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Original Article

ORIGINAL ARTICLE

Pre-diagnostic copper and zinc biomarkers and colorectal cancer risk in the European Prospective Investigation into Cancer and Nutrition cohort

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Abstract

Adequate intake of copper and zinc, two essential micronutrients, are important for antioxidant functions. Their imbalance may have implications for development of diseases like colorectal cancer (CRC), where oxidative stress is thought to be etiologically involved. As evidence from prospective epidemiologic studies is lacking, we conducted a case-control study nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort to investigate the association between circulating levels of copper and zinc, and their calculated ratio, with risk of CRC development. Copper and zinc levels were measured by reflection X-ray fluorescence spectrometer in 966 cases and 966 matched controls. Multivariable adjusted odds ratios (OR) and 95% confidence intervals (CI) were calculated using conditional logistic regression and are presented for the fifth versus first quintile. Higher circulating concentration of copper was associated with a raised CRC risk (OR = 1.50; 95% CI: 1.06, 2.13; *P*-trend = 0.02) whereas an inverse association with cancer risk was observed for higher zinc levels (OR = 0.65; 95% CI: 0.43, 0.97; *P*-trend = 0.07). Consequently, the ratio of copper/zinc was positively associated with CRC (OR = 1.70; 95% CI: 1.20, 2.40; *P*-trend = 0.0005). In subgroup analyses by follow-up time, the associations remained statistically significant only in those diagnosed within 2 years of blood collection. In conclusion, these data suggest that copper or copper levels in relation to zinc (copper to zinc ratio) become imbalanced in the process of CRC development. Mechanistic studies into the underlying mechanisms of regulation and action are required to further examine a possible role for higher copper and copper/zinc ratio levels in CRC development and progression.

Abbreviations

BMI	body mass index
CRC	colorectal cancer
EPIC	European Prospective Investigation into Cancer and Nutrition
HRT	hormone replacement therapy
hsCRP	high sensitive C-reactive protein
IGF1	insulin-like growth factor 1
ROM	reactive oxygen metabolites
ROS	reactive oxygen species

Introduction

Colorectal cancer (CRC) is the third most common cancer in men and the second in women worldwide and is most prevalent in the developed regions (1). Geographical variations in the incidence rates of CRC suggest an aetiology linked to lifestyle and dietary patterns. An important mechanism of development of colorectal and several other cancers is oxidative damage due to overproduction of reactive oxygen species (ROS) and impaired DNA repair mechanisms (2,3). Some lifestyle habits, such as smoking or alcohol drinking, may promote oxidative stress, whereas consumption of nutrients rich in dietary antioxidants may protect against oxidative stress (4).

Copper and zinc are essential micronutrients whose intra- and extracellular concentrations are tightly regulated; however, they may vary based on their dietary intake levels (e.g. high zinc can decrease copper absorption), efficiency of absorption and excretion, and the presence of other dietary components that may promote or impair their absorption (5). They are both cofactors for a wide range of enzymes, including copper/zinc superoxide dismutase 1 (Cu/Zn-SOD), a powerful intracellular antioxidant defence enzyme essential for eliminating highly reactive superoxides. However, excess circulating copper levels may also promote ROS production (6). Therefore both relatively low zinc and relatively high circulating copper levels may result in increased oxidative stress or impaired antioxidant capacity (5,6). Moreover, altered intake of only one of the two micronutrients may lead to an imbalance in their levels and competition for absorption (5). Other important functions of zinc include immune functions, regulation of apoptosis and improvement of intestinal epithelial barrier function (7). Additionally, both mechanistic and human data suggest that copper plays a central role in angiogenesis enabling tumour growth, invasion and metastasis (8). Therefore both of these micronutrients may play important roles in carcinogenesis.

Several case-control studies have shown that cancer patients have significantly higher circulating levels of copper, lower zinc concentration or an altered ratio of the two micronutrients (9–14). The authors of one of these studies suggested that the Cu/Zn ratio could be used as a clinical indicator for diagnosis of digestive cancers (14). Data from the Paris Prospective Study, a small French cohort of men (15) and the US Second National Health and Nutrition Examination Survey (16) both suggest an association between high circulating levels of copper with

cancer mortality. Other studies investigated dietary intakes of these micronutrients based on dietary questionnaire data. For dietary zinc a meta-analysis of six case-control studies concluded there was an inverse dose-response association (17). A French case-control study has shown a positive association for dietary copper and CRC risk (18). Data from a prospective Japanese cohort showed no association with dietary zinc (19), whereas findings from the Iowa Women's Health Study cohort in the USA showed a decreased cancer risk in the colon with high dietary zinc intake (20).

To date, a comprehensive analysis of the role of copper and zinc in CRC aetiology is missing, particularly from prospective settings and using circulating biomarkers as opposed to dietary intake data. In this study based on the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort, we utilized detailed subject data and biological samples collected prior to diagnosis to assess CRC risk associations for circulating concentrations of copper, zinc and their calculated ratio (Cu/Zn). The large number of subjects allowed for analyses by anatomical subsite and follow-up time, and consideration of differences of associations between men and women.

Materials and methods

Study design and participants

EPIC is a multicentre prospective cohort study from 23 centres in 10 European countries (Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden, and the United Kingdom) (21). Over 520 000 men and women were recruited between 1992 and 2000. Sociodemographic, dietary (using validated country-specific food frequency questionnaires), lifestyle and anthropometric information were collected at baseline. Biological samples were also collected from approximately 80% of all participants and are stored in –196°C liquid nitrogen at the International Agency for Research on Cancer (IARC, Lyon, France), except Denmark (at –150°C under nitrogen vapour) and Sweden (–80°C freezers). Written informed consent was collected from all participants. Approval for the EPIC study was obtained from the IARC ethical review committee and the relevant ethical review boards of the participating institutions.

Cancer incidence and case ascertainment

Incident cases were identified during follow-up based on population cancer registries (Denmark, Italy, Netherlands, Spain, United Kingdom), or by a combination of different methods (insurance records, pathology registries, direct contact with participants or next-of-kin; France, Germany, Greece).

CRC was defined as the first incident colon and rectal cancer cases, identified according to the 10th revision of the International Statistical Classification of Diseases, Injury and Causes of Death (ICD10) and the second revision of the International Classification of Disease for Oncology (ICD-O-2). Colon cancers were tumours in the cecum, appendix, ascending colon, hepatic flexure, transverse colon, splenic flexure, descending and sigmoid colon (C18.0–C18.7) and overlapping or unspecified site tumours (C18.8 and C18.9). Rectal cancers were defined as tumours at the recto-sigmoid junction (C19) or rectum (C20). Anal canal cancers (C21) were excluded.

Nested case-control study design

The present analysis is based on a nested case-control subset of the EPIC cohort with available biological samples from all participating countries except for Norway (blood samples only recently collected; few CRCs diagnosed after blood donation) and Sweden (no available serum samples). Cases were subjects who developed histologically confirmed first incident CRC after the recruitment until the censoring date. The follow-up censoring date in this study ranged between June 2002 and 2003. Each case was matched by incidence density sampling to a cohort participant free of cancer, by: age at blood collection (±1 year), sex, study centre, time of the day at blood collection (±3 h), fasting status at blood collection (<3, 3–6,and >6 h); among women, additionally by menopausal status (pre-, peri-, and post-menopausal), and hormone replacement therapy (HRT) use at time of blood collection (yes/no). Copper and zinc were measured in 966 CRC case-control sets (569 colon and 370 rectal cancers).

Laboratory assays

Bench-top total reflection X-ray fluorescence (TXRF) spectrometer (Picofox™ S2, Bruker Nano GmbH, Berlin, Germany) was used to analyse the serum samples at Charité University Medical School (Berlin, Germany), as previously described (22). Fluorescence of copper and zinc were determined from the emission spectrograms recorded during simultaneously performed selenium analyses (23). For quality-control, case-control status was blinded during the trace element analyses. Copper and zinc measurements were controlled with two commercial standard serum preparations (Level 1 and Level 2, Seronorm, Billingstad, Norway). The coefficient of variation in between each analysis plate was 5.4% (copper) and 8.1% (zinc), respectively. The Cu/Zn ratio was computed by dividing the concentration of copper by that of zinc.

Statistical analyses

Baseline subject characteristics were compared between cases and controls using paired *t*-test or Wilcoxon rank sum test for continuous and Fisher's exact test for categorical variables. As there was no evidence for deviation from normal distribution of the exposure variables, no data transformation was performed. Partial Spearman correlations (adjusted for centre, sex, fasting status, age at recruitment and body mass index, BMI) were calculated in all controls for circulating copper, zinc and their ratio with subjects' baseline characteristics as well as existing measures of relevant biomarkers of oxidative stress (reactive oxygen metabolites, ROM, and ferric reducing ability of plasma, FRAP) (2), inflammation (high sensitive C-reactive protein, hsCRP), lipid and sugar metabolism (TG, HDL, LDL, C-peptide, HbA1c) (24–26), insulin-like growth factor 1 (IGF1) and IGF binding proteins (27), circulating vitamins A, C, D, E (28), and the trace mineral selenium (23). Only correlations where the correlation coefficient was higher than ±0.15 are reported in the results section. Generalised linear models stratified by sex, and adjusted for country were used to compare copper and zinc levels among controls by baseline subject characteristics and other available existing biomarker measures (adjusted by country-, sex-, age- and BMI), in order to investigate the possible influence on the circulating concentrations of copper and zinc of dietary and lifestyle factors.

Conditional logistic regression models were used to calculate odds ratios (OR) and 95% confidence intervals (95% CI) for baseline serum copper and zinc levels and Cu/Zn ratio, in relation to risk of CRC. Crude models were conditioned on the matching criteria. Multivariable models were additionally adjusted for *a priori* selected confounders: categories of physical activity, smoking status and highest level of education attained (see Table 1 for the categories), as well as continuous variables (BMI, total energy consumed, alcohol intake, red and processed meats, fruit and vegetable intake at recruitment). Additional adjustment for the intakes of other dietary variables that have been previously linked to CRC risk, i.e. fish, dietary fibre, and calcium or adjustment for red and processed meats and fruits and vegetables separately, did not alter the estimates and were not included in the model.

Statistical analyses for quintile categories were conducted for all subjects and separately for men and women, using the first category as the reference value. Quintile cut-points were based on the distribution of concentrations in control subjects only. For each variable, *P*-values for trend were calculated using continuous models based on a median value of each exposure category of the relevant variable.

The potential non-linearity of the relation between copper and zinc and CRC risk was examined using restricted cubic splines (29). Tests for linearity used the likelihood ratio test, comparing the model with only the linear term to the model with the linear and the cubic spline terms. The minimum value was chosen as a reference and curves were fixed based on automatically selected four knots. Where a significant linear association was observed, continuous analyses were performed per 1 SD level in all controls of each biomarker (30 μg/dl for copper, 20 μg/dl for zinc and 0.4 units for the Cu/Zn ratio) and the results are presented in Figure 1. Heterogeneity of effects by anatomical subsite of the colon and rectum was assessed by chi-square statistic.

Table 1. Baseline demographic and lifestyle characteristics of CRC cases and their matched controls in the case-control study nested within EPIC cohort

	Colorectal cancer		
	Cases	Controls	*P*-value
N	966	966	
Copper (Cu, µg/dl), mean (SD)	138.6 (30.5)	135.8 (30.8)	0.01
Zinc (Zn, µg/dl), mean (SD)	96.4 (22.6)	97.1 (21.8)	0.37
Cu/Zn ratio, mean (SD)	1.49 (0.43)	1.45 (0.39)	0.001
Age at blood collection (year)[a], mean (SD)	58.6 (7.1)	58.6 (7.1)	
BMI (kg/m^2), mean (SD)	26.8 (4.4)	26.4 (3.8)	0.02
Follow-up from blood collection (year), mean (SD)	3.8 (2.1)	9.11 (1.68)	
Use of HRT (in women; *n*, %)[b]			
No	431 (87.4)	434 (86.7)	0.90
Yes	62 (12.6)	61 (12.3)	
Physical activity (*n*, %)[b]			
Inactive	152 (15.8)	122 (12.8)	0.16
Moderately inactive	283 (29.4)	290 (30.5)	
Moderately active	426 (44.3)	419 (44.0)	
Active	101 (10.5)	121 (12.7)	
Smoking status (*n*, %)[b]			
Never	378 (39.1)	408 (42.2)	0.33
Former	321 (33.2)	317 (32.8)	
Smoker	260 (26.9)	231 (23.9)	
Education (*n*, %)[b]			
None	51 (5.46)	43 (4.57)	0.16
Primary school completed	323 (34.6)	366 (38.8)	
Technical/professional school	241 (25.8)	249 (26.4)	
Secondary school	146 (15.6)	118 (12.5)	
Longer education	173 (18.5)	167 (17.7)	
Daily dietary intake, median (5, 95%)			
Energy (kcal)	2095.9 (1241.3, 3391.5)	2074.4 (1310.9, 3316.7)	0.69
Alcohol (g)	9.5 (0.0, 64.9)	9.4 (0.0, 56.5)	0.06
Red and processed meat (g)	110.6 (33.3, 240.3)	107.8 (33.5, 227.5)	0.21
Fruits and vegetables (g)	365.3 (113.2, 877.1)	398.3 (125.1, 889.9)	0.002

For continuous variables Means (SD) or Medians (5, 95%) are presented. Categorical variables are expressed as *n* (%) per category. Paired *t*-test or Wilcoxon rank sum test for continuous and Fisher's exact test for categorical variables were used to calculate the *P*-value.

[a]Matching factor.

[b]Missing values rectal cancer: use of HRT 8 cases/7controls, physical activity 4 cases/ 14 controls, unknown smoking status 10 cases/ 7 controls, education 23 cases/32 controls.

To test the robustness of the results and to assess the possibility of reverse causation bias, sensitivity analyses were conducted. Analyses by follow-up time categories for cases and their matched controls (≤2 years and >2 years in categorical and ≤2, >2–4, >4–6 and >6 years in continuous analyses) were performed. To test if the observed associations were modified by follow-up time, sex, age at diagnosis, BMI, smoking, alcohol consumption, fasting status, and HRT use in women, interaction tests were performed using the likelihood ratio test. Additionally interactions between categories of exposure (copper, zinc and Cu/Zn ratio) and biomarkers previously associated with CRC that correlated with copper or zinc in this study were also measured to evaluate them as potential effect modifiers. Sensitivity and effect modification analyses were investigated for CRC only.

Analyses were conducted using SAS version 9.2 (SAS Institute, Cary, NC). All statistical tests were two-sided and *P*-values < 0.05 were considered statistically significant.

Results

Baseline characteristics of the study participants

Cases had higher levels of copper, Cu/Zn ratio, BMI, alcohol intake, and lower fruit and vegetable consumption compared to controls (Table 1). Possible micronutrient deficiency in all subjects was 7.5% (79 cases and 66 controls) for zinc and <1% (one case and two controls) for copper (i.e. estimated copper or zinc circulating concentration below 63 µg/dl and 70 µg/dl, respectively, based on recommended daily intakes values (30)). Differences in copper and zinc levels among subject characteristics categories in controls are presented in Supplementary Table 1.

In the control subjects, we observed statistically significant negative correlations between serum copper levels and circulating levels of vitamin C (r = –0.16) and IGF-1 (r = –0.18) and positive correlations with ROM (r = 0.62) and hsCRP (r = 0.33). Similar relationships were observed for the Cu/Zn ratio, except for IGF1, where no association was evident. All biomarkers correlated positively with calcium levels (r for Cu = 0.36, Zn = 0.17, Cu/Zn ratio = 0.16). A positive correlation was also found between zinc and copper (r = 0.26) and between zinc (r = 0.20) and copper (r = 0.17) with selenium levels. No statistically significant correlations were found with dietary variables, circulating vitamins, lipids, cholesterol and apolipoproteins. In the Generalised linear model country-, sex-, age- and BMI-adjusted models copper concentrations and the Cu/Zn ratio were significantly higher with increasing quartiles of ROM (P < 0.0001 and P < 0.0001, respectively) and hsCRP (P < 0.0001 and P < 0.0001, respectively) and lower with increasing circulating vitamin C quartiles (P = 0.0005

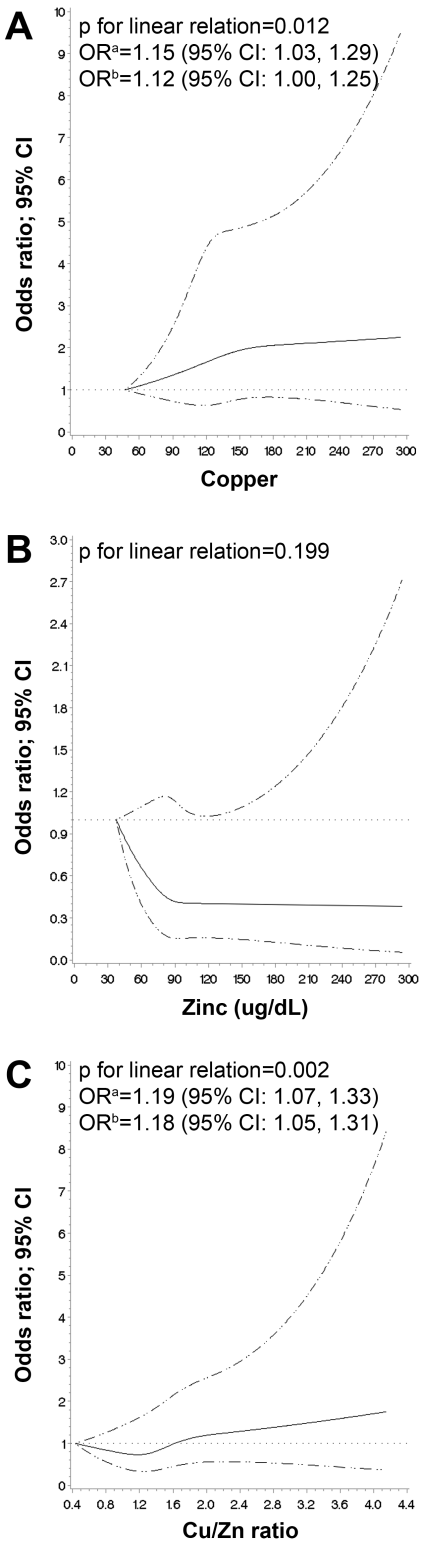

Figure 1. Cubic spline modelling for copper (Cu), zinc (Zn) and Cu/Zn ratio in relation to CRC. Cubic spline shows multivariable adjusted OR (continuous line) and 95% CI (dotted line) for the continuous levels of biomarkers. Reference value was set at minimum value for all subjects. OR (95% CI) for crude[a] and multivariable[b] adjusted continuous models are presented per 1 SD increment.

and $P < 0.0001$, respectively). Copper but not the Cu/Zn ratio was higher with higher IGF1 ($P = 0.004$) quartile levels. Both zinc ($P < 0.0001$) and copper ($P < 0.0001$) were increasing with higher dietary calcium categories, and zinc was lower with increasing c-peptide levels ($P = 0.02$) (data not shown).

Associations with CRC risk

Associations between zinc, copper and Cu/Zn ratio with CRC risk are shown in Table 2. Modelling of associations is illustrated in Figure 1, where ORs (95% CI; per 1SD: i.e. 30 µg/ml for copper, 20 µg/ml for zinc, and per unit for the ratio) are presented only for the biomarkers for which a linear association was found.

Higher serum copper levels were positively associated with CRC risk in crude (fifth versus first quintile OR = 1.60; 95% CI: 1.14, 2.24; *P*-trend = 0.005) and multivariable adjusted models (OR = 1.50; 95% CI: 1.06, 2.13; *P*-trend = 0.021). There was no heterogeneity of the results by sex ($P = 0.948$). As illustrated by Figure 1A, for a 1 SD increase in serum copper a positive linear ($P = 0.012$) association with CRC was noted: OR = 1.15 (95% CI: 1.03, 1.29) and OR = 1.12 (95% CI: 1.00, 1.25) for crude and multivariable adjusted models, respectively. By visual inspection, levels above 150 µg/dl appear to be the threshold for increasing CRC risk, after which the cubic spline indicated a plateau. For zinc, overall an inverse association was suggested with CRC risk. There was a borderline significant inverse association between zinc and CRC in crude (OR = 0.69; 95% CI: 0.47, 1.02; *P*-trend = 0.122) and multivariable adjusted models (OR = 0.65; 95% CI: 0.43, 0.97; *P*-trend = 0.07) for the fifth versus first quintile; although, we observed heterogeneity by sex ($P = 0.023$) (Table 2). The inverse association for zinc appeared not to be linear in men and women combined ($P = 0.249$, Figure 1B) or in men only ($P = 0.732$) but linear in women ($P = 0.013$) (Supplementary Figure 1). Overall, following a gradual decrease in the CRC risk for zinc values up to 90 µg/dl a flattening of the spline was observed beyond this concentration. Higher values for the Cu/Zn ratio were associated with an increased CRC risk in both crude (OR = 1.75, 95% CI: 1.25, 2.46; *P*-trend: 0.0003) and fully adjusted models (OR = 1.70, 95% CI: 1.20, 2.40; *P*-trend = 0.001). There was no heterogeneity of the results by sex ($P = 0.469$). Cu/Zn had a positive dose-response association with CRC ($P = 0.002$, Figure 1C). Each 1 SD increase in the ratio was associated with an increased CRC risk of 19% (95% CI: 7, 33) and 18% (95% CI: 5, 31) in the crude and adjusted models, respectively.

Associations with colon and rectal cancer risk separately

Tests for heterogeneity of findings by anatomical subsite were not statistically significant (copper $P = 0.66$, zinc $P = 0.56$ and Cu/Zn ratio $P = 0.47$). Nevertheless, risk estimates by anatomical subsite were calculated for each analyte and are presented in Supplementary Tables 2 and 3, because previous studies have shown differences in aetiology between colon and rectal cancers (31). Comparing the fifth quintile to the reference category, an increased risk of colon cancer was observed for all subjects with higher copper levels (OR = 1.59; 95% CI: 1.01, 2.50; *P*-trend = 0.034) whereas a borderline lower risk of colon cancer was found for higher zinc concentrations (OR = 0.60; 95% CI: 0.35, 1.00; *P*-trend = 0.078). For the Cu/Zn ratio, a higher risk of colon cancer was observed with similar estimates in crude and adjusted models (OR adjusted for fifth versus first quintile = 1.91; 95% CI: 1.20, 3.04; *P*-trend = 0.001). For rectal cancers, none of the estimates reached statistical significance for copper, zinc or the Cu/Zn ratio, although the number of cases was smaller.

Sensitivity and effect modification analyses

There was an interaction by the time of follow-up category (≤2 years) for copper ($P = 0.003$) and Cu/Zn ratio ($P = 0.0001$), but

Table 2. Odds ratio and 95% confidence intervals (OR, 95% CI) for the association of quintiles of circulating zinc, copper and their ratio with CRC risk

		All subjects			Men[a]			Women[a]	
Biomarker (μg/dl or unit)	*n* cases/ controls	Crude[b]	Adjusted[c]	*n* cases/ controls	Crude[b]	Adjusted[c]	*n* cases/ controls	Crude[b]	Adjusted[c]
Copper (Cu)									
47.3–111.1	162/193	Ref	Ref	118/139	Ref	Ref	44/54	Ref	Ref
111.2–124.5	170/193	1.09 (0.81, 1.46)	1.09 (0.80, 1.48)	107/120	1.07 (0.75, 1.54)	1.07 (0.74, 1.57)	63/73	1.11 (0.65, 1.89)	1.09 (0.63, 1.90)
124.6–139.3	213/193	1.41 (1.04, 1.91)	1.35 (0.99, 1.84)	111/98	1.37 (0.94, 1.99)	1.29 (0.87, 1.90)	102/95	1.46 (0.86, 2.47)	1.41 (0.81, 2.46)
139.3–156.6	198/193	1.36 (0.99, 1.87)	1.28 (0.92, 1.79)	79/67	1.49 (0.96, 2.32)	1.41 (0.89, 2.24)	119/126	1.29 (0.77, 2.16)	1.23 (0.72, 2.12)
156.7–294.2	223/194	1.60 (1.14, 2.24)	1.50 (1.06, 2.13)	50/41	1.57 (0.94, 2.62)	1.55 (0.91, 2.66)	173/153	1.61 (0.95, 2.71)	1.48 (0.85, 2.57)
P-trend		0.005	0.021		0.026	0.052		0.077	0.184
Zinc (Zn)									
48.4–80.2	215/192	Ref	Ref	104/79	Ref	Ref	111/113	Ref	Ref
80.3–89.1	181/194	0.80 (0.60, 1.08)	0.80 (0.59, 1.08)	83/105	0.57 (0.36, 0.88)	0.56 (0.35, 0.89)	98/89	1.10 (0.73, 1.64)	1.12 (0.73, 1.72)
89.2–99.0	190/194	0.83 (0.60, 1.14)	0.82 (0.59, 1.13)	81/97	0.61 (0.39, 0.96)	0.64 (0.39, 1.03)	109/97	1.12 (0.71, 1.77)	1.14 (0.70, 1.85)
99.1–113.2	209/194	0.88 (0.62, 1.24)	0.83 (0.58, 1.19)	104/95	0.82 (0.49, 1.35)	0.82 (0.49, 1.38)	105/99	0.96 (0.59, 1.55)	0.91 (0.55, 1.52)
113.2–293.8	171/192	0.69 (0.47, 1.02)	0.65 (0.43, 0.97)	93/89	0.78 (0.44, 1.38)	0.74 (0.41, 1.36)	78/103	0.60 (0.35, 1.04)	0.55 (0.31, 0.99)
P-trend		0.122	0.070		0.831	0.774		0.052	0.030
Cu/Zn ratio									
0.43–1.11	171/193	Ref	Ref	122/138	Ref	Ref	49/55	Ref	Ref
1.12–1.30	171/193	1.06 (0.79, 1.42)	1.01 (0.75, 1.37)	99/114	1.05 (0.73, 1.51)	1.00 (0.68, 1.46)	72/79	1.07 (0.65, 1.75)	1.00 (0.60, 1.67)
1.31–1.48	176/193	1.13 (0.82, 1.55)	1.06 (0.77, 1.48)	99/90	1.38 (0.91, 2.11)	1.39 (0.90, 2.15)	77/103	0.89 (0.54, 1.48)	0.80 (0.47, 1.35)
1.49–1.71	202/194	1.35 (0.97, 1.87)	1.33 (0.95, 1.86)	76/72	1.32 (0.84, 2.08)	1.28 (0.80, 2.06)	126/122	1.29 (0.78, 2.13)	1.22 (0.72, 2.05)
1.72–3.96	246/193	1.75 (1.25, 2.46)	1.70 (1.20, 2.40)	69/51	1.82 (1.09, 3.04)	1.79 (1.04, 3.08)	177/142	1.63 (0.99, 2.69)	1.55 (0.92, 2.60)
P-trend		0.0003	0.001		0.014	0.022		0.007	0.009

[a]OR and 95% CI estimated by conditional logistic regression conditioned on the matching factors. Heterogeneity of the results by sex was tested by likelihood ratio test. *P*-heterogeneity: copper (*P*-crude = 0.963, *P*-adjusted = 0.948), zinc (*P*-crude = 0.026, *P*-adjusted = 0.0.023), Cu/Zn ratio (*P*-crude = 0.575, *P*-adjusted = 0.469).

[b]Crude model: matching factors only, age at blood collection (1 year), sex, study centre, time of the day at blood collection (3 h), fasting status at blood collection (<3,3–6,and >6 h), among women, additionally by menopausal status (pre-, peri-, and postmenopausal), and hormone replacement therapy use at time of blood collection (yes/no).

[c]Adjusted model: matching factors+ smoking status (categorical), education (categorical), sex-specific physical activity (categorical), baseline BMI (continuous kg/m^2), alcohol, fruit and vegetable, and red and processed meat intake (continuous, g/day).

not zinc (P = 0.235) in the fully adjusted models. In the analyses limited to cases diagnosed at least 2 years after recruitment, which comprised 732 CRC cases (346 men and 386 women), no significant associations were found for copper (fifth versus first quintile, OR = 1.04; 95% CI: 0.69, 1.56; P-trend = 0.99), zinc (fifth versus first quintile: OR = 0.72; 95% CI: 0.45, 1.15; P-trend = 0.326) or the Cu/Zn ratio (OR = 1.11; 95% CI: 0.74, 1.67; P-trend = 0.48) (Table 3). In contrast, higher risk of CRC was observed for copper and the Cu/Zn ratio among those cases diagnosed within the first 2 years for copper and the ratio (fifth versus first quintile, OR = 4.00; 95% CI: 1.74, 9.16 and OR = 7.44; 95% CI: 3.00, 18.47, respectively), whereas a borderline inverse association with CRC risk was estimated for zinc (OR = 0.39; 95% CI: 0.16, 0.98). In additional continuous analyses, similarly to the categorical analyses within each follow-up time category (≤2, >2–4, >4–6, >6 years) no statistically significant results were found for any biomarker with follow-up time longer than 2 years (data not shown).

No interactions were evident for the quintile categories of copper, zinc and the ratio with baseline BMI (per 1 kg/m^2; P = 0.263, 0.219, 0.988, respectively) and alcohol intake (per 1 g/day; P = 0.498, 0.832, 0.812), and categories of fasting status (P = 0.522, 0.689, 0.728) or categories of HRT use in women (P = 0.275, 0.816, 0.612). In additional analyses, we found no interactions between circulating baseline levels of copper, zinc and the ratio with ROM (P = 0.61, 0.42, 0.51), hsCRP (P = 0.98, 0.65, 0.53), IGF1 (P = 0.10, 0.45, 0.48), vitamin C (P = 0.14, 0.87, 0.36), calcium (P = 0.37, 0.65, 0.65) and selenium (P = 0.70, 0.47, 0.71).

Discussion

In this study, we observed an increased risk of CRC with higher circulating levels of copper, either independently or in relation to lower zinc levels. However, these associations were evident only in subjects having a CRC diagnosed within the first 2 years of follow-up after cohort enrolment. Results were not statistically significant upon exclusion of these cases. Thus reverse causality may underlie these observations. However, as we elaborate below, there are several plausible biological mechanisms that could also contribute to our results. In anatomical subsite analyses the significant associations were in concordance with those for CRC, but limited to colon cancer with larger sample size.

One of the potential pathways of CRC carcinogenesis may be related to the chronic inflammatory response of the gastrointestinal tract leading to overproduction of ROS and subsequent DNA damage (32). Cu/Zn-SOD is involved in the neutralisation of ROS and its activity was shown to be increased in colorectal tumours as compared to healthy tissue (33). Sufficient and adequate copper levels in relation to zinc are important for oxidative defence and functioning of the Cu/Zn-SOD antioxidant enzyme (34). The Cu/Zn ratio was previously identified as a diagnostic and prognostic factor of inflammation in several cancers (35–37). Therefore maintenance of adequate copper and zinc levels is very important and tightly regulated at absorption/excretion level (38). However, in some physiological conditions, such as altered dietary intakes or impaired gastrointestinal functions (5), there may be a disruption of their homeostasis. High copper levels may have pro-oxidative properties. Increased levels of circulating copper have been observed previously in response to inflammatory stimuli in rats (39) and excess copper has been associated with increased oxidative stress due to its role in catalysing redox reactions, which can lead to ROS production (40). In the present study we observed increased CRC risk with higher circulating copper and positive correlations between copper and both hsCRP, an inflammation marker, and ROM, an oxidative stress biomarker. hsCRP and ROM levels increased with each category of circulating copper levels, which is in line with the previously observed potential role of copper in inflammatory and oxidative pathways (6). This previous cross-sectional study in asymptomatic adults found that hsCRP and levels of plasma nitrotyrosine, another oxidative stress biomarker, increased

Table 3. Odds ratio and 95% confidence intervals (OR, 95% CI) for the association of quintiles of circulating zinc, copper and their ratio with colorectal cancer risk by the time of follow-up (≤2 years) in both men and women

	≤2 years of follow-up		> 2 years of follow-up		
	N cases/controls[a]	Multivariable adjusted model	N cases/controls[a]	Multivariable adjusted model	P-heterogeneity
Copper					
47.3–111.1	37/60	Ref	125/133	Ref	
111.2–124.5	27/47	1.02 (0.50, 2.10)	143/146	1.06 (0.74, 1.51)	
124.6–139.3	54/48	2.09 (1.04, 4.21)	159/145	1.13 (0.78, 1.63)	
139.3–156.6	48/38	2.33 (1.11, 4.89)	150/155	1.01 (0.69, 1.49)	
156.7–294.2	68/41	4.00 (1.74, 9.16)	155/153	1.04 (0.69, 1.56)	
P-trend		0.0048		0.9903	0.003
Zinc					
48.4–80.2	58/44	Ref	157/148	Ref	
80.3–89.1	42/40	0.87 (0.44, 1.73)	139/154	0.82 (0.58, 1.17)	
89.2–99.0	43/50	0.52 (0.24, 1.13)	147/144	0.94 (0.64, 1.37)	
99.1–113.2	54/59	0.40 (0.17, 0.91)	155/135	0.99 (0.66, 1.49)	
113.23–293.8	37/41	0.39 (0.16, 0.98)	134/151	0.72 (0.45, 1.15)	
P-trend		0.2364		0.3262	0.235
Cu/Zn ratio					
0.43–1.11	35/55	Ref	136/138	Ref	
1.12–1.30	34/52	1.29 (0.59, 2.79)	137/141	0.98 (0.69, 1.38)	
1.31–1.48	34/43	1.45 (0.64, 3.29)	142/150	0.97 (0.67, 1.41)	
1.49–1.71	57/52	3.25 (1.35, 7.78)	145/142	1.06 (0.72, 1.56)	
1.72–3.96	74/32	7.44 (3.00, 18.47)	172/161	1.11 (0.74, 1.67)	
P-trend		<0.0001		0.4802	0.0001

[a]In these analyses, controls were censored at the time of diagnosis of their matched case.

from the lowest to the highest tertile of serum copper concentration (6). This suggests that inflammatory and oxidative changes could be involved in CRC development. Indeed, previous analyses based on the same nested case-control study as reported here indicated that the 'ROM/CRP' biomarker pattern was positively associated with CRC risk (24). Similar to our findings for copper this association was no longer statistically significant after exclusion of the first 2 years of follow-up. However, we did not observe an interaction between copper and ROM or hsCRP in relation to CRC risk.

Although copper possesses both pro- and antioxidant properties, zinc is mainly known for its anti-oxidative role and lack of direct participation in redox reactions (41). Although not always statistically significant, a meta-analysis of six prospective studies reported that the highest category intake of zinc was associated with a lower CRC risk (RR = 0.83; 95% CI = 0.72, 0.94) (17). Similar to our findings, the Nurses' Health Study and Health Professionals Follow-up Study observed an inverse association in women but not in men (42), although this was based on dietary estimations and not circulating zinc levels. In our study we observed an inverse association with CRC risk only for the highest category of circulating zinc (levels above 113 µg/dl), although visual inspection of the cubic spline models suggested a plateau of the association at levels above 90 µg/dl. The role of chance in these observations cannot be eliminated and thus more data from other prospective studies are needed to further explore the potential role of zinc in colorectal carcinogenesis.

The observation that the significant associations were most evident closer to time of diagnosis may indicate that the absorption or metabolism of copper and zinc may be altered by processes of CRC development, such as during the adenoma to cancer transformation. In keeping with this hypothesis, a Korean case-control study reported significantly higher circulating copper levels, but not zinc, in patients with colorectal adenomas or tumours compared to controls (43,44). Higher levels of circulating copper in CRC patients has been attributed to increased levels of pro-inflammatory cytokines that stimulate hepatic synthesis of acute-phase reactive protein ceruloplasmin (45). Ceruloplasmin is a major copper transporter that binds copper released for circulation (38). Another factor in our observation could be that cases may have modified their dietary intakes prior to cancer diagnosis, resulting in altered intake of dietary sources of these micronutrients or dietary inhibitors of their absorption. This is a potential reverse causation bias that requires further investigation in other settings.

The limitations of this study include lack of availability of dietary intake data for comparison of copper and zinc intake between the groups, as they were not selected as priority micronutrients in dietary assessment. However, circulating zinc and copper are accepted biomarkers of intake of these micronutrients and provide accurate serum measurements compared to estimations based on dietary intake questionnaires. There was only a single measure of exposure that could have been modified during the follow-up either at the intake or physiological level and only total circulating copper and zinc levels were measured thus not their protein bound and free form which differ in their biological activities (46). Finally, we have no information on the presence of adenomas in this cohort. The strengths of our study comprise measurement of copper and zinc levels in a large number of pre-diagnostic blood samples and its prospective design enabling estimation of the differences in biomarker status years before diagnosis. Availability of detailed information on lifestyle factors, including smoking habits, alcohol and dietary intakes, as well as data on multiple pertinent biomarkers allowed for better control of confounding and the careful investigation of potential effect modifiers.

In conclusion, increased circulating copper and its imbalance in relation to zinc, as illustrated by the Cu/Zn ratio, appeared to be associated with increased risk of CRC, but only within the 2 years preceding disease diagnosis. There was no evident association of circulating zinc with CRC risk. Further studies are needed to confirm these possible links and potential mechanisms. If our results are replicated in other studies, it may indicate a potential use of copper in relation to zinc levels as an early indicator of CRC development.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

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For information on how to submit an application for gaining access to EPIC data and/or biospecimens, please follow the instructions at http://epic.iarc.fr/access/index.php.
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