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Circulating copper and zinc levels and risk of hepatobiliary cancers in Europeans

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Background: Copper and zinc are essential micronutrients and cofactors of many enzymatic reactions that may be involved in liver-cancer development. We aimed to assess pre-diagnostic circulating levels of copper, zinc and their ratio (Cu/Zn) in relation to hepatocellular carcinoma (HCC), intrahepatic bile duct (IHBD) and gall bladder and biliary tract (GBTC) cancers.

Methods: A nested case–control study was conducted within the European Prospective Investigation into Cancer and Nutrition cohort. Serum zinc and copper levels were measured in baseline blood samples by total reflection X-ray fluorescence in cancer cases (HCC $n = 106$, IHDB $n = 34$, GBTC $n = 96$) and their matched controls (1:1). The Cu/Zn ratio, an indicator of the balance between the micronutrients, was computed. Multivariable adjusted odds ratios and 95% confidence intervals (OR; 95% CI) were used to estimate cancer risk.

Results: For HCC, the highest vs lowest tertile showed a strong inverse association for zinc (OR = 0.36; 95% CI: 0.13–0.98, $P_{trend} = 0.0123$), but no association for copper (OR = 1.06; 95% CI: 0.45–2.46, $P_{trend} = 0.8878$) in multivariable models. The calculated Cu/Zn ratio showed a positive association for HCC (OR = 4.63; 95% CI: 1.41–15.27, $P_{trend} = 0.0135$). For IHBC and GBTC, no significant associations were observed.

Conclusions: Zinc may have a role in preventing liver-cancer development, but this finding requires further investigation in other settings.

Hepatocellular carcinoma (HCC) and intrahepatic bile duct (IHBC) cancers are two subtypes of liver cancer, where HCC is the most common (Page et al, 2014). The prognosis for liver cancer is poor with overall mortality to incidence rate reaching 95%, making it the second deadliest cancer worldwide (Globocan, 2012). Main known risk factors for liver cancers include hepatitis infection, aflatoxin exposure, heavy alcohol intake, smoking, body fatness and non-alcoholic fatty liver disease (NAFLD; Gomaa et al, 2008; Augustine and Fong, 2014). In addition to the established factors, chronic inflammatory and oxidative pathways may be involved in liver-cancer development as a consequence of free radical damage to hepatocytes (Bishayee, 2014).

Zinc and copper are essential micronutrients whose levels are controlled by their rate of absorption in the intestine and excretion

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with bile (Hotz *et al*, 2003; Iakovidis *et al*, 2011; Osredkar, 2011). The richest food sources of zinc and copper include meat, shellfish, offal, seeds and nuts, whole grains, beans and chocolate (Ma and Betts, 2000). Both zinc and copper are cofactors for many enzymatic reactions, including the copper–zinc-dependent super-oxide dismutase (Cu/Zn-SOD) with antioxidant functions that requires adequate zinc and copper to support cellular defence systems against reactive oxygen species (ROS; Osredkar, 2011). It is suspected that the disruption of copper–zinc homoeostasis and overproduction of ROS may lead to DNA damage, protein modification and possibly to cancer development (Osredkar, 2011). Although copper deficiency may diminish Cu/Zn-SOD activity and impair antioxidant defences (Aigner *et al*, 2015), at high levels copper may exhibit toxic effects, particularly in the liver where it is stored (Iakovidis *et al*, 2011). Excess circulating copper in relation to zinc has been shown to increase lipid peroxidation and deplete glutathione (GSH) reserves in rat liver (Ozcelik *et al*, 2003).

A disruption of zinc and/or copper homoeostasis has been observed in different stages of liver diseases (Sawa, 1990; Hyun *et al*, 1992; Lin *et al*, 2006; Guo *et al*, 2013) and was linked to altered production of carrier proteins for these micronutrients in response to inflammatory signals or because of impaired liver synthesis (Deshmukh *et al*, 1985; Brown *et al*, 2004). In fact, information from case–control studies indicates higher circulating copper and lower zinc levels in patients with hepatitis C (Ko *et al*, 2005) and liver cirrhosis (Rahelic *et al*, 2006; Nangliya *et al*, 2015). Additional evidence suggests a link between hepatitis C infection and higher antioxidant enzyme Cu/Zn-SOD activity which has also been observed to be high in liver biopsies of NAFLD patients (Ko *et al*, 2005; Perlemuter *et al*, 2005). Both of these disorders are related to oxidative stress and inflammatory pathways (Satapati *et al*, 2015; Piciocchi *et al*, 2016) and are risk factors for liver cancers (Augustine and Fong, 2014). Specific to liver cancers, two case–control studies have reported decreased levels of zinc and increased levels of copper and Cu/Zn ratio in patients with gall bladder carcinoma (Gupta *et al*, 2005) and HCC (Poo *et al*, 2003). The only existing evidence in a prospective setting, the Shanghai Women's Health Study and the Shanghai Men's Health Study, found no significant liver-cancer risk associations (Ma *et al*, 2017). But the study was based on dietary zinc and copper intake estimates which are likely to have considerable measurement errors. A prospective study based on biomarkers of copper and zinc status is lacking in the current literature.

In this case–control study nested within a large prospective multicentre cohort, we measured baseline circulating levels of these two microelements and related them to the later incidence of cancers located within the liver (HCC, IHBC) and the anatomically related cancers of the gall bladder and biliary tract (GBTC). Additional understanding on possible underlying mechanisms of liver-cancer development may provide greater insight into prevention strategies and modifiable determinants.

MATERIALS AND METHODS

Study design and participants. The European Prospective Investigation into Cancer and Nutrition (EPIC) study 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) (Riboli and Kaaks, 1997). Between 1992 and 2000 over 520 000 men and women were recruited for whom socio-demographic, dietary (using validated country-specific food frequency questionnaires), lifestyle, anthropometric data and biological samples (from approximately 80% participants) were collected at baseline.

Samples from all countries are stored in $-196\,^{\circ}$C liquid nitrogen at the International Agency for Research on Cancer (IARC, Lyon, France), except Denmark (at $-150\,^{\circ}$C under nitrogen vapour) and Sweden ($-80\,^{\circ}$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. For this specific analysis, determination of cancer incidence is complete up to December 2006, conducted by record linkage with regional cancer registries. Exceptions are France, Germany and Greece where a combination of methods was used (health insurance records, contacts with cancer and pathology registries, active follow-up) and is complete up to June 2010.

Cases were identified according to the 10th revision of the International Statistical Classification of Diseases, Injury and Causes of Death (ICD10) and the 2nd edition of the International Classification of Diseases for Oncology (ICD-O-2). HCC was defined as C22.0. GBTC were defined as tumours in the gall bladder (C23.9), extrahepatic bile ducts (C24.0), ampulla of Vater (C24.1) and biliary tract (C24.8 and C24.9). IHBD were defined as C22.1. For each identified case, the histology and the methods used for diagnosis were reviewed and metastatic cases or other types of primary liver cancer were excluded. In detail, from all recruited cohort participants, 23 818 subjects with prevalent cancer other than non-melanoma skin cancer, 4380 with incomplete follow-up data or missing information on the date of diagnosis, 6192 with missing dietary information, 60 with missing lifestyle information, and 9596 who were at the top or bottom 1% of the distribution of the ratio of reported energy intake to energy requirement, and 78 with metastasis in the liver or ineligible histology code were excluded from the above classifications, which were conducted on 477 206 eligible participants.

Nested case–control study design. The present analyses are based on a nested case–control subset of the EPIC cohort (for full description see (Trichopoulos *et al*, 2011)), and include 106 HCC subjects with available biological samples from seven out of ten European countries participating in EPIC (excluding France, Sweden and Norway). Additional analyses were conducted for 34 IHBC and 96 GBTC cases. Each case was matched to a cohort participant who was free of cancer and alive at the time of diagnosis of the case. Control subjects were matched (1:1) using incidence density sampling from all cohort members 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 postmenopausal), and hormone replacement therapy use at time of blood collection (yes/no).

Laboratory assays. Duplicate samples of 20 μl of serum were analysed at Charité University Medical School (Berlin, Germany) in a bench-top total reflection X-ray fluorescence (TXRF) spectrometer (Picofox S2, Bruker Nano GmbH, Berlin, Germany), as described earlier (Hughes *et al*, 2016). Fluorescence of copper and zinc were determined from the emission spectrograms, and the concentrations were determined in relation to a gallium standard added as internal calibrator. The Seronorm Trace Elements Serum L-2 (LOT 0903107, SERO AS, Billingstad, Norway) was used as standard to verify recovery of copper and zinc within the reference concentrations. Samples for which the coefficient of variation (CV) was higher than 10% ($n = 24$ and 39 for copper and zinc, respectively) were re-analysed. Two serum samples of known concentration per each biomarker were used as controls in each plate. Inter-plate CVs were computed and evaluated using GraphPad Prism 6.01 (La Jolla, CA, USA). The coefficients were 9% for copper and 6.9% for zinc.

The present analysis made secondary use of existing biomarker measurements of hepatitis infection status (measured by HBsAg and anti-HCV assays), inflammatory marker high sensitivity CRP (hsCRP), alpha-fetoprotein (AFP), liver function biomarkers (gamma-glutamyltransferase, GGT; alanine aminotransferase, ALT; aspartate aminotransferase, AST; alkaline phosphatase, ALP; total bilirubin and albumin) available for a subset of participants. Circulating levels of these biomarkers at recruitment were previously determined using standard protocols at the Centre de Biologie République laboratory, Lyon, France, as described elsewhere (Stepien et al, 2016b). Measurements of amino acids for the calculation of the Fischer's ratio (ratio of branched chain (isoleucine, leucine, valine) to aromatic (phenylalanine + tyrosine) amino acids; a clinical indicator of severity of liver dysfunction (Ishikawa, 2012) was formerly performed using a targeted metabolomics approach (Biocrates Life Sciences, Innsbruck, Austria), as previously detailed (Stepien et al, 2016a).

Statistical analyses. Comparisons of the baseline subject characteristics for continuous variables were conducted with the t-test for continuous and Fisher's exact test for categorical variables other than the matching factors. The Cu/Zn ratio was computed by dividing the concentration of copper by that of zinc. Crude and sex-, country- and fasting status-adjusted general linear models (GLM) were used to compare biomarker levels among controls by each potential confounder category. Threshold for the Fischer's ratio median category was based on median values for control subjects for each cancer subsite.

Conditional logistic regression models were used to calculate odds ratios (OR) and 95% confidence intervals (95% CI) for baseline serum copper (per $25 \mu g \, dl^{-1}$), zinc (per $20 \mu g \, dl^{-1}$) and Cu/Zn levels (per 0.33 unit), as an approximation of a standard deviation (1 s.d.) increase, in relation to risk of HCC, IHBC and GBTC separately. Categorical analyses for HCC and GBTC were conducted by tertiles of copper, zinc and Cu/Zn ratio. Thirds were defined using sex-specific tertile cut-points in the distribution of concentrations in control participants for each cancer subsite. Sex-specific categories were used because of an apparent heterogeneity of circulating levels between men and women and limited power for sub-group analyses based on low sample size, particularly of women. Crude models were conditioned on the matching criteria (model 1). Multivariable models were additionally adjusted for the following a priori selected confounders: body mass index (BMI), physical activity, alcohol intake at recruitment, lifetime alcohol intake pattern, smoking status, highest level of attained education (model 2; see Table 1 for additional information). Other potential confounders were considered but not kept in the model because they did not modify risk estimates by more than 10%, including daily intakes of: meat and meet products, fruits, nuts and seeds, legumes, cereal products, fish and shellfish, coffee, and self-reported diabetes status at baseline. For HCC, a third model was also applied with additional adjustment for hepatitis B/C infection status (model 3). Additional analyses were performed with model 2, adjusting additionally also for: the liver function score, the Fischer's ratio category (see footnotes f and g in Table 1, respectively, both of which are indicative of possible liver impairment/damage), diabetes status, inflammatory marker hsCRP and circulating albumin levels, which is the carrier protein for zinc in the circulation and whose production may be altered in the event of liver impairment. Cut-offs for categories of hsCRP and albumin were $3 \, mg \, l^{-1}$ and $34 \, g \, l^{-1}$, respectively, as previously employed (Stepien et al, 2016b). To maintain all subjects in the analyses, a category of missing data was created for all the additional variables. Sensitivity analyses excluding the missing category were performed. Restricted cubic spline curves were constructed to visualise the dose–response association between biomarker levels and IHBC and GBTC risk. Minimum

value for all participants was chosen as a reference and three knots were employed. The OR (95% CI) are presented on log10 scale in relation to continues values of the biomarkers ($\mu g \, dl^{-1}$ or units).

As an attempt to control for potential reverse causation, subjects with a short follow-up of <2 years after blood collection were excluded. As hepatitis infection, an established risk factor for liver cancers may alter liver function and hence circulating copper and zinc levels, additional sensitivity analyses were performed by excluding hepatitis positive subjects. Sub-group analyses based on hepatitis negative subjects were conducted using continuous unconditional logistic regression models adjusted for sex, country and age at recruitment. Additional sensitivity analyses were performed excluding samples from Denmark and Sweden, where bio-sample storage conditions differed from those at the IARC biobank.

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

RESULTS

Baseline characteristics. Anthropometric and lifestyle characteristics by HCC status are displayed in Table 1. More HCC cases were current smokers ($P = 0.0054$) and former alcohol drinkers ($P = 0.0025$), had abnormal liver function tests ($P = 0.005$), belonged to lower median for the Fischer's ratio ($\leqslant 0.0001$), indicating possible impairment of their liver functions, and had higher Cu/Zn ratio ($P = 0.0001$). Five HCC cases had zinc levels in the range of deficiency (below $70 \mu g \, dl^{-1}$), none were copper deficient ($<63 \mu g \, dl^{-1}$) and 10 cases had low albumin levels ($<34 \, g \, l^{-1}$). Results for GBTC and IHBC are presented in Supplementary Table 1; there were no significant differences between cases and controls. For the levels of copper, zinc and their ratio by matching factors and potential confounder categories see Supplementary Table 2. In brief, in the GLM sex, country and fasting status- adjusted models zinc levels were significantly different among education level, physical activity, and hepatitis and diabetes status categories. Both copper and its ratio to zinc varied between smoking, physical activity, hsCRP categories, but only the Cu/Zn ratio was different among categories of liver function indices (score and the Fischer's ratio).

Associations with HCC risk. Associations between zinc, copper and Cu/Zn ratio with HCC risk are shown in Table 2. Higher serum zinc levels were inversely associated with HCC risk in crude (model 1, OR = 0.39; 95% CI: 0.19–0.81 for 3rd vs 1st tertile, $P_{trend} = 0.0072$) and multivariable adjusted models (model 2, OR = 0.36; 95% CI: 0.13–0.98 for 3rd vs 1st tertile, $P_{trend} = 0.0123$). Each $20 \mu g \, dl^{-1}$ increase in circulating zinc was associated with 45% (OR = 0.55; 95% CI: 0.39–0.78) and 47% (OR = 0.53; 95% CI: 0.33–0.84) lower HCC risk in models 1 and 2, respectively. For copper, no significant associations were observed with HCC risk in any of the analytic models. For the Cu/Zn, the third tertile was significantly associated with increased HCC risk compared with the reference category (model 1: OR = 4.75; 95% CI: 1.86, 12.12, $P_{trend} = 0.0029$; model 2: OR = 4.63; 95% CI: 1.41, 15.27, $P_{trend} = 0.0135$). Each 1 s.d. increase of Cu/Zn ratio was associated with higher HCC risk (model 1: OR = 2.29; 95% CI: 1.52, 3.46; model 2: OR = 2.53; 95% CI: 1.44, 4.46). Further adjustment for hepatitis status (model 3) attenuated the strength of effects and associations lost statistical significance in the top category (Table 2). Additional adjustment of the model 2 for the liver function score, the Fischer's ratio, hsCRP, diabetes status and albumin (for zinc) categories resulted in similar findings

Table 1. Baseline demographic and lifestyle characteristics of hepatocellular carcinoma (HCC) cases and their matched controls in the case–control study nested within European Prospective Investigation into Cancer and Nutrition (EPIC) cohort

	Cases (N = 106)	Matched controls (N = 106)	P*
Men, N (%)[a]	73 (68.9)	—	
Age at blood collection (years),[a] mean ± s.d.	60.3 ± 7.6	—	
Follow-up from blood collection (years), median (5, 95%)	6.5 (0.7, 12.1)	—	
Body, mass index (BMI; kg m^{-2}), mean ± s.d.	27.6 ± 4.2	28.3 ± 4.40	0.5801
Physical activity (mets), mean ± s.d.	87.4 ± 55.3	86.2 ± 49.1	0.2197
Education (n, %)[c]			0.3851
None/primary	54 (50.9)	55 (51.9)	
Technical/professional	31 (29.3)	21 (19.8)	
Secondary	5 (14.2)	8 (7.6)	
University or higher	15 (14.2)	19 (17.9)	
Smoking status (n, %)[d]			0.0054
Never smokers	32 (30.2)	49 (46.2)	
Former smokers	33 (31.1)	36 (34.0)	
Current smokers	40 (37.7)	20 (18.9)	
Alcohol intake pattern (N, %)			0.0025
Never drinkers	8 (7.6)	9 (8.5)	
Former drinkers	19 (17.9)	3 (2.8)	
Drinkers only at recruitment	4 (3.8)	7 (6.6)	
Always drinkers	75 (70.8)	87 (82.1)	
Hepatitis infection (n, %)[e]			7.1 × 10^{-6}
Yes	26 (24.5)	4 (3.8)	
No	44 (41.5)	66 (62.3)	
Diabetes status (N, %)[f]			0.1818
Yes	15 (14.2)	8 (7.6)	
No	82 (77.4)	89 (84.0)	
Liver function score, N (%)[b,g]			0.005
0	22 (20.8)	56 (52.8)	
1	47 (44.3)	14 (13.2)	
Fischer's ratio, N (%)[h]			<0.0001
⩽2.98	78 (73.6)	41 (38.7)	
>2.98	27 (25.5)	58 (54.7)	
Baseline serum biomarker levels, mean ± s.d.			
Copper (Cu; μd dl^{-1})	140.6 ± 27.5	136.6 ± 26.1	0.5857
Zinc (Zn; μd dl^{-1})	104.6 ± 21.2	113.6 ± 18.5	0.1695
Cu/Zn Ratio	1.39 ± 0.38	1.22 ± 0.26	0.0001
Albumin[b] (g l^{-1})	41.4 ± 2.9	38.7 ± 4.1	0.0051
C-reactive protein[b] (hsCRP; mg l^{-1})	7.32 ± 12.32	3.01 ± 3.39	<0.0001

[a]Matching factor.
[b]Number of cases plus controls with missing variable value: n = 74; cut-off for categorical analyses: albumin = 34 g l^{-1}, hsCRP = 3 mg l^{-1}.
[c]Number of cases plus controls with missing/not specified information: n = 4.
[d]Number of cases plus controls with missing information: n = 2.
[e]Self-reported at baseline, number of cases plus controls with missing information: n = 72.
[f]Number of cases plus controls with missing information value: n = 18.
[g]On the basis of abnormal liver function tests, 0 if none were above the clinical threshold (ALT > 55 U l^{-1}, AST > 34 U l^{-1}, GGT men >64 U l^{-1}, GGT women > 36 U l^{-1}, ALP > 150 U l^{-1}, albumin < 34 g l^{-1}, total bilirubin > 20.5 μmol l^{-1}; based on the values provided by the laboratory).
[h]Number of cases plus controls with missing variable value: n = 8.
Missing values were not excluded from percentage calculations, thus the sum of percents across sub-groups may not add up to 100%. *P-value estimated based on independent sample t-test or Fisher's exact test. Categorical variables are presented as numbers (percentages). Continuous variables are presented as mean ± standard deviations (s.d.).

as obtained from the hepatitis adjusted model 3 (Supplementary Table 3). Exclusion of missing categories for the above variables did not modify the findings.

Additional analyses on the associations between zinc and copper and IHBC and GBTC risks are presented in Supplementary Figure 1. For these cancer sites, no statistically significant associations were observed for any of the biomarkers measured. Exclusion of the two countries that did not store their samples in IARC (n = 15 and 12 case–control sets for HCC and GBTC, respectively) did not change the risk estimates.

Sensitivity analyses. In sensitivity analyses, exclusion of cases and matched controls (n = 19 case-sets) with a follow-up period of <2 years did not significantly modify the findings in continuous

analyses (model 2 for zinc: OR = 0.60; 95% CI: 0.36, 1.02 and Cu/Zn ratio: OR = 7.80; 95% CI: 1.07, 56.84, Supplementary Table 4A). For copper, null associations were observed (OR = 1.00; 95% CI: 0.66, 1.51). Similar risk estimates were obtained after exclusion of 68 subjects with <4 years of follow-up, although these analyses are based on low number of subjects (n = 72 case-sets) (data not shown).

Restricting these analyses to hepatitis negative participants (44 cases, 66 controls, not matched, Supplementary Table 4B), we observed a statistically significantly higher risk of HCC per 1 s.d. increase for Cu/Zn ratio (OR = 2.23; 95% CI: 1.17, 4.29) and no apparent change in the magnitude of the effect estimates for zinc (OR per 20 μg dl^{-1} = 0.55; 95% CI: 0.29, 1.02) and copper (OR per 25 μg dl^{-1} = 1.15; 95% CI: 0.73, 1.83) in fully adjusted model.

Table 2. The association for hepatocellular carcinoma (HCC) risk with zinc, copper and their ratio for tertiles and in continuous models

	Tertile 1 Reference	Tertile 2 OR (95% CI)	Tertile 3 OR (95% CI)	P_{trend}	OR (95% CI)
Zinc (Zn, μg dl^{-1})					**Per 20 μg dl^{-1}**
No. of cases/controls	59/36	21/35	26/35		106/106
Model 1	1.00	0.33 (0.16, 0.70)	0.39 (0.19, 0.81)	0.0072	0.55 (0.39, 0.78)
Model 2	1.00	0.18 (0.06, 0.51)	0.36 (0.13, 0.98)	0.0123	0.53 (0.33, 0.84)
Model 3	1.00	0.20 (0.06, 0.65)	0.50 (0.16, 1.61)	0.1029	0.62 (0.38, 1.03)
Copper (Cu, μg dl^{-1})					**Per 25 μg dl^{-1}**
No. of cases/controls	32/36	31/34	43/36		106/106
Model 1	1.00	1.06 (0.55, 2.02)	1.38 (0.70, 2.71)	0.3184	1.22 (0.90, 1.64)
Model 2	1.00	1.00 (0.42, 2.36)	1.06 (0.45, 2.46)	0.8878	1.23 (0.84, 1.79)
Model 3	1.00	0.94 (0.36, 2.46)	1.02 (0.39, 2.64)	0.9449	1.26 (0.83, 1.92)
Cu/Zn ratio					**Per 0.33 units**
No. of cases/controls	16/35	40.36	50/35		106/106
Model 1	1.00	3.22 (1.36, 7.65)	4.75 (1.86, 12.12)	0.0029	2.29 (1.52, 3.46)
Model 2	1.00	2.35 (0.86, 6.43)	4.63 (1.41, 15.27)	0.0135	2.53 (1.44, 4.46)
Model 3	1.00	1.90 (0.63, 5.80)	3.41 (0.92, 12.65)	0.0698	2.20 (1.21, 3.98)

OR and 95% CI estimated by conditional logistic regression conditioned on the matching factors (model 1) and additional adjustments in models 2 and 3. Continues estimates are based on values close to 1 standard deviation of relevant exposure. Cut-off values for categories: zinc—male ($\leqslant$105.6, >105.6–119.2, >119.2), female ($\leqslant$105.9, >105.9–121, >121). Copper—male ($\leqslant$116.3, >116.3–135.6, >135.6), female ($\leqslant$144.2, >144.2–162.3, >162.3). Cu/Zn ratio- male ($\leqslant$1.02, >1.02–1.22, >1.22), female ($\leqslant$1.20, >1.20–1.54, >1.54). Model 1: matching factors: age at blood collection ($\pm$1 year), sex, study centre, time of the day at blood collection ($\pm$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). Model 2: model 1+ smoking status (categorical), baseline (continuous, g d^{-1}) and lifetime alcohol intake pattern (categorical), education (categorical), body mass index (BMI, continuous kg m^{-2}), and physical activity (PA, mets). Model 3: model 2+plus hepatitis status.

In crude models adjusted only for the matching factors, zinc was significantly negatively (OR = 0.56; 95% CI: 0.35, 0.90) and Cu/Zn ratio positively (OR = 2.45; 95% CI: 1.49, 4.05) associated with HCC, whereas for copper the association was not significant (OR = 1.35; 95% CI: 0.92, 1.98).

DISCUSSION

In this prospective study we observed an inverse association between circulating levels of zinc with risk of development of HCC, but not IHBC nor GBTC. The association with HCC may not be fully independent of hepatitis status or extent of liver dysfunction. Although higher copper levels were not associated with risk of hepatobiliary cancers, its increased levels in relation to zinc demonstrated a positive HCC risk association. To our knowledge this is the first prospective investigation on these associations to date.

The possible roles of zinc and copper in development of cancers at several anatomical sites have been previously explored in experimental settings (Theophanides and Anastassopoulou, 2002; Franklin and Costello, 2009). A meta-analysis of six case–control studies predominantly conducted among Asian populations which showed that an imbalance between circulating levels of copper and zinc may be associated with bladder cancer development (Mao and Huang, 2013) was in line with our findings. Differences in the Cu/Zn ratio have also been noted comparing blood and tissue samples in malignant vs benign ovarian (Lightman et al, 1986) and lung (Diez et al, 1989) tumours. Other data from a small French cohort study (Leone et al, 2006) and the US NHANES study (Wu et al, 2004) also suggest an association between high circulating levels of copper and lower zinc with cancer mortality. A prospective cohort from Iran indicated an inverse association between dietary zinc and non-linear tend for copper with oesophageal cancer risk (Hashemian et al, 2015).

Zinc is thought to have an important role in development and progression of chronic liver diseases and HCC (Costello and Franklin, 2014). Lower circulating zinc levels have been observed in these disorders (Costello and Franklin, 2014), possibly due to impaired hepatic function and lower production of albumin, the carrier protein for zinc (Brown et al, 2004). Our observed zinc-HCC association was attenuated by statistical adjustment for hepatitis status, liver function markers, and albumin levels, particularly at the top tertile. This suggests that liver dysfunction and hepatitis infection may have a confounding or mediating effect on zinc-HCC risk association. For this reason, the potential of reverse causality in our observations cannot be completely ruled out, despite the prospective nature of our study and our lag analyses suggesting consistent associations. However, it is well established that zinc has numerous important physiologic and metabolic roles. Its functions in antioxidant pathways are central to its purported cancer protective effects (Osredkar, 2011). In addition, clinical studies of zinc supplementation indicate that it also acts to improve gut barrier function, decrease pro-inflammatory cytokine production and improve liver function in patients with liver diseases (Mohammad et al, 2012). This implies that maintenance of adequate zinc levels may be an important factor to support hepatocytes from cancer development in patients with underlying liver disease, but this needs to be confirmed in further studies.

In our study, copper was not directly associated with HCC development, but its level in relation to zinc (i.e. a higher Cu/Zn ratio) was strongly associated with increased HCC risk. This observation is likely to be derived more from the influence of zinc in calculation of the ratio. Nevertheless, copper itself has important metabolic roles whose impairment is relevant to cancer development. For example, excess copper may be pro-oxidative and pro-inflammatory (Bo et al, 2008), particularly in the liver where it is stored (Perlemuter et al, 2005). Data from animal studies show that copper excess, imbalanced by lower zinc level, increases lipid peroxidation and depletes GSH reserves (Ozcelik et al, 2003) suggesting a pro-oxidative effect. Interestingly, in the present study circulating copper was significantly increased in those with higher hsCRP levels, a marker of chronic inflammation, suggesting that higher serum copper is linked to inflammatory processes. Such processes may in turn activate production of ceruloplasmin, the copper carrier protein (Deshmukh et al, 1985), thus increasing its availability to bind copper within hepatocytes to release it to circulation. Liver disease progression and concomitant hepatocyte destruction can also cause the release of copper from the liver into the circulation by the damaged cells (Osredkar, 2011). However, as

copper was not associated with HCC in our prospective study, it seems unlikely that this mechanism is an early event in liver tumorigenesis.

It is well recognised that copper to zinc ratio has a significant role in oxidative stress (Osredkar, 2011). The ratio has been previously studied as a measure of susceptibility or progression for a range of neurological, developmental and metabolic disorders, including diabetes and cardiovascular disease (Osredkar, 2011). The Cu/Zn ratio has been observed to be elevated in patients with hepatitis, liver cirrhosis or HCC, and appears to be correlated with the severity of liver disease (Nakayama et al, 2002; Lin et al, 2006). The role of Cu/Zn-SOD, whose functions are dependent on the adequate presence of both copper and zinc, is to catalyse ROS degradation originating from metabolic pathways to less reactive compounds, which are then removed by other antioxidant enzymes such as GSH peroxidase, thus reducing oxidative stress (Johnson and Giulivi, 2005). Maintenance of adequate copper to zinc status is important for supporting anti-oxidative functions of Cu/Zn-SOD and protection from damage.

The gall bladder, bile ducts and biliary tract are anatomically related to liver and liver metabolism. Excess body copper and zinc, are excreted with bile (Iakovidis et al, 2011; Osredkar, 2011), potentially exposing the biliary system to these microelements. However, copper and zinc status is highly regulated and excess levels are not common (Osredkar, 2011), suggesting low exposure of GBTC in the population studied. Although there is some existing evidence that gall bladder cancer patients have decreased serum zinc, increased copper and increased Cu/Zn ratio compared with healthy controls (Shukla et al, 2003; Gupta et al, 2005) our observations did not show any associations with development of GBTC or IHBD tumours. This may be indicative of no aetiological role for these micronutrients, but it may also be a consequence of our low sample size for these rarer tumours. In our previous study a pro-inflammatory cytokine, interleukin 6 that was linked to hypozincemia in a mouse model (Liuzzi et al, 2005), was also positively associated with HCC, but not other hepatobiliary cancers risk (Aleksandrova et al, 2014).

Despite being the second most common cause of death worldwide, indicating the need of more research to better understand its aetiology, liver cancer is relatively uncommon in more developed world regions with age-standardised rates of <10 per 100 000, compared with >20 in East and South-East Asia (Globocan, 2012). For this reason the main limitation of this study conducted on European populations was a relatively small sample size, especially with regard to the less prevalent type of liver cancer, IHBC. We also have no information on evolution of the biomarkers or lifestyle/dietary changes during the follow-up. Oxidative stress has been linked to initiation and progression of cancers (Hjelmeland and Zhang, 2016). Whether oxidative stress-related malignant changes are a consequence or a cause of underlying liver diseases, and specifically of HCC, still needs to be clearly established. Another limitation of the study is that we have measurements of total zinc and copper and cannot differentiate between bound and free forms; both free circulating zinc and copper represent the biologically active forms (Walshe, 2003; Twomey et al, 2005; Maret, 2013). Also, serum zinc and copper are only short-term biomarkers of status. However, given lack of gold standard to measure zinc and copper status, their serum levels are considered useful biomarkers (de Benoist et al, 2007; Harvey et al, 2009). Importantly, potential residual confounding with smoking and alcohol consumption cannot be discounted, nor can the possibility of reverse causality given the long term nature of HCC development and role of the liver in metabolism of these micronutrients. The strengths of our study include its prospective design, being conducted in separate labs with personnel blinded to the clinical identity of the samples and possibility to study early differences in biomarker status years before cancer diagnosis. In addition, availability of additional measures and information on liver-cancer associated disorders (diabetes, hepatitis B and C infection), indices of liver function (liver function tests, Fischer's ratio) and inflammation status (hsCRP), and detailed information on relevant lifestyle factors, including body size, alcohol intake and smoking, allowed us to explore multiple factors in relation to biomarker levels, although this information was not available for all case-sets.

In conclusion, this study indicates an inverse association between pre-diagnostic zinc but not copper levels and HCC risk. Copper imbalance in relation to zinc concentration was in turn positively associated with HCC risk. If these results are confirmed in other prospective cohort studies and different population groups, they would suggest that maintenance of adequate circulating levels of zinc and copper may have an important role in preventing development of liver cancer.

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DISCLAIMER

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

All named authors have read the manuscript, have agreed to the submission and have participated

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