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ORIGINAL ARTICLE

Prediagnostic levels of urinary 8-*epi*-prostaglandin $F_{2\alpha}$ and prostaglandin E_2 metabolite, biomarkers of oxidative damage and inflammation, and risk of hepatocellular carcinoma

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Abstract

Chronic inflammation and oxidative stress play pivotal roles in the pathogenesis of hepatocellular carcinoma (HCC). We conducted a nested case–control study of 347 HCC cases and 691 matched controls within a prospective cohort of 18 244 Chinese men in Shanghai, China. The concentrations of 8-*epi*-prostaglandin $F_{2\alpha}$ (8-*epi*-$PGF_{2\alpha}$), a biomarker of oxidative stress, and prostaglandin E_2 (PGE_2) metabolite (PGE-M), a biomarker of the inflammation mediator PGE_2, were determined in baseline urine samples using validated mass spectrometry assays. 8-*epi*-$PGF_{2\alpha}$ levels were significantly higher in HCC cases than control subjects (geometric means 0.92 versus 0.80 pmol/mg creatinine, $P < 0.001$). The relative risks of developing HCC for the highest relative to the lowest quartile of 8-*epi*-$PGF_{2\alpha}$ were 2.55 (95% confidence interval = 1.62–4.01, $P_{trend} < 0.001$). This positive 8-*epi*-$PGF_{2\alpha}$–HCC risk association was independent of smoking status, alcohol consumption and hepatitis B or liver cirrhosis and was present 10 years before the clinical manifestation of HCC. This study did not find any significant association between urinary PEG-M and HCC risk. This study provides direct evidence in support of the critical role of oxidative stress in the development of HCC regardless of its underlying causes.

Introduction

Primary liver cancer in both men and women combined is the sixth most frequently diagnosed cancer worldwide and the fourth most frequent cause of cancer death (1). In the USA, hepatocellular carcinoma (HCC) is the major histological form, accounting for 75% of total primary liver cancer cases (2). In high-risk populations, important risk factors for HCC include chronic infection with hepatitis B virus and dietary aflatoxin (3). In relatively low-risk populations, hepatitis C, excessive alcohol intake, cigarette smoking, diabetes and obesity play a prominent role in HCC development (4–6). Both incidence and mortality rates of HCC in the USA have been rapidly and continuously increasing in the past 40 years (7–9). Liver cancer was the sixth leading cause of cancer death in the USA in 2017 (10).

Chronic inflammation, regardless of underlying causes, causes persistent liver injury and consecutive regeneration, potentially leading to fibrosis and cirrhosis, and consequently, to the development of HCC. There is substantial evidence that obesity is associated with chronic low-grade systemic

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Abbreviations

AFB_1	aflatoxin B_1
BMI	body mass index
CI	confidence interval
COX-2	cyclooxygenase-2
8-*epi*-$PGF_{2\alpha}$	8-*epi*-prostaglandin $F_{2\alpha}$
HBsAg	hepatitis B surface antigen
HCC	hepatocellular carcinoma
NASH	non-alcoholic steatohepatitis
NAFLD	non-alcoholic fatty liver disease
OR	odds ratio
PGE_2	prostaglandin E_2
PGE-M	prostaglandin E_2 metabolite

inflammation, which is believed to contribute to metabolic disorders, and the progression from hepatic steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and finally to HCC (11). With the increasing prevalence of obesity in most developed countries over the past three decades, obesity and obesity-associated biomarkers have been increasingly recognized as a major independent risk factor for HCC (12–14). It is estimated that among overweight individuals, the prevalence of non-alcoholic fatty liver disease (NAFLD) was 28% in the USA (15). Among patients with NAFLD, 30–69% were confirmed as having NASH (16,17). The incidence rate of HCC was estimated to be 0.44 (per 1000 person-years) among individuals with NAFLD and 5.29 among subjects with confirmed NASH, and the estimated liver-specific mortality rates were 11.77 and 25.56, respectively. Compared with subjects without NAFLD, subjects with NAFLD had relative risk of 1.94 (95% confidence interval [CI] = 1.26–2.92) liver-specific mortality. Similarly, subjects with NASH had 64.6 times (95% CI = 35.43–117.8) the risk of liver-specific mortality than those without NASH (18).

Oxidative stress, which results from the generation of reactive oxygen species by environmental risk factors or cellular mitochondrial dysfunction, may be involved in the progression of chronic liver disease to the development of HCC (19,20). Oxidative stress could be induced *via* chronic hepatic inflammation regardless of etiology. Oxidative stress has been reported to play a critical role in many liver diseases, including viral hepatitis, alcoholic liver injury and NASH (21–23). Biomolecules such as lipid, protein and DNA are the targets of reactive oxygen species, resulting in DNA damage and in lipid peroxidation (24).

8-*epi*-Prostaglandin $F_{2\alpha}$ (8-*epi*-$PGF_{2\alpha}$) is a product of lipid peroxidation, which originates from cell membranes in every tissue of the human body. 8-*epi*-$PGF_{2\alpha}$ has been recognized as a specific, chemically stable, quantitative marker of systemic and integrated measure of oxidative stress (25). It can be detected in peripheral blood and in urine (26–28). Its levels were significantly elevated in urine samples of smokers and significantly associated with urinary cotinine (29–31). Urinary levels of 8-*epi*-$PGF_{2\alpha}$ also were found to be significantly associated with urinary levels of aflatoxin B_1 (AFB_1), an established hepatocarcinogen (32). Measurement of 8-*epi*-$PGF_{2\alpha}$ in urine or plasma has also been shown to reflect the oxidative stress of patients with a variety of disease conditions. Previous studies have reported that urinary levels of 8-*epi*-$PGF_{2\alpha}$ were significantly associated with a higher risk of several cancers including breast cancer (33) and lung cancer (31). There has been only one case–control study (74 HCC cases) nested in a cohort of Taiwanese that found that baseline urinary levels of 15-F_{2t}-isoprostane (the same as 8-*epi*-$PGF_{2\alpha}$) were related to HCC risk (32).

Consistent with the strong association between chronic inflammation and hepatocarcinogenesis, studies have shown that mediators of inflammation, such as prostaglandins, have an important role in hepatocarcinogenesis (34). Prostaglandin E_2 (PGE_2) is one of the major end products of the cyclooxygenase-2 (COX-2) pathway, an enzyme that is an important mediator of inflammation. Previous studies have shown that the amount of PGE_2 was significantly increased in HCC tissue and adjacent non-cancerous tissues relative to normal liver tissue (35). Catabolism of PGE_2 results in a stable end metabolite (PGE-M), which is excreted in the urine (36) and is used as an index of systemic PGE_2 levels (37,38). Previous studies have shown that urinary levels of PGE-M were significantly elevated in smokers (39), overweight or obese individuals (40), and patients with breast cancer (41), colorectal cancer (42), gastric cancer (43) or head and neck cancer (44), possibly, reflecting their high COX-2 activity. There have been no epidemiologic data reported on PGE-M and risk of HCC.

Using the resources of the Shanghai Cohort Study, we examined prospectively the associations for urinary levels of 8-*epi*-$PGF_{2\alpha}$ and PGE-M with the risk of developing HCC. The uniqueness of this study was using both oxidative and inflammatory biomarkers in urine samples collected many years before the diagnosis of HCC that would minimize the potential impact of the presence and progression of HCC on these biomarkers as usually is the case in the retrospective and cross-sectional studies.

Material and methods

Study population

Subjects were drawn from the Shanghai Cohort Study (45). Briefly, the study consisted of 18 244 men enrolled from 1 January 1986 to 30 September 1989, who were 45–64 years of age and resided in the city of Shanghai, China. Participants were interviewed in person using a structured questionnaire for information on demographic characteristics, use of tobacco and alcohol, usual adult diet and medical history. At completion of the interview, a 10 mL non-fasting blood sample and a single-void urine specimen were collected from each participant. Multiple aliquots of serum and urine from each study participant were then stored at −70°C or below. The Shanghai Cohort Study has been approved by the institutional review boards at the Shanghai Cancer Institute and the University of Pittsburgh. This study has been approved by the institutional review boards at the University of Minnesota and the University of Pittsburgh.

At recruitment, we asked each participant whether he had ever been told by a physician that he had any of the following diseases: hepatitis (infectious, serum or unknown type), liver cirrhosis, other liver disease, diabetes, gastric ulcer, duodenal ulcer, gallstones, other gallbladder conditions, tuberculosis, high blood pressure, asthma, emphysema, hemorrhoid, diverticulosis, polyposis coli, ulcerative colitis, adenomatous polyps, schistosomiasis, other parasitic diseases or cancer. If the answer was yes, he was asked to provide the year of the first diagnosis and the treatment for each disease. We also asked each participant whether he had ever drunk alcoholic beverages at least once a week for 6 months or more. If the answer was yes, he was asked to provide the typical amount of beer, wine and/or spirits (each) consumed. One drink was defined as 360 g of beer (12.6 g of ethanol), 103 g of wine (12.3 g of ethanol) or 30 g of spirits (12.9 g of ethanol) (46). Smokers were identified as those who smoked at least one cigarette per day for 6 months or more.

Case patients

Identification of incident cancer cases and deaths among cohort participants was accomplished through annual in-person reinterviews of all surviving cohort members and routine review of reports from the population-based Shanghai Cancer Registry and the Shanghai Municipal Vital Statistics Office. To date, losses to follow-up totaled 839 individuals (4.6%) of the entire cohort after ~28 years of study.

As of 2015 (the cutoff date of this study), the study had accumulated 507 264 person-years of observation. A total of 347 cohort participants who were free of cancer at recruitment had developed HCC. These patients were diagnosed with HCC by histopathologic analysis (n = 58), elevated serum α-fetoprotein with consistent clinical and radiologic history (n = 84), positive computerized axial tomography scan and/or ultrasonography with consistent clinical history (n = 177) or by death certificate only (n = 28).

Control subjects

For each case patient, we attempted to choose two cancer-free control subjects, who were individually matched to the index case patients by date of birth (within 2 years), date of urine collection (within 1 month) and neighborhood of residence at recruitment, were randomly chosen from the cohort. All except three cases were matched with two control subjects for this study whereas only one control subject was found to each of these three cases, resulting in a total of 691 individually matched controls.

Laboratory measurements

Urine samples of all study subjects were retrieved from the biospecimen bank. Specimens from matched control subjects and their index cases were always assayed in the same batch. All urine aliquots were identified only by unique codes, and laboratory personnel had no knowledge of the case/control status of the test samples. The assay for quantifying 8-*epi*-$PGF_{2\alpha}$ in urine was described previously (31,47). Briefly, urine (0.2 ml) was mixed with the internal standard [D_4]8-*epi*-$PGF_{2\alpha}$ (2 ng), and the sample was acidified with 20 μL 88% formic acid, then applied to Agilent Bond Elut C18 solid phase extraction plates, for enrichment as described previously, before the analysis by liquid chromatography–electrospray ionization–tandem mass spectrometry, as described previously. The limit of detection was 0.04 pmol/ml. The interday precision of the assay was 5.9% relative standard deviation.

The assay for quantifying PGE-M in urine was performed essentially as described previously (38). The limit of detection was 2.2 pmol/ml. The interday precision of the assay was 3.4% relative standard deviation. We used High Performance Liquid Chromatography methods to measure serum concentrations of retinol and carotenoids (48), and urinary biomarkers of dietary exposure to AFB_1 as previously reported (49,50). Urinary creatinine (Cr) was using a Kodak Ektachem 500 chemistry analyzer.

Of the 347 HCC cases and 691 individually matched controls, 325 cases and 667 controls had measurement of urinary 8-*epi*-$PGF_{2\alpha}$ after excluding 46 subjects (22 cases and 24 controls) due to missing data—18 due to chromatographic interference and 28 due to the depletion of urine samples after previous studies (49–51). Similarly, we excluded 88 subjects (57 cases and 31 controls) due to missing data on urinary PGE-M because of the urine depletion after measurement of 8-*epi*-$PGF_{2\alpha}$ and other urinary biomarkers in previous studies, resulting in 290 cases and 660 controls that were included in the analysis for PGE-M. The distributions of risk factors for HCC including age, hepatitis B surface antigen (HBsAg), smoking and alcohol consumption status among HCC cases and control subjects excluded were similar to those among HCC cases and control subjects included, respectively.

Statistical analysis

Urinary 8-*epi*-$PGF_{2\alpha}$ and PGE-M were expressed in pmol per mg Cr to correct for varying water contents of individual spot urine samples. Given the markedly skewed distributions of the urinary biomarkers, formal statistical testing was performed on logarithmically transformed values, and geometric (as opposed to arithmetic) means and 95% CIs are presented. We used χ^2 and t-test statistics to examine the difference in the distributions of selected variables between HCC cases and controls. The analysis of covariance method was used to compare the difference in the Cr-adjusted mean concentrations of urinary 8-*epi*-$PGF_{2\alpha}$ and PGE-M among control subjects across different categories of exposures such as (i) body mass index (BMI; <18.5, 18.5–<25.0, 23.0–<27.0, ≥27.5 kg/m^2), (ii) smoking status (never, former and current smokers), (iii) regular drinkers of alcoholic beverages (no, yes), and (iv) serologic status of HBsAg (negative, positive). We also used analysis of covariance method to compare the differences in urinary concentrations of 8-*epi*-$PGF_{2\alpha}$ and PGE-M between HCC case patients and control subjects with adjustment for potential confounding factors.

We analyzed the data using both conditional and unconditional logistic regression methods to examine the association between urinary levels of 8-*epi*-$PGF_{2\alpha}$ and PGE-M and HCC risk. The results derived from conditional and unconditional logistic regression models were almost identical. To maximize the sample size by including all cases and controls with available urinary measurement of 8-*epi*-$PGF_{2\alpha}$ and PGE-M that were no longer matched, the results presented were based on unconditional logistic regression models with adjustment for matching variables including age, neighborhood of residence and year of biospecimens collection. Study subjects were grouped into quartile categories based on the distributions of urinary analytes. Given that the urinary levels of 8-*epi*-$PGF_{2\alpha}$ were significantly different between smokers and never smokes, we used the cutoff values for quartile groups among current smokers, former smokers or never smokers separately. The relationship between urinary 8-*epi*-$PGF_{2\alpha}$ or PGE-M levels and HCC risk was assessed by odds ratios (ORs) and associated 95% CIs and P values. The linear trend tests were computed by treating the quartiles of urinary analytes as ordinal variables in the logistic regression models. To adjust for potential confounding effects of established risk factors for HCC, the multivariate logistic regression models also included the following variables: cigarette smoking (never, former and current smokers), alcohol consumption (non-drinkers, <4 drinks per day and ≥4 drinks per day), BMI (<18.5, 18–<23, 23–<27 and 27+ kg/m^2), self-reported history of physician-diagnosed liver cirrhosis (no, yes), and serological HBsAg status (negative, positive). Given the extremely low prevalence of hepatitis C in the study population (e.g. 1.3% in HCC cases and 0.2% in control subjects) (52), serological status of anti-hepatitis C virus was not determined on all study subjects included in this study, and thus not included in the multivariate regression models.

Statistical analyses were carried out using SAS software, version 9.3 (SAS Institute, Cary, NC). All P-values reported are two-sided, and those that were <0.05 were considered to be statistically significant.

Results

The mean age (± standard deviation) of cases at diagnosis of HCC was 68.4 (±8.1) years whereas the corresponding age of control subjects at the time of case's diagnosis was 68.2 (±7.8) years. The average time interval between urine collection and cancer diagnosis among cases was 11.6 (± 7.0) years (range, 1 month–27 years). Table 1 shows the distributions of selected baseline characteristics and risk factors for HCC in cases and control subjects. The prevalence of ever smokers (61.1%), self-reported history of physician-diagnosed cirrhosis (9.5%) and positive serology of HBsAg (57.0%) were statistically significantly higher among HCC cases than control subjects. There were no differences in BMI, levels of alcohol consumption or prevalence of self-reported history of physician-diagnosed type 2 diabetes at baseline between HCC cases and controls.

There was a moderate correlation between urinary 8-*epi*-$PGF_{2\alpha}$ and PGE-M (ρ = 0.14, P = 0.0003). Among control subjects, urinary levels of 8-*epi*-$PGF_{2\alpha}$ were significantly higher in current smokers and heavy drinkers of alcoholic beverages whereas urinary levels of PGE-M were inversely associated with BMI (Table 2). Among current smokers, levels of 8-*epi*-$PGF_{2\alpha}$ were positively associated with number of cigarettes per day (ρ = 0.16, P =0.004). Urinary levels of PGE-M were not associated with smoking or alcohol intake (Table 2). In addition, we examined the association between urinary levels of 8-*epi*-$PGF_{2\alpha}$ and PGE-M and urinary levels of AFB_1 biomarkers among 100 control subjects with available measurements from a previous study and did not find any difference between AFB_1-positive and AFB_1-negative subjects (both P's > 0.45; data not shown). We also examined the correlation between urinary levels of 8-*epi*-$PGF_{2\alpha}$ and PGE-M and serum levels of antioxidants among 408 control subjects.

Table 1. Baseline demographic and lifestyle characteristics of study participants who developed hepatocellular carcinoma (cases) and those who remained cancer free (controls), the Shanghai Cohort Study

Characteristic	Cases	Controls	P[a]
Number of subjects	347	691	
Age (year), mean ± SD	56.8 ± 5.2	56.6 ± 5.1	0.624
Body mass index (kg/m^2), %			
Mean ± SD	22.0 ± 3.2	22.1 ± 3.0	0.676
<18.5	9.8	10.0	0.758
18.5–<23.0	54.8	53.8	
23.0–<27.0	28.5	30.7	
≥27.0	6.9	5.5	
Highest level of education, %			
No formal education	4.1	5.2	0.649
Primary	26.2	24.8	
Secondary and above	69.7	70.0	
Cigarette smoking, %			
Never smokers	38.9	47.2	0.039
Former smokers	8.1	6.5	
Current smokers	53.0	46.3	
Alcohol drinking, %			
Nondrinkers	59.9	57.0	0.615
<4 drinks/day	31.7	34.7	
≥4 drinks/day	8.4	8.3	
Self-reported history of physician-diagnosed type 2 diabetes, %			
No	99.7	99.1	0.281
Yes	0.3	0.9	
Self-reported history of physician-diagnosed liver cirrhosis, %			
No	90.5	98.8	<0.001
Yes	9.5	1.2	
Hepatitis B surface antigen serology, %			
Negative	42.4	88.7	<0.001
Positive	57.6	11.3	

[a]Two-sided *P* values were based on *t*-test for continuous variables or chi-square test for categorical variables.

Table 2. Concentrations of urinary 8-*epi*-$PGF_{2\alpha}$ and PGE-M among all control subjects and subgroups stratified by body mass index, smoking status, alcohol intake and serological status of HBsAg, the Shanghai Cohort Study

	8-*epi*-$PGF_{2\alpha}$ (pmol/mg creatinine)		PGE-M (pmol/mg creatinine)	
Subject group	N[a]	Geometric mean (95% CI)[b]	N[a]	Geometric mean (95% CI)[b]
All control subjects	667	0.78 (0.66, 0.90)	660	25.8 (19.5, 34.0)
By body mass index (kg/m^2)				
<18.5	67	0.72 (0.60–0.86)	68	23.9 (17.2–3.2)
18.5–<23.0	358	0.80 (0.68–0.94)	353	27.7 (20.9–36.7)
23.0–<27.0	206	0.76 (0.64–0.88)	204	23.6 (17.6–31.5)
≥27.0	36	0.68 (0.56–0.84)	35	20.2 (13.8–29.5)
P		0.297		0.0580
By smoking status				
Never smokers	311	0.68 (0.58–0.80)	308	25.0 (18.8–33.2)
Former smokers	44	0.76 (0.62–0.94)	44	28.4 (19.7–40.9)
Current smokers	312	0.88 (0.76–1.04)	308	26.5 (19.9–35.3)
P		<0.001		0.393
By alcohol consumption				
Nondrinkers	380	0.76 (0.64–0.88)	372	25.8 (19.4–34.3)
<4 Drinks/day	230	0.78 (0.66–0.90)	232	25.8 (19.3–34.5)
≥4 Drinkers/day	57	0.90 (0.74–1.08)	56	25.6 (18.3–35.9)
P		0.033		0.976
By HBsAg status				
Negative	592	0.74 (0.64–0.88)	585	25.1 (19.1–33.0)
Positive	75	0.80 (0.68–0.96)	75	26.5 (19.4–36.1)
P		0.244		0.581

[a]Twenty-four subjects were excluded due to missing 8-*epi*-$PGF_{2\alpha}$, and 31 subjects were excluded due to missing PGE-M (the details were described in the Methods).
[b]Adjusted for age, year of biospecimen collection, neighborhood of residence, body mass index (<18.5, 18–<23, 23–<27, 27+ kg/m^2), cigarette smoking (never, former, current smoker), alcohol consumption (non-drinker, <4 drinks per day, ≥4 drinks per day), self-reported history of physician-diagnosed liver cirrhosis (no, yes) and serological status of HBsAg (negative, positive).

8-*epi*-$PGF_{2\alpha}$ was inversely correlated with α-carotene (ρ = –0.17, *P* = 0.0006), β-carotene (ρ = –0.14, *P* = 0.0040), β-cryptoxanthin (ρ = –0.20, *P* < 0.0001) and lycopene (ρ = –0.22, *P* < 0.0001). PGE-M was positively correlated with retinol (ρ = 0.14, *P* = 0.0044), α-tocopherol (ρ = 0.13, *P* = 0.0116) and total tocopherol (ρ = 0.12, *P* = 0.0146).

The geometric mean of creatinine-corrected 8-*epi*-$PGF_{2\alpha}$ in HCC cases was significantly higher (0.92 pmol/mg Cr) than that in controls (0.80 pmol/mg Cr) after adjustment for smoking status, alcohol consumption, history of liver cirrhosis and HBsAg serology positivity in all subjects (*P* < 0.001; Table 3). Higher levels of 8-*epi*-$PGF_{2\alpha}$ in cases than controls were present in never and current smokers, in non-drinkers and drinkers and in HBsAg-negative and HBsAg-positive subjects. The geometric mean of urinary PGE-M in HCC cases was similar to that in control subjects, regardless of their status of smoking, alcohol consumption or HBsAg status (Table 3).

Higher levels of urinary 8-*epi*-$PGF_{2\alpha}$ were significantly associated with a higher risk of HCC. Compared with the lowest quartile of 8-*epi*-$PGF_{2\alpha}$, the multivariable-adjusted OR of developing HCC for the highest quartile was 2.55 (95% CI = 1.62–4.01; *P* for trend < 0.001; Table 4). This positive association was present in all subgroups stratified by smoking, alcohol consumption or HBsAg status. We did not find any significant association of urinary levels of PGE-M with the risk of HCC overall or in any subgroups (Table 4). BMI did not modify the association between urinary levels of 8-*epi*-$PGF_{2\alpha}$ and PGE-M and risk of HCC (data not shown).

We further examined the association between urinary levels of 8-*epi*-$PGF_{2\alpha}$ and PGE-M and HCC risk with additional adjustment for levels of serum antioxidants available for 213 HCC cases and 408 controls from a previous study (48). In this subset, ORs of HCC for the highest relative to the lowest quartile of urinary 8-*epi*-$PGF_{2\alpha}$ were 3.11 (95% CI = 1.74–5.56, *P* for trend < 0.001) without further adjustment for antioxidant variables. Further adjustment for serum antioxidants including β-cryptoxanthin, lycopene and total carotenes did not materially change the association between 8-*epi*-$PGF_{2\alpha}$ and risk of HCC; the antioxidants-adjusted OR was 2.83 (95% CI = 1.57–5.10, *P* for trend < 0.001). The adjustment for antioxidants did not change the null association between urinary PGE-M and HCC risk (data not shown).

The potential impact of disease progression on 8-*epi*-$PGF_{2\alpha}$ and PGE-M was examined among all HCC cases by evaluating the time interval from biospecimen collection to HCC diagnosis. Urinary levels of 8-*epi*-$PGF_{2\alpha}$ decreased with increasing number of years from urine collection to HCC diagnosis (ρ = –0.19, *P* < 0.001) whereas urinary PGE-M level remained constant over the same time interval (ρ = –0.05, *P* = 0.399). Compared with the lowest quartile, the highest quartile of 8-*epi*-$PGF_{2\alpha}$ was associated with OR of 5.29 (95% CI = 1.92–14.54) for HCC diagnosed within 5 years after urine collection. Although the OR for HCC with longer duration of follow-up was reduced, it remained statistically significant, even for those ≥ 10 years after urine collection Table 5). The association between urinary PGE-M and HCC risk remained null regardless of the duration of follow-up (Table 5).

Discussion

In this nested case–control study within the Shanghai Cohort Study, we found a statistically significant association between urinary level of 8-*epi*-$PGF_{2\alpha}$ and HCC risk. This association was robust and independent of cigarette smoking, alcohol consumption, hepatitis B and the presence of liver cirrhosis. The overall risk of HCC was more than doubled for persons in the highest quartile of 8-*epi*-$PGF_{2\alpha}$ than those in the lowest

Table 3. Concentrations of urinary 8-*epi*-$PGF_{2\alpha}$ and PGE-M in hepatocellular carcinoma cases and control subjects in total and subgroups stratified by smoking status, alcohol intake, and serological status of HBsAg, the Shanghai Cohort Study

	8-*epi*-$PGF_{2\alpha}$ (pmol/mg creatinine)[a]			PGE-M (pmol/mg creatinine)[a]		
Subject group	Cases	Controls	*P*	Cases	Controls	*P*
Total subjects, *n*	325	667		290	660	
Mean (95% CI)[b]	0.92 (0.86–1.00)	0.80 (0.72–0.86)	<0.001	22.2 (19.3–25.6)	23.4 (20.1–27.1)	0.442
By smoking status						
Never smokers, *n*	126	311		114	308	
Mean (95% CI)[b]	0.82 (0.74–0.90)	0.68 (0.62–0.76)	0.001	21.9 (18.3–26.0)	22.7 (19.3–26.6)	0.696
Former smokers, *n*	26	44		21	44	
Mean (95% CI)[b]	0.82 (0.68–0.98)	0.78 (0.66–0.90)	0.619	23.3 (16.5–32.9)	24.9 (19.0–32.7)	0.746
Current smokers, *n*	173	312		155	308	
Mean (95% CI)[b]	1.04 (0.96–1.16)	0.90 (0.82–0.98)	0.002	22.4 (18.9–26.6)	23.8 (20.2–28.0)	0.471
By alcohol intake						
Nondrinkers, *n*	195	380		178	372	
Mean (95% CI)[b]	0.88 (0.82–0.98)	0.78 (0.70–0.86)	0.001	22.3 (19.1–26.0)	23.4 (19.9–27.4)	0.551
Drinkers, *n*	130	287		112	288	
Mean (95% CI)[b]	0.96 (0.86–1.06)	0.82 (0.74–0.90)	0.004	22.1 (18.3–26.7)	23.0 (19.8–27.4)	0.561
By HBsAg status						
Negative, *n*	137	592		123	585	
Mean (95% CI)[b]	0.86 (0.78–0.96)	0.76 (0.70–0.82)	0.003	24.0(20.0–28.7)	23.7 (20.5–27.4)	0.865
Positive, *n*	188	75		167	75	
Mean (95% CI)[b]	0.96 (0.88–1.06)	0.80 (0.72–0.92)	0.004	21.0 (18.0–24.6)	24.9 (20.1–31.0)	0.116

[a]Forty-six subjects (22 cases and 24 controls) were excluded due to missing 8-*epi*-$PGF_{2\alpha}$, and 88 subjects (57 cases and 31 controls) were excluded due to missing PGE-M (the details were described in the Methods).

[b]Geometric means (95% CIs) were adjusted for age, year of biospecimen collection, neighborhood of residence, cigarette smoking (never, former or current smoker), alcohol consumption (non-drinker, <4 drinks per day or ≥4 drinks per day), body mass index (<18.5, 18–<23, 23–<27, 27+ kg/m^2), self-reported history of physician-diagnosed liver cirrhosis (no or yes), and serological status of HBsAg (negative, positive).

Table 4. Urinary levels of 8-*epi*-prostaglandin $F_{2\alpha}$ and prostaglandin E_2 metabolite in relation to risk of hepatocellular carcinoma in all subjects and subgroups stratified by smoking status, alcohol intake and serological status of HBsAg, the Shanghai Cohort Study

	8-*epi*-prostaglandin $F_{2\alpha}$[b]		PGE-M[b]	
Urinary levels of biomarker in quartile[a]	Ca/Co	OR (95% CI)[c]	Ca/Co	OR (95% CI)[c]
All subjects				
First quartile	52/167	1.00	70/165	1.00
Second quartile	64/167	1.25 (0.76–2.05)	70/165	0.88 (0.55–1.41)
Third quartile	61/167	1.08 (0.66–1.79)	83/165	1.19 (0.76–1.89)
Fourth quartile	148/166	2.55 (1.62–4.01)	67/165	0.88 (0.55–1.42)
P for trend		<0.001		0.949
Never smokers				
First quartile	17/78	1.00	30/79	1.00
Second quartile	29/78	1.95 (0.88–4.29)	20/77	0.57 (0.26–1.24)
Third quartile	22/78	1.10 (0.48–2.50)	36/79	1.50 (0.74–3.03)
Fourth quartile	58/77	2.45 (1.17–5.16)	28/73	0.86 (0.41–1.81)
P for trend		0.053		0.675
Ever smokers				
First quartile	35/89	1.00	40/86	1.00
Second quartile	35/89	0.93 (0.49–1.79)	50/88	1.09 (0.60–1.98)
Third quartile	39/89	1.12 (0.59–2.12)	47/86	1.00 (0.54–1.85)
Fourth quartile	90/89	2.68 (1.50–4.81)	39/92	0.87 (0.47–1.64)
P for trend		<0.001		0.615
Non-drinkers of alcohol				
First quartile	32/94	1.00	43/92	1.00
Second quartile	41/99	1.31 (0.69–2.49)	40/95	0.79 (0.42–1.48)
Third quartile	39/101	1.01 (0.53–1.93)	52/95	1.13 (0.62–2.06)
Fourth quartile	83/86	2.56 (1.40–4.69)	43/90	1.03 (0.55–1.93)
P for trend		0.004		0.642
Drinkers of alcohol				
First quartile	20/73	1.00	27/73	1.00
Second quartile	23/68	1.12 (0.51–2.46)	30/70	0.86 (0.41–1.78)
Third quartile	22/66	1.19 (0.53–2.67)	31/70	1.19 (0.57–2.48)
Fourth quartile	65/80	2.40 (1.20–4.82)	24/75	0.65 (0.31–1.39)
P for trend		0.006		0.430
HBsAg negative and no cirrhosis				
First quartile	23/152	1.00	28/150	1.00
Second quartile	28/149	1.33 (0.72–2.44)	27/140	1.06 (0.58–1.91)
Third quartile	28/141	1.36 (0.74–2.49)	34/146	1.51 (0.85–2.69)
Fourth quartile	49/144	2.30 (1.31–4.04)	26/143	1.04 (0.56–1.91)
P for trend		0.004		0.604
HBsAg positive or had cirrhosis				
First quartile	29/15	1.00	42/15	1.00
Second quartile	36/18	1.01 (0.41–2.47)	43/25	0.59 (0.26–1.35)
Third quartile	33/26	0.63 (0.26–1.51)	49/19	0.84 (0.37–1.93)
Fourth quartile	99/22	3.17 (1.36–7.39)	41/22	0.64 (0.28–1.46)
P for trend		0004		0.503

[a]Quartile cutoff values of 8-*epi*-prostaglandin $F_{2\alpha}$ were <0.48, 0.48–0.64, 0.65–0.84 and ≥0.85 pmol/mg creatinine for non-smokers; <0.56, 0.56–0.74, 0.75–0.95 and ≥0.96 pmol/mg creatinine for former smokers; <0.67, 0.67–0.86, 0.87–1.12 and ≥1.13 pmol/mg creatinine for current smokers; Quartile cutoff values of prostaglandin E_2 metabolite were <14.8, 14.8–22.8, 22.9–35.6 and ≥35.7 pmol/mg creatinine.

[b]Forty-six subjects (22 cases and 24 controls) were excluded due to missing 8-*epi*-$PGF_{2\alpha}$, and 88 subjects (57 cases and 31 controls) were excluded due to missing PGE-M (the details were described in the Methods).

[c]ORs (95% CIs) were derived from unconditional logistic regression models with adjustment for age, year of biospecimens collection, neighborhood of residence, cigarette smoking (never, former, current smokers), alcohol consumption (nondrinkers, <4 drinks per day, ≥4 drinks per day), body mass index (<18.5, 18–<23, 23–<27, 27+ kg/m^2), self-reported history of physician-diagnosed liver cirrhosis (no, yes), and serological status of HBsAg (negative, positive).

quartile. In this study, we did not find any statistically significant association between urinary PGE-M level and HCC risk overall or in subgroup analysis by status of smoking, alcohol consumption or HBsAg serology.

Oxidative stress has been discussed as a common pathogenic mechanism in various liver diseases. 8-*epi*-$PGF_{2\alpha}$ is considered a gold standard biomarker for oxidative damage *via* lipid peroxidation. Several epidemiological studies have reported positive associations between urinary 8-*epi*-$PGF_{2\alpha}$ levels and risk of cancers at different sites including breast (33), liver (32), lung (31,53) and prostate (54). In the only reported study involving 74 HCC cases from a community-based Cancer Screen Program cohort in Taiwan, higher levels of 8-*epi*-$PGF_{2\alpha}$ in prediagnostic urine samples were associated with a statistically significant higher risk of HCC (P_{trend} < 0.0001); the multivariable-adjusted OR for HCC was 6.27 (95% CI = 2.17–18.13) for the highest compared with the lowest tertile of 8-*epi*-$PGF_{2\alpha}$ (32). Our results, based on a much larger sample size, were consistent with those from the previous study.

Table 5. Urinary levels of 8-*epi*-$PGF_{2\alpha}$ and PGE-M in relation to risk of hepatocellular carcinoma by years between biospecimen collection and cancer diagnosis, the Shanghai Cohort Study

Urinary levels of biomarker in quartile	Controls	<5 years		5–<10 years		≥10 years	
		Cases	OR (95% CI)[b]	Cases	OR (95% CI)[b]	Cases	OR (95% CI)[b]
8-*epi*-$PGF_{2\alpha}$[a]							
First quartile	167	6	1.00	18	1.00	28	1.00
Second quartile	167	9	1.53 (0.47–4.99)	14	0.87 (0.39–1.94)	41	1.26 (0.69–2.29)
Third quartile	167	13	2.16 (0.71–6.51)	21	1.21 (0.57–2.56)	27	0.68 (0.35–1.30)
Fourth quartile	166	39	5.29 (1.92–14.54)	40	2.41 (1.21–4.80)	69	2.21 (1.27–3.86)
P for trend			<0.001		0.005		0.012
PGE-M[a]							
First quartile	165	7	1.00	19	1.00	44	1.00
Second quartile	165	5	0.56 (0.14–2.14)	20	1.17 (0.55–2.46)	45	0.83 (0.48–1.43)
Third quartile	165	15	1.98 (0.64–6.12)	28	1.57 (0.76–3.25)	40	0.87 (0.50–1.52)
Fourth quartile	165	9	1.09 (0.31–3.85)	20	1.15 (0.53–2.50)	38	0.78 (0.44–1.36)
P for trend			0.374		0.557		0.428

[a]Forty-six (22 cases and 24 controls) were excluded due to missing 8-*epi*-$PGF_{2\alpha}$, and 88 subjects (57 cases and 31 controls) were excluded due to missing PGE-M (the details were described in the Methods).

[b]ORs (95% CIs) were derived from unconditional logistic regression models with adjustment for age, year of biospecimens collection, neighborhood of residence, cigarette smoking (never, former, current smokers), alcohol consumption (non-drinkers, <4 drinks per day, ≥4 drinks per day), body mass index (<18.5, 18–<23, 23–<27, 27+ kg/m^2), self-reported history of physician-diagnosed liver cirrhosis (no, yes), and serological status of hepatitis B surface antigen (negative, positive)

Our results and those from the previous study (32) both showed that higher levels of 8-*epi*-$PGF_{2\alpha}$ were associated with significantly higher risk of HCC among individuals who were free of chronic infection with hepatitis B virus (i.e. negative HBsAg serology), the major causal factor for HCC in these two study populations, suggesting that oxidative stress may play an important role in non-viral HCC. Obesity, diabetes and metabolic syndrome are underlying causes for NAFLD. NAFLD can remain benign, but can also potentially progress over time into an inflammatory and fibrotic liver disease known as NASH. NASH can further progress to liver cirrhosis and ultimately HCC (55,56). In the USA, the prevalences of NAFLD and NASH were 46% and 12.2%, respectively, among adults without a prior diagnosis of NAFLD (16). With efficient treatments now available against hepatitis B and hepatitis C, it is expected that NASH will rapidly become the leading cause of HCC in the USA and other developed countries. Previous studies reported that both urinary and blood levels of 8-isoprostanes were significantly elevated in patients with NASH (57,58). The findings of this study may have significance for public health implication that urinary 8-*epi*-$PGF_{2\alpha}$ may be developed as a biomarker for identifying high-risk individuals, especially those without traditional risk factors such as chronic hepatitis and heavy alcohol consumption, for the development of HCC.

We did not find that urinary levels of PGE-M, a major urinary metabolite of PGE_2, were significantly associated with the risk of HCC. PGE_2 is one of major structurally related prostanoids generated from arachidonic acid by COX enzymes. However, PGE_2 is an unstable compound that is rapidly metabolized *in vivo* to the stable PGE-M. Thus, measurement of excreted urinary PGE-M is the best way to quantify systemic PGE_2 production *in vivo*. PGE_2 is the most abundant prostaglandin found in various types of human malignancies including colorectal, lung, head and neck cancers (59). COX-2-derived PGE_2 has been shown to play an important role in the pathogenesis of various liver diseases (60). The amount of PGE_2 was significantly increased in HCC tissue and adjacent non-cancerous tissues relative to normal liver tissue (35). Epidemiological studies have shown that the use of aspirin was significantly associated with lower risk of HCC (61,62). However, these studies did not find a statistically significant association for HCC risk with use of non-aspirin non-steroidal anti-inflammatory drugs, which have a more direct and stronger effect on inhibition the COX-2 enzymes, key enzymes for the production of PGE_2. Our null findings on the urinary levels of PGE-M and the risk of developing HCC are consistent with results of these earlier observational studies.

This study has several strengths. The prospective study design minimized potential effects of HCC development and progression on 8-*epi*-$PGF_{2\alpha}$ and PGE-M. Furthermore, a long duration of follow-up for study subjects after baseline urine collection further diminished the potential impact of subclinical stage and progression of disease (i.e. HCC) on urinary levels of 8-*epi*-$PGF_{2\alpha}$ and PGE-M, thus is less likely to influence the association between 8-*epi*-$PGF_{2\alpha}$ or PGE-M and HCC risk. In addition, this study showed that serum levels of multiple antioxidants including α- and β-carotenes, β-cryptoxanthin and lycopene were inversely correlated with urinary 8-*epi*-$PGF_{2\alpha}$. Further adjustment for these antioxidants did not materially change the association between urinary 8-*epi*-$PGF_{2\alpha}$ and HCC risk. The study has the largest sample size so far with >340 HCC cases and 680 individually matched controls allowing for subgroup analysis stratified by major risk factors for HCC, eliminating their potential confounding effect on the association between the urinary biomarkers and HCC risk. The study had several limitations. Urinary levels of aflatoxin metabolites were available only for a small subset of study subjects, thus preventing us from conducting more detailed statistical analysis. However, we did not find any significant difference in 8-*epi*-$PGF_{2\alpha}$ between AFB_1-positive and AFB_1-negative subjects. Furthermore, the previous study reported that adjustment for AFB_1 strengthened, rather weakened, the association between urinary levels of 8-*epi*-$PGF_{2\alpha}$ and HCC risk (32). Therefore, the observed positive association between 8-*epi*-$PGF_{2\alpha}$ and HCC risk is less likely due to the non-adjustment for AFB_1. Another limitation of this study was including men only, so the results may not be applicable to women.

In summary, using prospectively collected urine samples from participants in the Shanghai Cohort Study, we demonstrated that high urinary levels of 8-*epi*-$PGF_{2\alpha}$ at baseline were associated with a statistically significant, more than doubled risk of

HCC. The positive association was robust and independent of major risk factors including chronic infection with hepatitis B virus, alcohol consumption and history of liver cirrhosis. The public health implication is that urinary 8-*epi*-$PGF_{2\alpha}$ may be developed as a biomarker for identifying high-risk individuals without traditional risk factors for the development of HCC.

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