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Endogenous versus exogenous exposure to N-Nitroso compounds and gastric cancer risk in the European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST) study.

Paula Jakszyn [1], Sheila Bingham [2], Guillem Pera [1], Antonio Agudo [1], Robert Luben [3] Ailsa Welch [3], Heiner Boeing[4], Giuseppe del Giudice[5], Domenico Palli[6], Calogero Saieva [6], Vittorio Krogh [7], Carlotta Sacerdote [8], Rosario Tumino[9], Salvatore Panico[10], Göran Berglund[11], Henrik Simán[11], Göran Hallmans[12], María José Sanchez[13], Nerea Larrañaga [14], Aurelio Barricarte[15], María Dolores Chirlaque [16], José R. Quirós[17], Timothy J. Key[18], Naomi Allen [18], Eiliv Lund[19], Fátima Carneiro[20] Jakob Linseisen [21], Gabriele Nagel[21], Kim Overvad[22], Anne Tjonneland[23], Anja Olsen [23], H. Bas Bueno-de-Mesquita[24], Marga O. Ocké [24], Petra HM.Peeters[25], Mattijs E. Numans[25], Françoise Clavel-Chapelon[26], Antonia Trichopoulou [27], Claus Fenger [28], Roger Stenling[29], Pietro Ferrari[30], Mazda Jenab[30], Teresa Norat[30], Elio Riboli [30,31], Carlos A. Gonzalez [1]

1. Department of Epidemiology, Catalan Institute of Oncology, Barcelona, Spain
2 Medical Research Council, Dunn Human Nutrition Unit, Wellcome Trust/MRC Building, Cambridge ,UK
3. Strangeways Research Laboratory, Cambridge , UK
4. German Institute of Human Nutrition, Potsdam-Rehbücke, Germany
5. IRIS Research Center, Chiron-Vaccines, Siena, Italy
6. Molecular and Nutritional Epidemiology Unit. CSPO – Scientific Institute of Tuscany
7. Epidemiology Unit, Istituto Tumori, Milan, Italy
8. University of Torino, Italy.
9. Cancer Registry, Azienda Ospedaliera "Civile M.P. Arezzo", Ragusa, Italy
10. Dipartamento di Medicina Clinica e Sperimentale, Federico II University, Compagnia di San Paolo, Naples, Italy
11. Department of Medicine, University Hospital Malmo, Lund University Malmo, Sweden
12. Department of Nutritional Research, University of Umeå, Sweden
13. Andalusian School of Public Health, Granada, Spain
14. Department of Public Health of Guipuzkoa, San Sebastian, Spain
15. Public Health Institute of Navarra, Pamplona, Spain
16. Epidemiology Department, Health Council of Murcia, Spain
17. Public Health and Health Planning Directorate, Asturias, Spain
18. Cancer Epidemiology Unit. University of Oxford, UK
19. Institute of Community Medicine, University of Tromso, Norway.
20. Institute of Molecular Pathology and Immunology of the University of Porto and Medical Faculty, Porto, Portugal.
21. Division of Clinical Epidemiology, Deutsches Krebsforschungszentrum, Heidelberg, Germany

22. Department of Clinical Epidemiology, Aalborg Hospital, Aarhus University Hospital, Aalborg, Denmark

23. Institute of Cancer Epidemiology, Danish Cancer Society, Copenhagen, Denmark

24. Center for Nutrition and Health, National Institute for Public Health and the Environment, Bilthoven, The Netherlands
25. Julius Center for Health Sciences and Primary Care, University Medical Center, Utrecht, The Netherlands

26. INSERM, Institut Gustave Roussy, Villejuif, France
27. Department of Hygiene and Epidemiology, Medical School, University of Athens, Greece
28. Det Sundhedsvidenskabelige Fakultet, Syddansk Universitet, Denmark
29. Department of Medical Biosciences, University of Umea, Sweden
30 Nutrition and Hormones Group, International Agency for Research on Cancer, Lyon, France

31. Department of Epidemiology and Public Health Imperial College.London,Uk.

Requests for reprints: Paula Jakszyn, MPH**.** Epidemiology Departmen , Catalan Institute of Oncology, Av. Gran vía s/n km. 2,7 (089079 L´Hospitalet de Llobregat, Barcelona, Spain E.mail:paujak@iconcologia.catsalut.net

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ABSTRACT

The risk of gastric cancer (GC) associated with dietary intake of Nitrosodimethylamine (NDMA) and endogenous formation of Nitroso compounds (NOCs) was investigated in the European Prospective Investigation into Cancer and Nutrition (EPIC). The study included 521,457 individuals and 314 incident cases of GC that had occurred after 6.6 average years of follow up. An index of endogenous NOC formation (ENOC) was estimated using data of the iron content from meat intake and faecal apparent total NOCs formation according to previous published studies. Antibodies to Helicobacter pylori (Hp) and vitamin C levels were measured in a sub-sample of cases and matched controls included in a nested case-control within the cohort. Exposure to NDMA was less than 1 µg on average compared with 93 µg on average from ENOC. There was no

association between NDMA intake and GC risk (HR 1.00; 95 % CI 0.7-1.43). ENOC was significantly associated with non cardia cancer risk (HR 1.42; 95 % CI 1.14-1.78 for an increase of 40ug/d) but not with cardia cancer (HR 0.96; 95 % CI 0.69-1.33). Although the number of not infected cases is low, our data suggests a possible interaction between ENOC and Hp infection (p for interaction =0.09). Moreover, we observed an interaction between plasma Vitamin C and ENOC ($p < 0.01$). Endogenous NOC formation may account for our previously reported association between red and processed meat consumption and gastric cancer risk

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Introduction

Humans are exposed to N-Nitroso compounds (NOCs) from diet, tobacco smoke and other environmental sources, as well as from endogenous synthesis which contributes to 45-75 % of total exposure (1). Various NOCs have been found to be carcinogenic in multiple organs in at least 40 animal species including higher primates (1). Despite continuing concern that NOCs may be causally related to gastrointestinal cancer, the epidemiological literature has failed so far to support this link with any degree of conviction (2). Helicobacter pylori (Hp) infection and other dietary and environmental factors are thought to have a major role in gastric carcinogenesis. Our previous results show a positive association between red and processed meat intake and non-cardia gastric cancer, especially in Hp infected subjects (3).

Bingham et al, (4) have shown that red but not white meat diets markedly increase faecal NOCs (as Apparent Total NOCs (ATNC)) , and that there is a clear dose response with increasing red meat intake, suggesting that endogenous N-Nitrosation could explain the association between colorectal cancer and red and processed meat. Furthermore, there appears to be a specific role of haem on endogenous nitrosation

whereas non meat protein or inorganic iron has no effect (5). Under certain conditions, haems are known to be nitrosated, and act as nitrosating agents (6). Nitric oxide (NO) has been shown to react directly with hemoglobin and myoglobin to produce NOCs (7). The formation of N-nitrosoarginine by haem enzymes under anaerobic conditions has also been demonstrated (8). In addition, endogenous formation of NOCs is increased in the upper gastrointestinal tract following processed and red meat consumption (9). Endogenous production is substantiated by the markedly elevated levels of faecal output (in the order of 500 ug per day) compared with dietary intakes of only 13 ug per day (4)

In this large prospective study of diet and cancer, we have estimated the dietary intake of Nitrosodimethylamine (NDMA) and the endogenous formation of NOCs using an index of endogenous nitrosation (ENOC). Then, we assessed the effect of both on gastric cancer (GC) risk within the full cohort. Also, we have examined the effect modification of ENOC by plasma vitamin C and Hp infection in relation to non-cardia tumours in a nested case-control study.

Material and methods

Subjects: The rationale and methods of the EPIC study have been previously described in detail (10). Briefly, the EPIC cohort consists of *521,457* subjects *(368,010 women and 153,447 men),* from 10 European countries, aged 35-70 years, and recruited mostly between 1992 and 1998. Subjects from Norway (due to a short follow up period) and Greece (because the ENOC could not be calculated) were excluded. Finally, 314 incident GC cases confirmed as adenocarcinoma were included in the cohort analysis.

End points*:* The follow-up was based mostly on population cancer registries (10). GC includes cancers coded as C16 according to the 10th Revision of the International Statistical Classification of Diseases, Injuries and Causes of Death (ICD) (11).

Validation and confirmation of the diagnosis and classification of tumour site was carried out by a panel of pathologists (3). Of the 314 GC incident cases, 92 were classified as cardia cancer (including 19 gastro oesophageal junction cases), 155 as non-cardia and for 67 cases the site was unknown.

Diet and lifestyle questionnaires: Usual diet over the previous 12 months was measured at recruitment by country-specific validated questionnaires (10). Lifestyle questionnaires included questions on education, lifetime history of smoking and alcohol, occupation, reproductive history and use of hormones, history of previous illness and physical activity. Values for total energy and dietary iron were computed using country specific food composition tables.

***Nitrites and NDMA intakes*:** Dietary intake of nitrites and NDMA was estimated by matching food items on the country specific questionnaires with a food database of potential carcinogens (12). Since considerably more information is available for NDMA than for other nitrosamines found in foods only dietary intakes of NDMA were estimated. Given that food items could have several different source of information we selected country-specific values when they were available. For food items for which analysed values were not available, the value of the nearest comparable food was assigned. When there were several matches for a food item the mean of all the suitable values was assigned (< 5% of the food items).

Endogenous NOC: An index for endogenous NOC exposure (ENOC) was determined using data of iron intake from meat and faecal ATNC formation from published studies (4, 5, 9, 13,14). First we estimated the amount of iron from meat for diets administered in these studies, using the UK Food Composition Database (15). Then, the correlation between iron content and faecal ATNC levels was assessed. These estimations showed a very high correlation between intake of iron

from meat and ATNC formation (r=0.948), (Figure 1) whereas that from meat was substantially lower (r=0.576). Therefore, we used linear regression to predict the ENOC for each subject of the cohort using their consumption of iron from meat as independent variable (ENOC µg /day = 40.52 + 18.92 * iron from meat) for intakes of iron less than 8.5 mg/day. For iron intakes higher than 8.5 mg/day, we applied a fixed value of 201.41 obtained from the model (0.7 % of cases).

Nested case-control study: Plasma vitamin C and Hp infection: Plasma vitamin C levels and antibodies against Hp were determined in cases and controls selected for the nested case-control study within the EPIC cohort. For each incident GC case with available blood sample between two to four control subjects were randomly selected from the cohort, matched by sex, age group (+/- 2.5 years), centre and data of blood sample collection (+/- 45 days).

Statistical methods: The proportional hazard model (Cox regression) was used for analysis of the cohort data. It was stratified by age and by centre to control for potential confounding factors as differences in follow up procedures and questionnaire design. Age was used as the time scale variable in all models. Entry time was defined as age at recruitment and final time as age of diagnosis (cases) or age at censoring (at risk subjects). All models were adjusted for sex, height, weight, educational level, alcohol intake (g/day), status of smoking (never, former and current), daily cigarette smoking (in current smokers only), work physical activity (no activity, sedentary, standing, manual and heavy manual), leisure physical activity (as continuous METS-hour/week), energy intake (Kcal/day), consumption of total vegetables, fresh fruit and citrus fruit (g/day), and nitrites intake (mg/day). Intake was analyzed as continuous variables (increment of 1 µg/day and 40 µg/day for NDMA and ENOC respectively), log-transformed and as categorical variables, by EPIC-wide sex-specific tertiles. The increment of 40 µg/day of ENOC was chosen because this corresponded with 100g of meat. Categorical variables were scored from

1 to 3 and trend tests were calculated on these scores. The odds ratio for association of ENOC in Hp infected and non infected subjects and according with plasma levels of vitamin C were estimated by multiple unconditional logistic regression, including matching variables in the model. Interaction between predicted ENOC and HP infection and plasma vitamin C was tested by likelihood ratio test.

Results

The results reported here are from one of the largest cohorts of men and women specifically designed to examine the relationships between diet and cancer. There were 2,916,642 person-years in 6.64 average years of follow up. Baseline characteristics of the participants are given in Table 1. The exposure to NOCs estimated by ENOC was far greater than the dietary exposure according to NDMA intake estimations. GC cases had higher Hp infection rates and lower plasma vitamin C than controls.

Table 2 shows the hazard ratio (HR) of GC associated with dietary NDMA and ENOC exposure. Dietary NDMA was not associated with increased risk of GC (HR 1.00; 95% CI 0.70-1.43 for an increment of 1μg of NDMA). This lack of effect was observed in all the models performed and, for both, cardia and non-cardia tumour. ENOC was positively but not statistically significant associated with GC (HR 1.18; 95% CI 0.99-1.39 for an increment of 40 μg of EN). When analysed by tumour site, ENOC was not associated with cardia cancer risk (HR 0.96; 95 % CI 0.69 -1.33 for an increment of 40 μg of EN). However, it was significantly associated with non-cardia cancer risk (HR 1.42; 95% IC 1.14 - 1.78 for an increment of 40 μg of ENOC). Results were consistent in all models performed.

The risk of non-cardia tumour regarding ENOC stratified by Hp infection and plasma vitamin C is shown in Table 3. Among infected patients, ENOC was found to be associated with non cardia cancer in all models (OR 1.82; 95 % CI: 1.32-2.51 for

categorical trends), while in non-infected subjects we did not find any association However, the test for interaction was not statistically significant (P=0.09). In relation to plasma vitamin C, the positive association with ENOC was present only among those with low serum levels of vitamin C (OR 3.24; 95 % CI 1.77-5.93 for those with less than 40 micromol/l of plasma vitamin C). Among those with high levels of vitamin C, there was no association between ENOC and non- cardia GC risk. The test for interaction was statistically significant (p= 0.02) and consistent in all models performed. Moreover, the stronger effect of ENOC at low levels of plasma vitamin C persisted even after restricting the analysis to Hp infected subjects (OR 3.52; 95 % CI: 1.8-8.9). We analyzed also the interaction with tobacco smoking, but we did not observed any association (p for interaction=0.83).

Discussion

This is the first study reporting relationships between both endogenous and exogenous exposure to NOCs and GC risk. The exposure of NDMA from food was less than 1 μg per day, whereas that from ENOC was 93 μg/day (Table 1). We found that non cardia GC was positively associated with ENOC exposure but not with dietary NDMA. There is only one other cohort study which has investigated the association between NDMA and GC and no association was found (16). In the present study, we estimated intake of NDMA, the volatile nitrosamines most commonly detected in foods. Although this compound only represents a small fraction of total NOC exposure, it is considered an indicator of dietary exposure (17). The limitations of assigning values of NDMA to foods based on published reports include the limited availability of data of these compounds in some food items, and the variation of levels found in similar items across countries. During the second half of the last century reductions in the use of nitrates and nitrites for curing meat and the modifications of malting techniques in the brewing industry have resulted in significant reductions in the levels of NDMA in foods.

Therefore, it may be possible that current NDMA exposure in our population is below the biological level required to increase the risk of cancer

The contribution of endogenous nitrosation (ENOC) was estimated using content of iron from meat intake. The effect of red and processed meat on ENOC can be attributed to the haem content, but heam iron was not used to derive the index because standard data bases estimate haem iron as forty percent of iron in all meats, whereas newer methods of analysis indicate that the proportion should vary from 22 % to 79 % according to the type of meat (18). The use of an indirect measurement of endogenous exposure is a limitation but the correlation between iron and ENOC was very high (Figure 1), and it supports the rationale of our methodology. On the other hand, this methodology could be a useful tool in epidemiological studies where it is not possible to obtain direct estimations of endogenous nitrosation. Our results support the hypothesis that endogenous exposure is probably the major contributor to the overall burden of human exposure to NOC.

There is sufficient evidence that tobacco smoking increases the risk of gastric cancer (19). Our previous results suggest an increase of risk of 79% for GC in currents smokers (20). Although tobacco is a source of specific nitrosamines we did not observed any interaction with ENOC. It might suggest that dietary and tobacco related nitrosamines could have independent effects on the risk of GC.

It is well established that Hp infection increases the risk of developing distal non-cardia gastric cancer but is not associated with the cardia site (21). Infection with Hp is likely to increase NO production from macrophages in response to bacterial overgrowth, so that the availability of NO in HP infected individuals will be increased (22). Haem is readily nitrosated and can then nitrosate other substrates in the presence of NO. Table 2 shows that the effect of ENOC is only observed on non-cardia cancer and is restricted to infected individuals. This result is consistent with the previous reported association in

the same population between red meat intake and non cardia cancer in Hp infected subjects. (3).

Vitamin C may be protective against Hp associated gastric carcinogenesis by enhancing mucosal immune response, neutralizating free radicals, reducing the formation of gastric NOC, inhibiting cell proliferation and directly affecting Hp growth (23). Inflammation induced by Hp infection in the stomach not only causes significantly increased requirements for vitamin C, but also reduces secretion of the vitamin C into gastric lumen (23). We observed a positive association of ENOC among the individuals with low levels of plasma Vitamin C (Table 3). This may reflect lower vitamin C levels associated with Hp infection. Given the potential capacity of Hp to modulate the effect of vitamin C (or vice versa) we analyzed the interaction between ENOC and vitamin C stratifying by Hp infection. The results show that the interaction was even stronger among infected subjects. It is plausible there is synergy between Vitamin C and Hp which modifies the effect of NOCs on GC risk.

In conclusion, we have found a significant increase of non-cardia cancer risk associated with endogenous exposure to NOCs but not with preformed NDMA intake. Cardia cancer was not associated with any type of NOC exposure. Although the number of not infected cases in our study is low, the data suggests a possible interaction between ENOC and Hp infection. Moreover, we observed an interaction between plasma Vitamin C and ENOC. Further cohort studies with more cases and years of follow-up are needed to confirm these findings. Clarifications of the mechanisms of action of these compounds and their potential interactions with Hp and vitamin C levels are needed given the high prevalence of this infection in the general population.

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TABLE 1. Sample description.

	mean	sd	mean	sd
Full cohort	GC Cases		not cases	
N	314	-	439077	-
% male	56%	-	31%	-
Age at recruitment	59,20	7,48	51,58	10,05
Follow up (yrs)	3,73	2,22	6,64	1,72
EN (µg/day)	93,05	30,39	84,46	30,71
NDMA (µg/day)	0,26	0,34	0,19	0,31
Nested case-control **	GC cases		controls	
N	123		1086	
Hp positive (%)	83%	-	65%	-
Nested case-control **				
N	109		491	
Plasmatic Vitamin C (mmol/l)	39,85	25,63	41,12	

[†]EN: Endogenous Nitroscompounds Exposure Index

[‡] NDMA: Nitrosodimethylamine

[§] Hp: helicobacter pylori
Sd: standard deviation

TABLE 2. Endogenous Nitrosocompounds Exposure Index (ENOC) and dietary Nitrosodimethylamine (NDMA) exposure and the risk of stomach adenocarcinoma in the EPIC-EURGAST study *

		Tertiles†				Continuous			
	Cases	2		3		p	Original (‡)		Log-2	
Site	number	HR	(CI95%)	HR	(CI95%)	trend	HR	(CI95%)	HR	(CI95%)
NDMA										
Stomach	314	0.87	(0.64 - 1.2)	0.99	(0.69 - 1.41)	0.96	1	(0.7 - 1.43)	1.01	(0.9 - 1.12)
Cardia	92	0.74	(0.41 - 1.34)	0.68	(0.34 - 1.37)	0.29	0.73	(0.3 - 1.79)	1.01	(0.81 - 1.26)
Non-Cardia	155	1.04	(0.66 - 1.63)	1.09	(0.65 - 1.81)	0.75	1.09	(0.69 - 1.73)	0.96	(0.83 - 1.12)
EN										
Stomach	314	1.12	(0.83 - 1.51)	1.32	(0.94 - 1.84)	0.1	1.18	(0.99 - 1.39)	1.42	(1.06 - 1.92)
Cardia	92	1.43	(0.81 - 2.52)	1.29	(0.67 - 2.47)	0.5	0.96	(0.69 - 1.33)	1.04	(0.59 - 1.83)
Non-Cardia	155	1.22	(0.79 - 1.88)	1.61	(1.01 - 2.58)	0.04	1.42	(1.14 - 1.78)	1.93	(1.28 - 2.91)

† Tertiles are full cohort sex-specific. Cut points are: EN: Men (78 and 106), women (65 and 87); NDMA: men (0.12 and 0.28), women (0.06 and 0.11).

‡ Per 40 µg/d for ENand per 1 µg/d for NDMA.

* Full cohort analysis:

Stratified by center and age.

Adjusted by sex, height, weight, education level, tobacco smoking, cigarette smoking intensity, work and leisure physical activity, citrus and non citrus fruits intake, vegetables intake, alcohol intake, energy intake and nitrites.

TABLE 3. Risk of non-cardia adenocarcinoma regarding Endogenous Nitrosocompounds Exosure Index (ENOC) levels, stratified by Hp infection and plasma Vitamin C levels * in the EPIC-EURGAST study

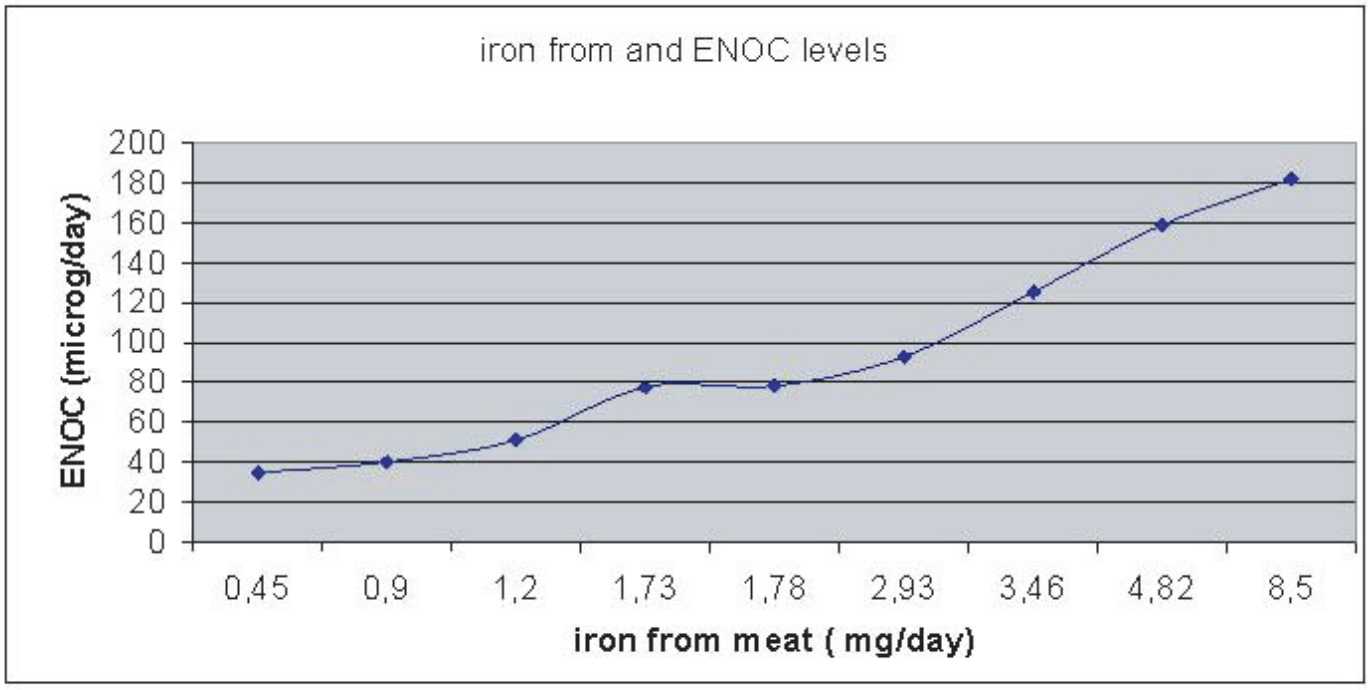
Stratified by Hp infection			Continuos	log-2
	Cases	**Controls**	**OR 95 % CI**	**OR 95 % CI**
infected	111	717	1.82 (1.32-2.51)	2.93 (1.63-5.29)
non-infected	12	369	0.15 (0.01-4.06)	0.22 (0.003-15.3)
p for interaction			0.09	0.13

Stratified by Plasma vitamin C			Continuos	log-2
	Cases	**Controls**	**OR 95 % CI**	**OR 95 % CI**
< 40 micromol/l	54	235	3.24 (1.77-5.93)	10.1 (3.25-31.1)
> 40 micromol/l	55	256	1.10 (0.63-1.93)	1.26 (0.52-3.08)
p for interaction			0.02	0.01

* 40 micromol/lMedian of plasmatic vitamin C

* Unmatched analysis. Adjusted by sex, age, center and date of blood extraction.
Also adjusted by height, weight, education level, tobacco smoking, cigarette smoking intensity, work and leisure physical activity, alcohol intake, energy intake and nitrites.
Hp models were also adjusted by citrus and non citrus fruits intake and vegetables intake.
Vitamin C models were also adjusted by Hp infection

FIGURE 1. Endogenous nitrosation (ENOC) formation* in relation to dietary iron from meat



* Data gathered from feeding studies from Bingham [6], Cross [9], Hughes [10], Silvester [11]