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Dietary N-Nitroso Compounds and Risk of Hepatocellular Carcinoma: A US-Based Study

Jiali Zheng, PhD[1,2], **Carrie R. Daniel, PhD**[2], **Rikita I. Hatia, MPH**[2], **Janice Stuff, PhD**[3], **Ahmed A. Abdelhakeem, MD**[4], **Asif Rashid, MD, PhD**[5], **Yun Shin Chun, MD**[6], **Prasun K. Jalal, MD**[7], **Ahmed O. Kaseb, MD**[4], **Donghui Li, PhD**[4], **Manal M. Hassan, MD, MPH, PhD**[2]

[1]Department of Epidemiology and Biostatistics, Shanghai Jiao Tong University School of Medicine, Shanghai, China

[2]Department of Epidemiology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

[3]USDA Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, TX, USA

[4]Department of Gastrointestinal Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

[5]Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

[6]Department of Surgical Oncology, Division of Surgery, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

[7]Michael E. DeBakey Department of Surgery, Division of Abdominal Transplantation and Hepatobiliary Surgery, Baylor College of Medicine, Houston, TX, USA

Abstract

Background & Aims: N-nitroso compounds (NOCs) are among the most potent dietary carcinogens. N-nitrosodiethylamine (NDEA), N-nitrosodimethylamine (NDMA), and N-nitrosopiperidine (NPIP) are abundant in foods and carcinogenic to the liver. We investigated the relationship between dietary NOCs and hepatocellular carcinoma (HCC) risk.

Approach & Results: In this large, hospital-based, case-control study of 827 pathologically or radiologically confirmed HCC cases and 1,013 controls, NOC intake was calculated by linking food frequency questionnaire-derived dietary data with a comprehensive NOC concentration database. The multivariable-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) of

Author contributions

J.Z. and M.M.H. designed the study and drafted the manuscript. J.Z. conducted the analyses. J.Z., J.S., C.R.D., D.L., and M.H. contributed to the design of the analyses and interpretation of results. J.S. designed the N-nitroso compound concentration database. D.L. and R.I.H. contributed to dietary data collection, management and initial data cleaning. D.L. contributed to control recruitment. A.O.K., P.K.J., and Y.S.C contributed to patient recruitment. R.I.H interviewed cancer patients for all the environmental factors and contacted patients for retrieving the diet questionnaires. A.R. contributed to the pathological definition of cirrhosis, and virus testing. A.A.A reviewed the radiology and pathology reports of patients for evidence of cirrhosis. All authors critically reviewed, revised, and approved the manuscript.

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HCC by quartiles of NOC consumption were estimated using logistic regression models, with the lowest quartile as the referent. We further investigated joint effects of consuming highest quartile of NOCs that were associated with increased HCC risk and hepatitis, diabetes, or alcohol drinking on HCC risk.

After adjustment for confounding factors, higher intake of NDEA from plant sources ($OR_{Q4\ vs.\ Q1}$=1.58; 95% CI=1.03–2.41), NDMA from plant sources ($OR_{Q4\ vs.\ Q1}$=1.54; 95% CI=1.01–2.34), and NPIP ($OR_{Q4\ vs.\ Q1}$=2.52; 95% CI =1.62–3.94) was associated with increased HCC risk. No association was observed for nitrate or total NOC intake and HCC risk. Higher consumption of HCC-inducing NOCs and positive hepatitis virus status jointly increased risk of developing HCC.

Conclusions: In conclusion, while some of our findings may indicate the presence of reverse causation owing to lower meat intake among cases with chronic liver diseases before HCC diagnosis, the potent dietary HCC carcinogens, NDEA, NDMA, and NPIP and their enhanced carcinogenic effects among chronic carriers of hepatitis virus warrant further prospective investigation.

Keywords

N-nitroso compounds; nitrosamine; diet; carcinogen; hepatocellular carcinomas

Introduction

Hepatocellular carcinoma (HCC), the most frequently occurring type of primary liver cancer, is the sixth most common cancer by incidence and the fourth most common cause of cancer death worldwide (1). Although the incidence rate of HCC is lower in the US than in much of East Asia and sub-Saharan Africa (2), in recent years it has risen steadily for both men and women and is expected to continue increasing in the upcoming decades (3,4).

HCC is a unique cancer that often gradually develops as a result of chronic liver diseases, including fatty liver disease and cirrhosis (5). Although some risk factors for HCC are well recognized, including infection with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV), excessive alcohol use, and exposure to aflatoxins, these risk factors cannot fully explain the etiology of HCC in the US. Therefore, other modifiable risk factors need to be identified to optimize prevention of this malignancy (6).

Given the central role of the liver in nutritional metabolism, diet is among the most promising modifiable lifestyle factors with the potential to impact cancer development (7). Among various dietary factors, N-nitroso compounds (NOCs) have garnered significant attention due to their ability to form mutagenic DNA adducts (8). Animal studies have provided strong evidence to support the carcinogenicity of NOCs in digestive organs, with liver cancer being reported most consistently (9, 10). Dietary exposure to NOCs, comprising both preformed NOCs from foods or food processing additives, as well as dietary nitrate and nitrite (precursors of endogenously formed NOCs) comprise the major source of human NOC exposure (11). However, evidence associating dietary NOCs and liver cancer is scarce. Only 1 prospective cohort study in the US assessed the association of nitrate and nitrite with

HCC and found no association (6). In addition, nearly all studies assessing NOC exposure with cancer outcomes focused only on total NOCs, nitrate and/or nitrite (12).

Therefore, the purpose of this large, case-control study was to examine the relationship between dietary intake of specific and total NOCs with HCC risk by using a validated and comprehensive NOC concentration database encompassing 21 different individual NOCs as well as nitrate and nitrite. We also assessed whether consuming higher level of NOCs that was associated with increased HCC risk in our study in conjunction with a known and major HCC risk factor (HBV or HCV infection, alcohol use, and diabetes) jointly raised the risk of developing HCC.

Materials and methods

Study population

Cases in this study were histologically or radiologically confirmed incident HCC cases who were treated in The University of Texas MD Anderson Cancer Center's gastrointestinal medical oncology and surgical oncology outpatient clinics. Patients with other types of primary liver cancer (cholangiocarcinoma, fibrolamellar carcinoma, or benign or unknown tumors) or a concurrent or past history of other cancers were excluded from the study. The controls were free of cancer at recruitment and they were spouses of cancer patients who were diagnosed with cancers other than liver, other gastrointestinal, lung, or head and neck cancers; these cancers were excluded to prevent selection bias from shared environmental and genetic HCC risk factors (13). Cases and controls were recruited simultaneously and consecutively from January 2004 to December 2018, and a total of 855 cases and 1018 controls were eligible for this study, of these, we removed participants with implausible total energy intake (i.e., 3 interquartiles above the 75th percentile or below the 25th percentile of sex-specific BOX-COX-transformed total energy intake)(n=4); and subjects who left more than half of food items blank on the food frequency questionnaire (FFQ) (n=29), which resulted in a total of 827 cases and 1013 controls in this analysis. This study was approved by The University of Texas MD Anderson Cancer Center's Institutional Review Board. Written informed consent was obtained from each participant.

Data collection

Validated and structured questionnaires were used by trained interviewers to collect information regarding demographics, lifestyle factors, family histories of cancers among first- and second-degree relatives, and personal medical histories from cases and controls[13]. No proxy interviews were conducted. We defined ever-alcohol drinkers as participants who had consumed at least 4 alcoholic drinks (e.g., beer, wine, and liquor) each month for at least 6 months in their lifetime. We further classified ever-alcohol drinkers into 2 groups (≤60 mL/day and >60 mL/day) according to their daily intake of ethanol. We used 60 mL of ethanol/day as the cut-off because this amount has been shown to be the threshold at which HCC risk increases (14). Participants self-reported their current heights and body weights and the heights and weights they had had at different ages. The body mass index (BMI) (weight [kg]/height $[m]^2$) was calculated and categorized based on the World Health

Organization criteria (15). Because obesity in early adulthood is a significant risk factor for HCC in this study population (13, 16), we used BMI reported in the 30's in our analyses.

HCC patients' clinical variables were retrieved from their medical records. Underlying cirrhosis was determined by pathological findings (diagnostic biopsies) and computed tomography scans. Blood samples from cases and controls were tested for HBV and HCV. HCV antibodies, hepatitis B surface antigen (HBsAg), and antibodies to hepatitis B core (HBc) antigen were detected with a third-generation enzyme-linked immunosorbent assay (ELISA) (Abbott Laboratories, North Chicago, IL).

Dietary assessment

Usual dietary intake over the past year was self-reported from cases and controls with two different (original and updated) versions of Harvard semi-quantitative FFQ in our study. The original and updated FFQs covered 84 and 131 food items, respectively, and most commonly consumed American foods were on both questionnaires (17). A total of 1257 individuals (301 cases and 956 controls) completed the original version of the FFQ and 583 individuals (526 cases and 57 controls) completed the updated version. On both questionnaires, participants reported their frequency of intake (ranging from never, less than once per month to 6+ times per day) for a specified portion of each food item. The validity of the original Harvard FFQ questionnaire was computed by comparing participants' FFQ-derived dietary intake with four 1-week diet records in a small sample of the Nurses' Health Study and the validity of the updated FFQ was tested against two 1-week diet records in the Health Professionals Follow-up Study (17, 18). For both questionnaires, reproducibility was tested by estimating the correlation between diet intake collected from same version of the FFQ at two time points in one year apart (17, 18). The results indicated that both original and updated questionnaires were reproducible and the self-reported dietary intake from FFQs had modest correlation coefficients with those derived from diet records. Specifically, r ranged from 0.36 to 0.75 for the original version and from 0.28 to 0.86 for the updated version of the FFQ (17, 18).

Calculation of NOC intake

To calculate participants' dietary NOC intake, we linked data from a validated and relatively comprehensive database of the concentrations of 21 NOCs, nitrate, and nitrite in 500 foods to diet data derived from the FFQ. To facilitate this linkage, 39 food subgroups were formed by aggregating the 500 foods based on their common usage and nutrient composition; each food subgroup received the same NOC concentration values (19). The details of the development of the NOC database have been previously described (19). Briefly, the database was constructed through a comprehensive internet search of food assays, publications, and government reports on the NOC content of foods. Due to assay complexities, assays for NOCs were not available for all foods, and no food had complete data for all the NOCs (19). The validity of this database was assessed in a previous cross-sectional study of 98 healthy controls where the NOC database was linked to a modified Block FFQ (20) and 7-day food records, which identified modest agreement between dietary NOC intake derived from these two dietary instruments (21).

To estimate our participants' dietary intake of NOCs, nitrate, and nitrite, we first translated portion sizes to weights (in grams) for each food item and multiplied the weights by the frequency of intake to derive the amount of food consumed per day (22). FFQ-specific calculations were conducted to account for differences in portion-to-gram translations and food items between the two FFQ versions (12, 22). We then calculated each participant's daily consumption of individual NOCs, nitrate, and nitrite by multiplying the daily amount of each food item by the NOC concentration values listed for each food item based on the food subgroup value in the NOC database and summing over all the food items. The daily total NOC intake was the sum of the values for all 21 NOCs plus nitrate and nitrite.

Statistical analysis

Demographic characteristics and HCC risk factors were described for cases and controls and compared using the Chi-square test in Table 1. The multivariable-adjusted association between each factor in Table 1 and the risk of HCC was calculated using unconditional logistic regression models after mutual adjustment for other factors. We categorized dietary intake of total NOC, each of the 21 NOCs, nitrite, and nitrate according to FFQ-specific, log-transformed, energy-adjusted quartile distributions of the control group. Unconditional logistic regression was used to estimate the multivariable-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) of HCC for participants in the higher versus those in the lowest quartiles of NOCs intake as referent group, with adjustment for confounders including total calorie intake, age, sex, race, education level, BMI at 30's, alcohol drinking, history of diabetes, smoking status, and family history of liver cancer. These confounding variables were selected *a priori* based on previous HCC epidemiologic studies in this population and a category of missing values was created for each of these confounders in the model to deal with the missing data issue (13, 23). Multivariable-adjusted associations for NOCs from plant foods and animal foods were investigated separately. To control confounding from plant vs. animal food sources, the total dietary intake of red and processed meat was adjusted for in the analysis of NOCs from plant foods; and dietary intake of fruits and vegetables was adjusted for the in analysis of NOCs from animal foods. Linear trend of HCC risk across quartiles was assessed by using the median value of each quartile in the multivariable-adjusted model, after linearity was confirmed by the restricted cubic spline function within the logistic model (24). If linearity did not hold ($P_{\text{non-linearity}}<0.05$), we did not report linear trend P values but noted the significant non-linearity.

Because we did not find significant gender difference in the NOCs and HCC associations, we performed all of our association analyses for men and women combined. In the main analyses, we focused on the associations of HCC with the intake of total NOCs, N-nitrosodiethylamine (NDEA), N-nitrosodimethylamine (NDMA), N-nitrosodibutylamine (NDBA), N-nitrosodipropylamine, N-nitroso-N-(1-methylacetonyl)-3-methylbutylamine (NMAMBA), N-nitrosopyrrolidine, N-nitrosopiperidine (NPIP), nitrate, and nitrite. Associations between HCC and other NOCs that did not have clear evidence of potential carcinogenicity, or consumed in very limited amounts by participants, or found in very few foods were listed in Supplemental Table 1 (11, 12, 25). We also identified the top 5 food groups contributing to NOC consumption among cases and controls separately by ranking the Spearman correlation coefficients between energy-adjusted NOC

intakes and the food groups in the NOC database. For those NOCs that were found to have significant positive associations with HCC in this study, we further calculated the multivariable-adjusted odds ratios with 95%CIs of HCC according to quartiles of consumption of major food contributors to these NOCs.to examine the main effects of these foods on HCC development.

We further evaluated the potential joint effects of consuming highest quartile of any NOC that was significantly associated with increased HCC risk in the main analysis in conjunction with known and major HCC risk factors including HBV and/or HCV infection, alcohol use, or history of diabetes using joint effect approach, given these risk factors may impact liver metabolism and enhance NOCs' carcinogenic effects. Specifically, we calculated the expected OR for the joint effect of two independent risk factors and compared this value with the observed OR for the joint association, under the null hypothesis that the observed OR would be less than or equal to the expected OR. The expected ORs were calculated as the product of the two independent effects derived from the multivariable-adjusted model. The observed ORs were calculated by comparing odds of HCC among participants having both risk factors versus those who had neither factor (26).

Several sensitivity analyses were performed. To account for the potential effects caused by diet modification and viral infection, we restricted main analyses to individuals without a history of diabetes and to individuals without HBV and/or HCV infection, respectively. Because some patients did not experience cirrhosis in their clinical progression to HCC, we also assessed associations between NOCs and noncirrhotic HCC risk where cases were individuals who did not experience cirrhosis in their clinical progression to HCC. In addition, we performed the analyses among Whites only to see if the inclusion of participants of other races could have impacted our overall findings. To demonstrate how two different FFQs would have impacted the results, we calculated FFQ-specific multivariable-adjusted relationships for HCC and NOCs in the study among subjects who completed the original FFQ, given the sample size of control subjects who had completed updated FFQs was not adequate ($n<15$) to generate stable estimates in the quartile analysis. We also conducted the interaction analyses between version of FFQ and each individual NOC with P values for interactions reported. Finally, we ran all analyses excluding participants with any missing data. All statistical analyses were conducted using SAS version 9.4 (Cary, NC, USA). All tests were 2-sided with P values<0.05 considered to be statistically significant if not otherwise noted.

Results

Compared to controls, HCC cases were more likely to be older, male, non-White, overweight or obese in their 30's, current or former smokers, less educated and to have a history of diabetes, HBV or HBC infection, and family history of liver cancer. After adjusting for confounders, older age (≥ 60), races other than White, African American and Hispanic, education less than high school, obesity at 30s, having diabetes (regardless of duration), ever-alcohol use, positive HBV or HCV status, and having family history of liver cancer were all risk factors for HCC in this population (Table 1).

As shown in Table 2, the highest versus lowest quartile of NDEA from plant sources ($OR_{Q4\ vs.\ Q1}$ =1.58; 95% CI =1.03–2.41), NDMA from plant sources ($OR_{Q4\ vs.\ Q1}$ =1.54; 95% CI=1.01–2.34), NMAMBA from plant sources ($OR_{Q4\ vs.\ Q1}$ =1.54; 95% CI=1.01–2.35), and NPIP which was entirely from animal sources ($OR_{Q4\ vs.\ Q1}$ = 2.52; 95% CI=1.62–3.94; P-trend = 0.0001) were associated with increased HCC risk, although associations appeared to be nonlinear for NDEA, NDMA, and NMAMBA from plant sources (all $P_{non\text{-}linearity}$<0.05) (Table 2). Higher intakes of NDBA ($OR_{Q4\ vs.\ Q1}$ = 0.39; 95% CI = 0.25–0.61; P-trend <0.001) and nitrite ($OR_{Q4\ vs.\ Q1}$ = 0.57; 95% CI = 0.37–0.86), two NOCs largely coming from animal sources, were associated with lower HCC risk. Correlations between food groups and NOCs are presented in Supplemental Table 2. As expected, red and processed meats, including beef, cured lunch meats, and bacon, were the greatest dietary contributors of nitrite and NDBA in this population. For NDEA, NDMA, and NMAMBA from plant sources, which were positively associated with HCC risk in this study, the most important dietary contributors were grains (not contained in vegetables, fruits, nachos, or mixed dishes), and roots (e.g., yams, potato), tofu, and vegetables were secondary important contributors. Cured lunch meats, fresh dairy products, and fermented cheese were highly correlated with NPIP consumption in this population. Based on the multivariable-adjusted associations between major food contributors to NDEA, NDMA, NMAMBA, NPIP and HCC, cases consumed higher level of grains than controls ($OR_{Q4\ vs.\ Q1}$=1.60, 95%CI=1.04–2.45), contributing to the observed positive associations of NDEA, NDMA, NMAMBA from plant sources with HCC; As a major food group contributor to NPIP, a greater amount of fresh dairy products was consumed by cases than controls ($OR_{Q4\ vs.\ Q1}$=4.98, 95%CI =3.20–7.77) (Supplemental Table 3). No associations were observed for NDEA, NDMA, and NMAMBA consumption from animal and plant sources combined or from animal sources only, nitrate, or total consumption of 23 NOC-related compounds (from either animal or plant sources) with HCC risk. Positive associations for NDEA, NDMA, and NMAMBA from plant sources with risk of noncirrhotic HCC were somewhat attenuated, while NPIP consumption remained significantly associated with noncirrhotic HCC risk (Supplemental Table 4). Compared to the NOCs and HCC associations we observed among total subjects, the associations among those completing original FFQ were generally weakened and became non-significant with wider 95%CIs, mainly due to the reduced sample size. None of the interactions between version of FFQ and each individual NOC on the odds of HCC were significant except NMAMBA (P-interaction=0.02), but NMAMBA either from plant source or from animal source did not significantly interacted with version of FFQ, indicating individual NOC consumption and HCC association was not significantly different between two FFQs (Supplemental Table 5). ORs did not change materially and there was no change in significance in any other sensitivity analyses (data not shown).

In the joint effect analyses, our results suggested that consuming highest quartile of NOCs associated with significantly elevated HCC risk in this population (i.e., highest quartile of NDEA, NDMA, or NMAMBA from plant sources or NPIP) and having HBV and/or HCV infection jointly increased risk of developing HCC. This was reflected by observed ORs that were more pronounced than the expected ORs for these two independent effects (i.e., 46.47>2.72×12.86=34.98, Table 3). However, there was no evidence to support a significant joint effect of NOCs and ever-alcohol drinking (i.e., 6.38<3.09×2.6, Table 3) or diabetes

history (48.57<2.87×23.02, Table 3), although the effect of having both exposures was greater than that of having only 1 of these risk factors.

Discussion

In this large case-control study assessing dietary NOC exposure and HCC, we observed that greater intake of NDEA, NDMA, and NMAMBA from plant sources and NPIP from animal sources was associated with a higher risk of HCC. Higher intake of NDBA and nitrite was associated with lower risk of HCC and no significant association was observed for nitrate and the total NOC intake. The risk of developing HCC was highest among individuals with high NOC intake and hepatitis infection, suggesting a potential joint or synergistic effect of these two conditions on HCC development.

NDEA and NDMA are the most prevalent NOCs identified in foods and are classified as class 2A carcinogens (those probably carcinogenic to humans) by the International Agency for Research on Cancer (25). These NOCs require metabolic activation by cytochrome P450 enzymes to form an electrophilic alkylating product that covalently binds to DNA to form a mutation-inducing DNA adduct (9). The liver usually has a higher capacity for metabolizing NOCs than extrahepatic tissues (27). The largest dose-response investigation on nitrosamines by Peto et al (28) indicated that NDMA and NDEA cause liver tumors in experimental animals. DNA adduct formation has also been detected in both animal and human livers after exposure to NDMA or NDEA (27, 29, 30). NPIP, a less potent class 2B carcinogen (possibly carcinogenic to humans) has induced liver tumors in some animals, including monkeys via a similar DNA damaging mechanism as NDEA and NDMA (31, 32). Compared to NDEA, NDMA, and NPIP, NMAMBA is not abundant in foods and its carcinogenicity is not clear (11). Our finding for NDEA, NDMA, and NMAMBA from plant sources and NPIP are in line with our prior findings for pancreatic cancer, another gastrointestinal cancer that shares pathophysiological characteristics with HCC (12), providing additional human evidence to support NOCs carcinogenicity in liver and other tumor types.

In this study, we found that cured meats (such as salami and kielbasa), fresh dairy, and fermented cheese were major contributors of dietary NPIP. Among these, fresh dairy products which referred to milk and ice cream in this study were consumed at a higher level by cases than controls, contributing to the observed significant positive association between NPIP consumption and HCC. The positive link between fresh dairy products and HCC we observed was also supported by two large prospective cohort studies: participants in the highest tertile of milk intake had a 1.51-fold significantly increased HCC risk compared to the lowest tertile group (P-trend=0.05) in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort study (33), and in the Nurses' Health Study(NHS) and Health Professionals Follow-Up Study (HPFS) where a total of 164 HCC cases were identified during up to 32 years of follow-up, higher dairy product intake was associated with an increased HCC risk ($HR_{T3vs.T1}$=1.85, 95% CI=1.19–2.88; P-trend=0.009) (34). On the other hand, as the most significant contributor to plant sources of NDEA, NDMA and NMAMBA, grains intake (contributed primarily by rice and pasta in this population) was found to be positively linked with HCC, which was in line with two previous case-control

studies and a cohort study that identified significantly higher HCC risk in association with higher level of intake of rice or pasta (35 – 37), with relative risks of HCC ranging from 1.21 for brown rice (37) to 4.34 for total rice consumption (35)The observed positive associations between NDEA, NDMA, and NMAMBA from plant sources and NPIP with HCC risk should be further investigated in a prospective cohort study. A deeper understanding of the mechanisms of exposure to NMAMBA and HCC development is also needed.

No associations between nitrate and total NOC intake and HCC risk were observed in this study. Consistent with our finding for nitrate, in the US's largest diet and health prospective cohort study (the National Institutes of Health-American Association of Retired Persons [NIH-AARP] study), the only known cohort study of NOC intake and HCC risk in the literature, Freedman et al. (6) also found that nitrate was not associated with HCC risk, although they identified significant positive associations between red meat and saturated fat intake and HCC.

The inverse associations we observed for NDBA and nitrite derived exclusively from animal foods with HCC paralleled with our previous observations in the pancreatic cancer study (12). In both case-control studies subjects were asked to recall their diets in the previous year of their cancer diagnosis. The preexisting medical conditions, such as diabetes or chronic liver diseases may impair the digestive or metabolic function and cause symptoms, such as decreased appetite, jaundice, abdominal pain, which force the patients to limit their fat intake (38 – 40). Thus, cases could have lower consumption levels of nitrite and some individual NOCs that come entirely from animal foods especially meat-related foods than did disease-free controls, driving the observed inverse associations. Such reverse causality phenomena are commonly seen in epidemiological studies of diet and gastrointestinal cancers and were even detected in a prospective cohort study in which diet change due to underlying diseases occurred several years before cancer diagnosis (41). In our study, however, we could not completely exclude the possibility that nitrite consumption reduces chance of developing HCC (42). A growing body of animal and clinical data suggests that nitrite can protect against various cardiovascular diseases by increasing blood flow, reducing inflammation, and reversing endothelial dysfunction. In addition, long-term dietary nitrate treatment has been shown to reverse metabolic syndrome in nitrogen oxide-deficient mice and this function depends on the nitrate-nitrite-nitrogen oxide pathway (42, 43).

Our epidemiologic study is among the first to investigate the interactions between potentially carcinogenic NOCs (defined in our study as those associated with increased HCC risk) and hepatitis infection, alcohol use, and diabetes in HCC development. Our results indicated that HBV or HCV infection could significantly enhance NOCs' carcinogenic effects on HCC, but that alcohol use or diabetes history did not act synergistically with carcinogenic NOCs to increase likelihood of developing HCC. In the NIH-AARP study, stratified analyses consistently showed that HCC risk estimates for baseline red meat intake did not vary by stratum of alcohol use and diabetes (both P-interaction>0.05), but no interaction analyses for viral hepatitis were performed because hepatitis data were unavailable (6). Earlier case-control studies in Thailand, where the high incidence of liver cancer was not associated with common risk factors such as hepatitis B infection or aflatoxin intake, suggested that endogenous nitrosamine from tobacco or preserved food may act as an

HCC-inducing carcinogen, especially when acting synergistically with HBV or liver fluke infection (44). In addition, woodchuck studies have shown that nitrate and nitrosamine synthesis was enhanced in woodchucks that were chronic carriers of the hepatitis virus (45). In addition to alcohol use, hepatitis infection, and diabetes history, some dietary factors modify endogenous nitrosation and play a role in modifying NOCs' carcinogenic effects in humans. Dietary intake of vitamins C and E and other micronutrients may inhibit nitrosation whereas the intake of red and processed meat may promote it (46). Due to data availability in our study, we analyzed the interactions between red and processed meat and NOCs but detected no significant interactions (data not shown). Gastric acidic conditions (e.g., gastric ulcers) or inflammatory conditions (e.g., inflammatory bowel disease) promote endogenous nitrosation (47). However, data on participants' existing diseases were not available in our study for interaction analyses. Tobacco products and prescribed drugs represent another major source of exogenous NOCs (27), but we did not find smoking status modified the NOC and HCC associations (data not shown). Future studies with adequate sample sizes are needed to confirm the findings of our joint effect analyses and explore other possible interactions with additional exposures.

Our study has several strengths. First, it had enough HCC cases to enable us to investigate the main associations between each individual NOC and HCC risk and conduct important two-factor joint effect analyses. Second, HCC was clearly defined and accurately ascertained in our study. Third, we used validated FFQs containing foods commonly consumed in the US and linked the FFQ data to a validated and relatively comprehensive NOC concentration database of 23 individual NOC-related compounds. This allowed us to conduct a relatively comprehensive assessment of dietary NOC intake. Fourth, our findings for NDEA and NDMA were supported by experimental studies of carcinogenic mechanisms and were consistent with findings from our case-control study on NOCs and pancreatic cancer. Fifth, our discovery that the hepatitis virus can act synergistically with the consumption of higher levels of HCC-inducing NOCs to enhance NOCs' carcinogenic effects has important clinical implications for high-risk liver cancer patients who have been infected with hepatitis. Our findings show that there is a critical need to inform these hepatitis individuals of their increased likelihood of developing HCC and to instigate dietary interventions to reduce added risk by carcinogenic NOCs. Finally, detailed information on HCC risk factors including important clinical features allowed for careful adjustment in the analyses. Because we had detailed information about the presence of cirrhosis in our HCC patients, we performed a restricted analysis of noncirrhotic HCC cases.

This study also had some limitations. Case-control studies have inherent study design-related biases, including recall biases and reverse causality biases, and this is particularly true in a case-control study focusing on the relationship between gastrointestinal cancer and diet in which diets were modified during the assessment period due to patients' cancer-related symptoms. Although we cannot completely eliminate the possibility that some NOCs reduce the risk of developing HCC, the fact that food contributors to these NOCs were mostly red and processed meat provided a consistent indication of the presence of reverse causality. Despite our case-control study design, no proxy responses were used in this study and we carefully controlled selection bias by selecting controls from population with similar demographic and socioeconomic features as cases and by avoiding control

selection related to HCC risk factors. However, cases who come to MD Anderson Cancer Center for diagnosis and treatment generally have high socioeconomic status, so our study results may have limited generalizability. In addition, incomplete coverage of food items and NOCs in the NOC database, our method of grouping foods in the database to assess NOC intake, FFQ-related measurement errors, and our use of a one-time assessment of diet may have contributed to inaccurate NOC intakes. However, this could have led to nondifferential misclassifications of NOC exposures and driven the association estimates towards null. Finally, as we mentioned previously, we lacked data on some potential effect modifiers such as existing comorbidities, dietary nitrosamine inhibitors, drinking water, and medication use, and this prevented adjustment of these factors in the model and examination of their interactions with NOCs. All these possible effect modifiers as well as other exposure pathways are partially responsible for the failure in identifying some individual NOCs as risk factors for HCC.

In summary, in this large, hospital-based, case-control study, we found positive associations between 3 potent dietary and liver carcinogens—NDEA, NDMA, and NPIP—and HCC risk. We also found that higher consumption of these NOCs and hepatitis infection synergistically increased risk of developing HCC; this finding may inform clinical practice related to dietary interventions for high-risk liver cancer patients. These findings need to be replicated in future large, prospective cohort studies involving a comprehensive list of foods containing NOCs and allowing sufficient time between diet assessments and the development of HCC early symptoms to largely avoid the possibility of reverse causality.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

FFQ	food frequency questionnaire
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus

NDBA	N-nitrosodibutylamine
NDEA	N-nitrosodiethylamine
NDMA	N-nitrosodimethylamine
NMAMBA	N-nitroso-N-(1-methylacetonyl)-3-methylbutylamine
NOC	N-nitroso compound
NPIP	N-nitrosopiperidine

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Table 1.

Demographics and risk factors of hepatocellular carcinomas for 827 cases and 1013 controls in the case-control study

Demographic/Risk Factors	Cases (n=827)	Controls (n=1013)	*P*-value [A]	Multivariable-OR (95% CI) [B]
	N (%)	N (%)		
Age groups (Years)			<.0001	
<50	74 (8.95)	174 (17.18)		1.00
50–59	188 (22.73)	292 (28.83)		0.92 (0.51–1.66)
60–69	304 (36.76)	349 (34.45)		1.77 (1.03–3.06)
≥70	261 (31.56)	198 (19.54)		3.71 (2.10–6.55)
Race			<.0001	
White	607 (73.40)	907 (89.54)		1.00
African American	63 (7.62)	29 (2.86)		2.36 (0.92–6.05)
Hispanic	110 (13.30)	69 (6.81)		1.32 (0.76–2.30)
Others [C]	47 (5.68)	8 (0.79)		16.47 (5.78–46.95)
Sex			<.0001	
Males	594 (71.83)	579 (57.16)		1.00
Females	233 (28.17)	434 (42.84)		0.99 (0.70–1.42)
Educational Level [D]			<.0001	
≤ High school	309 (37.36)	218 (21.52)		1.00
< College/Vocational School	253 (30.59)	316 (31.19)		0.65 (0.44–0.97)
College Graduate	137 (16.57)	294 (29.02)		0.53 (0.34–0.82)
Postgraduate	127 (15.36)	183 (18.07)		0.59 (0.37–0.96)
BMI Status at age 30s			<.0001	
Normal (18.5–24.9 kg/m^2)	360 (43.53)	406 (40.08)		1.00
Underweight (<18.5 kg/m^2)	17 (2.06)	24 (2.37)		0.69 (0.24–1.99)
Overweight (25–29.9 kg/m^2)	195 (23.58)	208 (20.53)		0.98 (0.66–1.46)
Obese (≥30 kg/m^2)	77 (9.31)	42 (4.15)		2.86 (1.53–5.36)
Missing	178 (21.52)	333 (32.87)		0.27 (0.18–0.41)

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Demographic/Risk Factors	Cases (n=827)	Controls (n=1013)	*P*-value [A]	Multivariable-OR (95% CI) [B]
History of Diabetes Mellitus [E]			<.0001	
No diabetes history	295(35.67)	906 (89.44)		1.00
Shorter duration (2–5 years)	94 (11.37)	48 (4.74)		7.23 (4.37–11.95)
Longer duration (≥6 years)	196 (23.70)	50 (4.94)		13.64 (8.72–21.35)
≤ 1-year duration	242 (29.26)	9 (0.89)		132.5 (60.89–288.31)
Cigarette Smoking Status [D]			<.0001	
Nonsmokers	276 (33.37)	545 (53.80)		1.00
≤ 20 Pack Years smoking	244 (29.50)	228 (22.51)		1.23 (0.83–1.83)
> Pack Years smoking	307 (37.12)	238 (23.49)		1.10 (0.75–1.60)
Alcohol Drinking Status			<.0001	
Never drinking	237 (28.66)	446 (44.03)		1.00
≤ 60g of Ethanol/day	426 (51.51)	516 (50.94)		1.92 (1.34–2.76)
>60g of Ethanol/day	164 (19.83)	51 (5.03)		3.90 (2.18–6.99)
HCV Status [F]			<.0001	
Negative	542 (65.54)	789 (77.89)		1.00
Positive	272 (32.89)	6 (0.59)		71.74 (29.71–173.23)
Missing	13 (1.57)	218 (21.52)		0.09 (0.01–0.75)
HBV Status [G]			<.0001	
Negative	664 (80.29)	770 (76.01)		1.00
Positive	146 (17.65)	25 (2.47)		3.14 (1.66–5.97)
Missing	17 (2.06)	218 (21.52)		0.62 (0.08–5.13)
Family History of Liver Cancer [D]			<.0001	
No	786 (95.04)	1000 (98.72)		1.00
Yes	41 (4.96)	11 (1.09)		3.50 1.17–10.50)

A. Chi-square test was used to compare the distribution of factors between cases and controls and P value was calculated

B. For each variable, multivariable-adjusted odds ratio and 95%CIs were calculated from the multiple unconditional logistic regression with all the other variables in this Table included

C. Other races include Asian, American Indian and other race/ethnicities

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[D] Data was missing on the variables of education level for 1 case and 2 controls, smoking status for 2 controls and family history of liver cancer for 2 controls

[E] Percentages may not add up to 100% because of rounding

[F] HCV status was determined by serum anti-HCV

[G] HBV status was determined by antibodies to hepatitis B core antigen and HBsAg

Table 2.

Multivariable-adjusted odds ratios and 95% CIs of HCC risk according to quartiles of dietary intake of N-Nitroso compounds (mcg per 1000 kcal/day)*

N-nitroso compounds from dietary sources	Quartiles of intake A				P-trend B
	Q1	Q2	Q3	Q4	
Total N-nitroso compounds C					
Mean (range)	21.44 (0.07–30.20)	35.11 (28.72–41.10)	46.13 (38.34–54.30)	78.48 (48.35–329.65)	
Case/control (n)	257/254	181/253	145/253	244/253	
Multivariable-adjusted OR (95% CI) D	1.00	0.91 (0.59–1.38)	0.61 (0.39–0.95)	1.16 (0.77–1.75)	0.78
Plant sources					
Case/Control (n)	251/254	179/253	160/253	237/253	
Multivariable-adjusted OR (95% CI) E	1.00	0.94 (0.61–1.45)	0.66 (0.42–1.04)	1.13 (0.75–1.71)	0.91
Animal sources					
Case/control (n)	303/254	144/253	160/253	220/253	
Multivariable-adjusted OR (95% CI) F	1.00	0.44 (0.28–0.68)	0.42 (0.27–0.65)	0.72 (0.48–1.08)	*
N-nitrosodiethylamine (NDEA)					
Mean (range)	0.04 (<0.001–0.05)	0.06 (0.05–0.07)	0.08 (0.06–0.09)	0.12 (0.09–0.32)	
Case/control (n)	164/254	163/253	235/253	265/253	
Multivariable-adjusted OR (95% CI) D	1.00	0.70 (0.44–1.11)	1.34 (0.88–2.03)	1.10 (0.72–1.69)	0.32
Plant sources					
Case/control (n)	168/254	150/253	190/253	319/253	
Multivariable-adjusted OR (95% CI) E	1.00	0.86 (0.55–1.35)	0.84 (0.54–1.31)	**1.58 (1.03–2.41)**	*
Animal sources					
Case/control (n)	213/254	170/253	205/253	239/253	
Multivariable-adjusted OR (95% CI) F	1.00	0.75 (0.48–1.16)	0.70 (0.45–1.08)	0.92 (0.60–1.41)	0.58
N-nitrosodimethylamine (NDMA)					
Mean (range)	0.28 (0.05–0.46)	0.46 (0.35–0.68)	0.61 (0.47–0.96)	1.20 (0.63–9.84)	
Case/control (n)	252/254	191/253	174/253	210/253	

N-nitroso compounds from dietary sources	Quartiles of intake [A]				P-trend [B]
	Q1	Q2	Q3	Q4	
Multivariable-adjusted OR (95% CI) [D]	1.00	0.89 (0.59–1.34)	0.67 (0.43–1.05)	0.80 (0.53–1.21)	0.22
Plant sources					
Case/control (n)	175/254	158/253	170/253	324/253	
Multivariable-adjusted OR (95% CI) [E]	1.00	0.87 (0.56–1.37)	0.87 (0.56–1.37)	**1.54 (1.01–2.34)**	*
Animal sources					
Case/control (n)	216/254	219/253	146/253	246/253	
Multivariable-adjusted OR (95% CI) [F]	1.00	1.10 (0.72–1.69)	0.84 (0.53–1.33)	1.26 (0.83–1.91)	0.39
N-nitrosodibutylamine (NDBA) [G]					
Mean (range)	0.75 (<0.001–1.36)	1.73 (1.27–2.23)	2.65 (1.94–3.48)	4.57 (2.95–12.27)	
Case/control (n)	306/254	182/253	205/253	134/253	
Multivariable-adjusted OR (95% CI) [D,F]	1.00	0.52 (0.34–0.79)	0.71 (0.47–1.08)	**0.39 (0.25–0.61)**	<0.001
N-nitrosodipropylamine (NDPA) [G]					
Mean (range)	0.04 (<0.001–0.07)	0.08 (0.06–0.11)	0.13 (0.10–0.19)	0.32 (0.17–1.84)	
Case/control (n)	259/254	170/253	205/253	193/253	
Multivariable-adjusted OR (95% CI) [D,F]	1.00	0.53 (0.34–0.83)	0.94 (0.61–1.44)	0.88 (0.56–1.39)	0.74
N-nitroso-N-(1-methylacetonyl)-3-methylbutylamine (NMAMBA)					
Mean (range)	0.006 (<0.001–0.01)	0.01 (0.008–0.014)	0.015 (0.01–0.02)	0.024 (0.013–0.065)	
Case/control (n)	181/254	144/253	221/253	281/253	
Multivariable-adjusted OR (95% CI) [D]	1.00	0.64 (0.41–1.01)	1.18 (0.78–1.80)	1.24 (0.81–1.87)	0.20
Plant sources					
Case/control (n)	171/254	137/253	211/253	308/253	
Multivariable-adjusted OR (95% CI) [E]	1.00	0.80 (0.51–1.27)	0.98 (0.63–1.51)	**1.54 (1.01–2.35)**	*
Animal sources					
Case/control (n)	280/294	198/213	201/253	148/253	
Multivariable-adjusted OR (95% CI) [F]	1.00	1.09 (0.67–1.76)	1.37 (0.91–2.06)	0.83 (0.53–1.28)	0.93

N-nitroso compounds from dietary sources	Quartiles of intake [A]				P-trend [B]
	Q1	Q2	Q3	Q4	
N-nitrosopyrrolidine (NPYR)					
Mean (range)	0.09 (0.02–0.11)	0.13 (0.10–0.14)	0.16 (0.14–0.19)	0.25 (0.19–0.79)	
Case/control (n)	237/254	200/253	229/253	161/253	
Multivariable-adjusted OR (95% CI) [D]	1.00	1.12 (0.73–1.72)	1.38 (0.90–2.10)	0.89 (0.58–1.37)	0.76
Plant sources					
Case/control (n)	270/254	160/253	182/253	215/253	
Multivariable-adjusted OR (95% CI) [E]	1.00	0.81 (0.53–1.24)	0.92 (0.61–1.41)	0.75 (0.49–1.16)	0.24
Animal sources					
Case/control (n)	228/254	191/253	186/253	222/253	
Multivariable-adjusted OR (95% CI) [F]	1.00	1.11 (0.73–1.71)	1.11 (0.71–1.74)	1.04 (0.68–1.59)	0.88
N-nitrosopiperidine (NPIP) [G]					
Mean (range)	0.005 (<0.001–0.007)	0.008 (0.006–0.01)	0.01 (0.009–0.015)	0.02 (0.01–0.09)	
Case/control (n)	132/254	167/253	157/253	371/253	
Multivariable-adjusted OR (95% CI) [D,F]	1.00	1.56 (0.97–2.51)	0.98 (0.59–1.63)	**2.52 (1.62–3.94)**	0.0001
Nitrate (NO_3) [*]					
Mean (range)	20.53 (0.06–29.43)	34.10 (27.77–40.10)	45.03 (37.03–53.44)	77.40 (47.37–329.04)	
Case/control (n)	257/254	175/253	148/253	247/253	
Multivariable-adjusted OR (95% CI) [D]	1.00	0.89 (0.58–1.36)	0.59 (0.38–0.92)	1.19 (0.79–1.79)	0.70
Plant sources					
Case/control (n)	251/254	179/253	160/253	237/253	
Multivariable-adjusted OR (95% CI) [E]	1.00	0.96 (0.62–1.47)	0.67 (0.43–1.04)	1.14 (0.76–1.73)	0.87
Animal sources					
Case/control (n)	313/254	144/253	171/253	199/253	
Multivariable-adjusted OR (95% CI) [F]	1.00	0.41 (0.26–0.65)	0.56 (0.37–0.86)	0.71 (0.47–1.08)	*
Nitrite (NO_2) [G*]					

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N-nitroso compounds from dietary sources	Quartiles of intake [A]				*P*-trend [B]
	Q1	Q2	Q3	Q4	
Mean (range)	0.36 (0.0001–0.65)	0.71 (0.56–0.84)	0.99 (0.82–1.18)	1.52 (1.09–4.38)	
Case/control (n)	281/254	155/253	172/253	219/253	
Multivariable-adjusted OR (95% CI) [D,F]	1.00	0.49 (0.32–0.75)	0.51 (0.33–0.79)	**0.57 (0.37–0.86)**	*

*Units for means and ranges of all NOCs consumption in this table are mcg/1000kcal/day, except three compounds: total N-nitroso compounds, Nitrate, Nitrite, which used unit of mg/1000kcal/day. Range values of four quartiles were overlapped due to intake from two FFQs

A. Quartile cut-off points were set as the energy-adjusted log-transformed FFQ-specific value based on control's distribution

B. P-trend was calculated by using the median value of each quartile of NOC consumption as a continuous variable in the multivariable-adjusted model. P-trend was noted as "*", If the linearity assumption was not met after assessing it with the restricted cubic spline function within the logistic model ($P_{non\text{-}linearity}<0.05$).

C. Intake of total N-nitroso compounds capture all the 23 compounds in the N-nitroso database, ie, 21 individual NOCs plus nitrite and nitrate.

D. The model was adjusted for age, total calorie, sex (males and females), race (whites, African Americans, Hispanics, others), education level (Less than high school/no formal education, high school graduate, some college/technical or vocational school, college graduate, postgraduate), BMI status (underweight, normal, overweight, obese, missing), alcohol level (never drinkers, current or past drinkers with <=60g of ethanol/day, current or past drinkers with>60g of ethanol/day), history of diabetes and duration combined (No diabetes, have diabetes history with duration< 6 years, have diabetes history with duration>=6 years, have diabetes history with unknown duration), smoking status and dose combined (non-smokers, Current or former smokers with <=20 pack years of smoking, Current or former smokers with>20 pack years of smoking), family history of liver cancer (yes, no), HCV status (negative, positive, missing), HBV status (negative, positive, missing).

E. Multivariable-adjusted model for NOCs from plant sources was additionally adjusted for red and processed meet intake

F. Multivariable-adjusted model for NOCs from animal sources was additionally adjusted for total vegetable and fruit intake

G. Total N-nitroso compounds consumption were all from animal sources.

Table 3.

Table 3. Joint effects of highest-quartile consumption of NOCs associated with increased HCC risk and hepatitis virus infection, alcohol use, or history of diabetes on HCC risk

	Dietary intake of highest quartile of NOCs associated with increased HCC risk [A]	**No. of Case/Control**	**OR (95% CI)** [B]	***P*-value** [C]
Hepatitis virus [D]				<.0001
Negative	No	141/421	1.00	
Negative	Yes	333/343	2.72 (1.93–3.84)	
Positive	No	97/17	12.86 (6.52–25.40)	
Positive	Yes	243/14	46.47 (23.98–90.05)	
Alcohol drink				<.0001
Never	No	66/218	1.00	
Never	Yes	171/228	3.09 (1.80–5.28)	
Ever	No	176/338	2.60 (1.50–4.51)	
Ever	Yes	414/229	6.38 (3.77–10.82)	
Diabetes history				<.0001
No	No	78/502	1.00	
No	Yes	217/404	2.87 (1.94–4.23)	
Yes	No	164/54	23.02 (13.82–38.35)	
Yes	Yes	368/53	48.57 (29.99–78.68)	

A. Based on results from table 2, If a subject consumed the highest quartile of NDEA from plant foods, NDMA from plant foods, NMAMBA from plant foods or NPIP which were associated with increased risk of HCC in our study, they are coded as “yes” otherwise “no” for this variable

B. In addition to all the variables we included in the multivariable-adjusted model, we adjusted for whether or not consuming highest quartile of NOCs that decreased HCC risk significantly based on results from table 2 (i.e, yes, no)

C. P value was calculated by adding the cross-product of two joint-effect factors into the multivariable-adjusted model.

D. Positive HBV status and/or positive HCV status was defined as positive Hepatitis virus, neither positive HBV or positive HCV status was defined as negative Hepatitis virus status.