UK) using a fluorometric analysis method, previously described in detail [38]. Plasma from blood initially drawn into citrate tubes was removed from storage, thawed and then stabilized with a standardized volume of freshly prepared meta-phosphoric acid. Samples were quickly refrozen to -70°C and shipped on dry ice to Addenbrookes Hospital for analysis. All samples were run in duplicate with matched case-control sets assayed in the same batch in order to minimize errors from batch to batch variations. Low (coefficient of variation=14.0%) and high (coefficient of variation=7.4%) concentration vitamin C quality control samples were run at the beginning and end of each analysis batch. Samples with more than 10% difference between duplicates were repeated. Laboratory technicians were blinded to the case/control status of all samples. No significant between-day drift was observed.

Statistical Methods. Differences between cases and controls in mean vitamin C levels and baseline covariates were tested by paired t-tests. The correlation between dietary and plasma vitamin C was assessed by way of a spearman rank correlation test, adjusted for age, smoking duration/status, body mass index, total energy intake, and Hp positivity.

Odds Ratios (OR) and 95% confidence intervals (95%CI) for GC in relation to plasma and dietary vitamin C concentrations were calculated by conditional logistic regression using the PHREG procedure (SAS statistical software, version 9, SAS Institute, Cary, NC), stratified by the case-control set. Risk estimates were computed both as "crude" (adjustment for matching variables only) and as "fully adjusted" with additional adjustments for potential confounders including total energy intake (in quartiles), body mass index (quartiles), Hp infection status (yes/no) and duration/status/intensity of smoking (variable categories: never-smokers, ex-smokers who smoked for <10 years, ex-smokers who smoked for ≥10 years, smokers who smoke <15 cig/d, smokers who smoke between 15-25 cig/d, smokers who smoke ≥25 cig/d, and missing). The effects of alcohol intake and the level of schooling (an indicator variable for socio-economic status) as potential confounding variables were examined, but they did not provide appreciable changes in risk estimates and were not included in the models. For both plasma and dietary vitamin C, risk associations were examined by quartiles with cut points based on the distribution of vitamin C in the GC control subjects. Models similar to the above were also run with plasma and dietary vitamin C measurements included in the model as continuous variables with the ORs estimated for the risk related to a change in the plasma or dietary level by one standard deviation of the distribution in control subjects (19.4 μmol/L for plasma and 72.9 mg/day for dietary vitamin C). For all models, tests for linear trend were performed using a score variable with values from 1 to 4, consistent with the quartile grouping.

The models for plasma vitamin C were run separately for GC, as well as by anatomical sub-site (cardia / non-cardia) and histological sub-type (diffuse / intestinal). The same quartile cut points used for analysis of all GCs were used in sub-group analyses. Tests for heterogeneity were also run for comparison of results by sub-site and sub-type. As described above, for a number of reasons, some GC cases could not be classified by anatomical sub-site or histological sub-type. For comparison purposes, ORs were also calculated for these cases and matched controls, using the same methods described above.

Potential modification of the effects of vitamin C by gender, Hp infection status and time of follow-up of 2 years or less and more than two years was tested using the likelihood ratio test to assess the statistical significance of a linear interaction. For the assessment of interaction for time of follow-up, each case-control set was assigned the value for years of follow-up of the case. No overall significant interactions were observed for any of these variables. In order to assess the effect of time of follow-up in greater detail, analyses were also performed for plasma vitamin C and GC risk, excluding cases diagnosed with less than 2 years of follow-up.

Hp infection status was used as a confounding variable because of its purported association with GC risk and its potential to alter the systemic bioavailability of vitamin C [39] and the vitamin C concentration of gastric juice [40;41]. To further explore the role of Hp infection status, unmatched case-control analyses stratified by this variable using plasma vitamin C modeled as a continuous variable were performed by using

unconditional logistic regression models adjusted for case-control matching variables, as well as the laboratory batch and all other variables described in the "fully adjusted" model above. In order to explore any role of smoking, a similar model to the above was used, stratified by smoking status (never, former, current, with missing in a separate category).

In order to correct for measurement errors in dietary vitamin C assessment, a linear calibration model was employed [42]. For this purpose, dietary vitamin C values derived from standardized 24-hour dietary recall measurements collected at baseline from an 8% subset of the EPIC cohort were taken as reference measurements [43]. They were linearly regressed on questionnaire measures of dietary vitamin C intake in order to compute a set of predicted values for all subjects [44]. The predicted values were used in the risk model to evaluate a corrected association between dietary vitamin C and gastric cancer on a continuous scale. The calibration model included center-specific terms, as well as a list of confounding variables identical to the "fully adjusted" model described above. A bootstrap sampling procedure with 300 repetitions was employed to compute the standard error of the corrected coefficient.

Since low levels of vitamin C and higher intake of red and processed meats are factors that may increase intra-gastric N-nitroso compound production, a potential interaction of effect of plasma vitamin C with dietary intake of red and processed meats was explored by modeling a smoothed dose-response relationship using the exposures and their interaction as continuous variables. The statistical significance of a linear interaction was assessed using the likelihood ratio test. Odds ratios were computed for different levels of plasma vitamin C and dietary red and processed meat intake, and the associated 95%CI were assessed through a bootstrap sampling procedure with 1000 repetitions [45]. The reference category was set as low level of plasma vitamin C and high intake of red and processed meats. In theory, this category would be expected to show the highest production of endogenous N-nitroso compounds in this population.

Results

Description of the Study Population. Table 1 shows the baseline characteristics and description of the study population. The mean age at recruitment of GC cases was (± standard deviation) 59.3±8.2 and controls was 59.4±8.2 (Table 1). On average, GC cases had 3.3 years between blood donation and diagnosis. GC cases had a higher percentage of Hp positivity than controls, while body mass index was similar between cases and controls (Table 1).

Plasma Vitamin C. Table 2 shows the mean plasma vitamin C values and standard deviations in cases and controls, as well as the p value for difference between cases and controls for all the GC groupings. For GC, the mean plasma vitamin C (± standard deviation) was 39.9±25.2 μmol/L in cases and 41.4±19.4 μmol/L in controls ($P_{difference}$=0.26). Plasma vitamin C values of cases and controls did not differ by the follow-up period (data not shown).

The association of plasma vitamin C with GC risk showed an inverse association which was significant in the highest versus the lowest quartile in both the crude (OR=0.53, 95%CI=0.31-0.90, P_{trend}=0.023) and fully adjusted models (OR=0.55, 95%CI=0.31-0.97, P_{trend}=0.043)(Table 3). A significant negative association was maintained after the exclusion of cases with less than 2 years of follow-up (OR of the highest versus the lowest quartile=0.40, 95%CI=0.19-0.83, P_{trend}=0.064; fully adjusted model).

The smoothed dose-response analysis of the interaction between plasma vitamin C and intake of red and processed meats showed that the decrease in GC risk with increasing plasma vitamin C concentration was more apparent in subjects with medium and high levels of red and processed meat intake (Table 4). The p value for interaction between plasma vitamin C levels and intake of red and processed meats was 0.058.

Hp Infection Status. Plasma vitamin C values of the controls were not significantly different between Hp positive (42.1±1.1 μmol/L) and Hp negative (39.4±1.8 μmol/L). The GC risk association of plasma vitamin C based on Hp infection status was explored via unconditional logistic regression models with vitamin C modeled as a continuous variable. In the fully adjusted model, the ORs were estimated for a 19.4 μmol/L increment (Hp negative=1.23, 95%CI=0.67-2.27, Hp positive=0.89, 95%CI=0.74-1.08).

Smoking Status. Plasma vitamin C values of the controls were not significantly different by smoking status, although values in never smokers (43.5±1.1 μmol/L; mean ± standard error) where slightly higher than former smokers (40.6±1.7 μmol/L) and current smokers (38.9±2.0 μmol/L). The GC risk association of plasma vitamin C stratified by smoking status was explored via unconditional logistic regression models with vitamin C modeled as a continuous variable and ORs were calculated for a 19.4 μmol/L increment (never smokers=1.13, 95%CI=0.86-1.47; former smokers=0.91, 95%CI=0.67-1.24; current smokers=0.67, 95%CI=0.43-1.06).

Dietary Vitamin C. The mean dietary vitamin C (± standard deviation) was 129.5±81.6 mg/day for GC cases and 128.1±72.9 mg/day for GC controls (Table 2). Dietary and plasma vitamin C levels were equally correlated in both the GC control subjects (r=0.18, p<0.001) and cases (r=0.17, p=0.016). Dietary vitamin C showed no significant associations with GC risk at any level of intake (Table 3). In the fully adjusted model, the OR for a 72.9 mg/day increase in dietary vitamin C intake was 1.09 (95%CI=0.90-1.33). Using the calibrated predicted values for dietary vitamin C, the calibrated OR for a similar increment in daily intake was reduced to 0.95 (0.66-1.37).

Grouping by Anatomical Sub-site and Histological Sub-type. There were no significant differences in mean plasma vitamin C concentrations between cases and controls by GC sub-site or sub-type (Table 2). Table 3 shows the ORs and confidence intervals for quartiles of increasing plasma vitamin C values and risk of GCs

by anatomical sub-site and histological sub-type. The p value for heterogeneity between the cardia and non-cardia sub-sites was 0.129 for the crude model and 0.091 for the fully adjusted model. For the diffuse versus the intestinal sub-type, the p value for heterogeneity was 0.455 for the crude model and 0.659 for the fully adjusted model. No statistically significant associations were observed with risk of either cardia or non-cardia GCs at any quartiles of plasma vitamin C, although the direction of effect was always negative, particularly in the cardia. Similarly, no significant associations were observed with risk of either the diffuse or intestinal sub-types, at any category of plasma vitamin C. For comparison purposes, the ORs for groups of cases of unknown anatomical sub-site (OR=0.83, 95%CI=0.51-1.32) or unknown/mixed histological sub-type (OR=0.76, 95%CI=0.46-1.25) were calculated for a 19.4 μmol/L increment in plasma vitamin C.

Discussion

This nested case-control study is one of the largest prospective analyses of the association of plasma and dietary vitamin C levels with GC risk ever performed on Western European populations. The results show that within the EPIC cohort, higher plasma vitamin C level is associated with a decreased risk of GC, and does not appear to be limited to a particular GC anatomical sub-site or histological sub-type. In contrast, dietary vitamin C showed no significant association with GC risk, even after linear calibration.

Vitamin C may plausibly be involved in GC prevention by way of its potential to modulate cell growth kinetics [46], its purported antimicrobial activity against Hp [7;47] and its antioxidant properties [9]. These mechanisms can all directly relate to GC risk [12-14] and a potential GC protective effect of higher dietary vitamin C intake has been observed in ecological [48;49] and case-control studies [3;18;19;25-27]. Although the demonstrated effect of vitamin C in these studies is quite strong, dietary data from case-control studies are nonetheless affected by recall bias, while biochemical values may be affected by the presence of the disease. It is for these reasons that data from prospective studies, particularly those using blood biomarkers, are often valuable in adding to the scientific knowledge in a given area. However, in the case of the association of blood vitamin C levels and GC, information from prospective studies is scarce, but existing data do show a generally inverse risk association in select European [31] and high risk Chinese populations [15;32] – although none stratified by sub-site or sub-type. In this regard, by way of its prospective design and use of both dietary and plasma vitamin C measures, the present study adds considerably to the degree of knowledge in the field.

Another key mechanism of vitamin C action is its inhibition of N-nitroso compound formation within the stomach [10;50]. In the present study, a borderline statistically significant interaction was observed between plasma vitamin C and dietary red and processed meats, whose higher intake is suggested to increase endogenous N-nitroso compound formation [51]. A smoothed dose-response analysis showed that the observed inverse GC risk association of higher plasma vitamin C concentration is more pronounced at higher intake levels of red and processed meats. In the future, the creation of databases concerning the level of

dietary consumption and endogenous production of N-nitroso compounds and their precursors will allow this observation to be assessed in greater detail.

It is well known that Hp infection is a major GC risk factor [52-54] and it can interact with vitamin C by reducing its systemic bio-availability [39] and its concentration in gastric juice [40;41]. In the present study, Hp infection status was determined for all cases and controls. Statistical tests for interaction between Hp positivity and the association of plasma vitamin C with GC risk were not significant, perhaps because a large percentage of Hp positive cases (86.5%) and controls (71.2%). Other studies have also observed no statistically significant interactions between Vitamin C (from the diet) and Hp infection in association with GC risk [55]. In another study, Hp positivity was shown to be a strong risk factor for GC at low levels of dietary vitamin C intake, but not at higher levels [19], implying that any interaction of Hp status and vitamin C may depend on the level of vitamin C. It may also pertain more to the concentration of vitamin C in gastric juice, since this has been shown to be significantly lower in Hp positive than Hp negative subjects, despite similar plasma vitamin C levels in the two groups [40]. Given the potential for Hp infection to modulate the effects of vitamin C (or vice versa), the present study explored the vitamin C-GC risk association stratifying by Hp infection status. Although the results were not statistically significant, they do show divergent GC risk associations based on Hp status (Hp positive OR=0.89; Hp negative OR=1.23), likely because of the low number of Hp negative cases and controls. Together, these results suggest a need to further explore this area, ideally with better powered studies.

By way of an international effort to collect tumor samples and pathology reports the present study can differentiate between GCs based on their anatomical localization and their histological sub-type. Both of these factors may play a role in GC etiology [20;21], but have seldom been previously explored. Information from previous case-control studies is mixed with some showing either no effect in the cardia sub-site [24] or an inverse association of dietary vitamin C with decreased risk of both anatomic sub-sites [25-27], while in a prospective setting, a stronger protective effect of dietary vitamin C has been observed in non-cardia GC [30]. An inverse association of dietary vitamin C with GC risk has also been observed to be equal in both histological sub-types [25] or to be stronger in the diffuse than in the intestinal [27]. Although in the present study, no significant associations were observed for plasma vitamin C by either anatomical sub-type or histological sub-site, the overall direction of effect was negative, particularly in the cardia sub-site. This is consistent with findings from a concurrent study based on the entire EPIC cohort showing a non-significant inverse association with the intake of citrus fruits, a rich source of dietary vitamin C, and risk of cardia GCs [5]. The observations of the present study may be due to either equal effect or low number of cases.

In the present study, GC risk was associated with plasma vitamin C concentration, but was not related to the level of dietary vitamin C intake. To some degree, this may be because of errors in food composition tables from which dietary vitamin C values were derived or the lack of information on vitamin C intake from

dietary supplements. But it could also be due to potential measurement errors in the assessment of dietary vitamin C intake. In order to better account for such errors, a calibration exercise was attempted in the present study. No significant GC risk association was observed, but calibration reduced the OR estimate of dietary vitamin C from 1.09 to 0.95, which is still not comparable to that obtained for plasma vitamin C. This may be because estimation of dietary vitamin C does not account for factors such as efficiency of uptake from the digestive tract or availability from different foods, which may influence overall plasma vitamin C levels. In addition, some cases may have lower plasma vitamin C levels prior to clinical diagnosis because of potential endogenous consumption of vitamin C by the process of tumor development [8;56] or possible changes in intake patterns of vitamin C rich foods induced by gastric discomfort from precancerous lesions. Furthermore, plasma vitamin C measures also reflect vitamin C intake derived from dietary supplements. Collectively these errors may in part account for some of the discrepancy in results observed here.

The relationship between dietary and plasma vitamin C is known to be non-linear [57]. Within a sub-group of the EPIC study [58], and elsewhere [59], it has been observed that the correlation of dietary and plasma vitamin C is lower at higher levels of vitamin C intake [58]. This suggests that the vitamin C-GC risk association may also be non-linear, and that the risk associated with dietary intakes that are lower on the plasma vitamin C curve may be more physiologically relevant. Although, this was explored via cubic spline flexible regression models in preliminary analyses for the present study, the results were not different from those presented here. An explanation for this may be found in data from controlled clinical studies showing that plasma vitamin C concentrations start to plateau at levels beyond 80 μmol/L and that dietary intakes above 1000 mg/day are associated with complete plasma vitamin C saturation [57] – both of which are higher than the average values of the highest quartiles of plasma and dietary vitamin C observed here. This suggests that in the present study, the vitamin C concentrations associated with an inverse GC risk are likely mostly in the linear part of the vitamin C pharmacokinetics curve, prior to any plateau of plasma values.

A potential limitation of the present study is the relatively short follow-up time. The mean number of years from blood donation to diagnosis was 3.3 years. Cases identified within a short period of time after the start of the study may have been experiencing symptoms leading to dietary changes and hence alterations in blood vitamin C levels. Here, interaction tests were run in order to assess if data in the first years of follow-up affected the association of plasma vitamin C and cancer risk. However, no statistically significant interactions were observed and elimination of the cases diagnosed in the first two years of follow-up did not change the observed negative association, suggesting that the short follow-up time is likely not a major factor in this investigation.

In summary, these results based on the prospective EPIC study, are in line with earlier observations that higher plasma vitamin C levels are inversely associated with GC risk. Further studies are also necessary to determine the mechanisms of vitamin C action and any potential interactions with Hp infection and smoking.

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Table 1: Baseline characteristics and description of the study population.

Gastric Cancer	Cases n=215	Controls n=416
Age at Recruitment	59.3 ± 8.2	59.4 ± 8.2
Age at Diagnosis	62.7 ± 8.6	NA
Mean Number of Years Between Blood Donation and Diagnosis	3.3 ± 2.2	NA
Percent Hp Positive	86.5	70.9
Body Mass Index	26.5 ± 3.9	26.5 ± 4.2
Number of Males	119	230
Number of Females	96	186
Grouping by Anatomical Sub-site:		
Cardia, no. of subjects	59	113
Non-cardia, no. of subjects	113	223
Unknown or Mixed Sub-site, no. of subjects	43	80
Grouping by Histological Sub-type:		
Diffuse, no. of subjects	86	166
Intestinal, no. of subjects	83	163
Unknown or Mixed Sub-type, no. of subjects	46	87

Values are either means ± standard deviation, or number of subjects, except for Hp positivity which is given as an overall percentage, as indicated. For gastric cancers, distribution of cases/controls by country: France=3/6, Germany=30/60, Greece=12/23, Italy=43/84, Netherlands=18/35, Spain=27/52, Sweden=54/102, United Kingdom=28/54.

Table 2: Means, Standard Deviation and Distribution of Plasma Vitamin C Levels Amongst Cases and Controls in Gastric Cancers and Adenocarcinoma of the Esophagus.

	Cases		Controls		P for Difference in Mean Vitamin C Levels †	5^{th} – 95^{th} Percentile Distribution of Vitamin C **	
	Number	Mean Vitamin C Level ± Stdev *	Number	Mean Vitamin C Level ± Stdev *		Cases	Controls
Gastric Cancers							
Plasma Vitamin C (µmol/L)*	215	39.9 ± 25.2	416	41.5 ± 19.4	0.26	11.0 – 82.0	12.0 – 75.0
Dietary Vitamin C (mg/day)*	215	129.5 ± 81.6	416	128.1 ± 72.9	0.67	41.0 – 279.1	42.7 – 248.7
Grouping of Gastric Cancers by Anatomical Sub-Site – Plasma Vitamin C (µmol/L)							
Cardia Gastric Cancers	59	37.0 ± 17.9	113	40.7 ± 18.3	0.08	10.0 – 79.0	15.0 – 77.0
Non-cardia Gastric Cancers	113	42.5 ± 27.7	223	42.2 ± 20.6	0.88	14.0 – 88.0	12.0 – 76.0
Grouping of Gastric Cancers by Histological Sub-Type – Plasma Vitamin C (µmol/L)							
Diffuse Gastric Cancers	86	43.8 ± 31.7	166	44.6 ± 20.3	0.81	9.0 – 88.0	17.0 – 86.0
Intestinal Gastric Cancers	83	37.9 ± 20.0	163	40.6 ± 18.7	0.25	10.0 – 82.0	12.0 – 67.0

* Values are means ± standard deviations.

** Values represent the lowest and highest plasma vitamin C values of all controls in each cancer grouping, in the 5^{th} and 95^{th} percentiles respectively.

† Two sided p values, paired t test, given for a difference between cases and controls.

Table 3: Odds ratios for quartiles of increasing levels of plasma and dietary vitamin C and risk of gastric cancers.

Gastric Cancer	Ref	Odds Ratios (OR) for Quartiles of Plasma or Dietary Vitamin C Levels § 2	3	4	Ptrend ‡	OR of One Std. Deviation Increase *
Plasma Vitamin C	*< 29.0*	*≥ 29.0 - < 40.0*	*≥ 40.0 - < 51.0*	*≥ 51.0* µmol/L		19.4 µmol/L
Cases/Controls	70/101	46/98	54/112	45/105		
Mean (µmol/L)**	19.1 ± 0.7	34.1 ± 0.3	44.9 ± 0.3	66.3 ± 1.6		
Crude	1.00	0.64 (0.40-1.03)	0.67 (0.42-1.05)	0.53 (0.31-0.90)	0.023	0.90 (0.75 - 1.08)
Fully Adjusted	1.00	0.73 (0.43-1.22)	0.70 (0.43-1.14)	0.55 (0.31-0.97)	0.043	0.93 (0.77 - 1.12)
Diet Vitamin C	< 78.0	*≥ 78.0 - < 111.5*	*≥ 111.5 - < 160.0*	*≥ 160.0* mg/day		72.9 mg/day
Cases/Controls	61/104	45/104	56/101	53/107		
Mean (mg/day)**	55.8 ± 1.5	96.9 ± 1.0	134.7 ± 1.4	223.3 ± 6.9		
Crude	1.00	0.75 (0.46-1.21)	0.94 (0.59-1.49)	0.85 (0.53-1.38)	0.719	1.02 (0.86 - 1.22)
Fully Adjusted	1.00	0.77 (0.45-1.30)	0.94 (0.57-1.58)	1.02 (0.60-1.74)	0.769	1.09 (0.90 - 1.33)
	Grouping of Gastric Cancers by Anatomical Sub-Site					
Plasma Vitamin C	*< 29.0*	*≥ 29.0 - < 40.0*	*≥ 40.0 - < 51.0*	*≥ 51.0* µmol/L		19.4 µmol/L
Cardia						
Cases/Controls	17/29	16/28	16/29	10/27		
Crude	1.00	0.84 (0.35-2.01)	0.79 (0.32-1.96)	0.47 (0.16-1.40)	0.209	0.71 (0.47 - 1.07)
Fully Adjusted	1.00	0.74 (0.25-2.16)	0.59 (0.21-1.66)	0.36 (0.10-1.33)	0.118	0.65 (0.40 - 1.06)
Non-cardia						
Cases/Controls	35/54	19/52	34/57	25/60		
Crude	1.00	0.53 (0.26-1.08)	0.91 (0.49-1.68)	0.57 (0.28-1.18)	0.334	1.02 (0.81 - 1.29)
Fully Adjusted	1.00	0.64 (0.30-1.39)	1.01 (0.51-2.03)	0.63 (0.28-1.42)	0.520	1.06 (0.82 - 1.37)
	Grouping of Gastric Cancers by Histological Sub-Type					
Plasma Vitamin C	*< 29.0*	*≥ 29.0 - < 40.0*	*≥ 40.0 - < 51.0*	*≥ 51.0* µmol/L		19.4 µmol/L
Diffuse						
Cases/Controls	27/30	17/44	21/43	21/49		
Crude	1.00	0.42 (0.20-0.91)	0.55 (0.27-1.14)	0.43 (0.19-1.00)	0.081	0.97 (0.75 - 1.25)
Fully Adjusted	1.00	0.50 (0.18-1.38)	0.65 (0.25-1.66)	0.36 (0.13-0.99)	0.091	0.96 (0.72 - 1.27)
Intestinal						
Cases/Controls	26/42	22/32	19/51	16/38		
Crude	1.00	1.02 (0.49-2.12)	0.58 (0.28-1.22)	0.58 (0.24-1.40)	0.101	0.83 (0.61 - 1.13)
Fully Adjusted	1.00	1.21 (0.53-2.75)	0.58 (0.26-1.32)	0.59 (0.20-1.73)	0.138	0.85 (0.59 - 1.21)

§ Values are ORs and 95% confidence intervals derived from models described above based on quartiles of plasma levels of vitamin C for GC and GC sub-group analyses, and quartiles of dietary vitamin C for GC.

‡ P of χ^2 test for trend using a continuous variable with 1 df.

* Values are ORs (95% confidence intervals), derived from models as described above, for a risk associated with an increment in vitamin C level equal to the standard deviation of the controls for GCs (Plasma: 19.4 µmol/L; Diet:72.9 mg/day).

** Values are means ± standard error calculated based on the control subjects in each quartile of the respective variable.

Table 4: Odds ratios for a smoothed dose response analysis of the interaction of increasing levels of plasma vitamin C and dietary intake of red and processed meats.

Plasma Vitamin C *(μmol/L)*	**OR (95% CI)** § **Dietary Red and Processed Meat Intake Level** *(g/day)*		
	High *≥ 95.0*	**Medium** *≥ 55.6 - < 95.0*	**Low** *< 55.6*
Low *< 32.0*	1.00	0.80 (0.54-1.11)	0.69 (0.36-1.18)
Medium *≥ 32.0 - < 46.0*	0.79 (0.58-1.00)	0.75 (0.46-1.06)	0.72 (0.37-1.20)
High *≥ 46.0*	0.58 (0.28-1.00)	0.69 (0.35-1.17)	0.76 (0.36-1.50)

§ Values are ORs (95% CI) derived from models described above based on a smoothed dose-response analysis with tertiles of plasma vitamin C and dietary intake of red and processed meats. 95%CI were assessed using a bootstrap sampling procedure.