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Association between serum 25(OH) vitamin D, incident liver cancer and chronic liver disease mortality in the Linxian Nutrition Intervention Trials: a nested case–control study

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Background: Although vitamin D deficiency has been noted in cross-sectional studies of chronic liver disease and laboratory studies suggest possible benefits of vitamin D in preventing liver cancer, little epidemiologic data are available.

Methods: We performed a nested case–control study in the Linxian Nutrition Intervention Trials on participants developing incident liver cancer or dying from chronic liver disease over 22 years of follow-up. Baseline serum 25(OH) vitamin D was measured for 226 incident liver cancer cases, 282 chronic liver disease deaths and 1063 age-, sex- and trial-matched controls. Unconditional logistical regression models were used to estimate odds ratios (ORs) and 95% confidence intervals (CIs).

Results: The median serum vitamin D level in controls was low (20 nmol l^{-1}). Compared with the lowest quartile, subjects in the fourth quartile had lower risk of chronic liver disease death (OR = 0.34, 95% CI = 0.21–0.55). For liver cancer incidence, risk estimates were below one, but were not statistically significant. Associations, however, were significant among participants with higher serum calcium levels (Q4 *vs* Q1, OR = 0.43, 95% CI = 0.21–0.89). Results for chronic liver disease did not vary by serum calcium level.

Conclusion: In a low vitamin D population, higher serum 25(OH) vitamin D concentrations were associated with significantly lower risk of chronic liver disease deaths, and among those with higher serum calcium, incident liver cancer. Our results suggest a possible protective role for vitamin D in these diseases.

Liver cancer is the fifth most common cancer in men and the seventh most common cancer in women worldwide, and it is the third most common cause of cancer mortality in both sexes combined (Ferlay *et al*, 2010). Nearly 85% of liver cancers occur in developing countries. In China, the incidence rate of liver cancer is 37.4 per 1 00 000 in men and 13.7 per 1 00 000 in women (Ferlay

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et al, 2010) and liver cancer ranks as the second most common cause of cancer deaths (Ministry of Health of the People's Republic of China, 2008). China has the greatest burden of liver cancer in the world (half of global new liver cancer cases in 2008 occurred in China), largely reflecting the elevated prevalence of chronic hepatitis B virus (HBV) infection, which affects approximately 7.2% of the general population (Liang *et al*, 2009).

Vitamin D, a hormone, can be obtained from the diet and from the sunlight, and it may be an important regulator of inflammation (Vanoirbeek *et al*, 2011). The circulating form, 25(OH) vitamin D, is made in the liver and is a best marker of vitamin D status (Rosen, 2011). 1α, 25$(OH)_2$ vitamin D is the most active form and mostly generated in the kidney, although can be made in many other organs as well. Growing evidence suggests that vitamin D may be important for bone health and other diseases, including those of the liver. In liver cell lines, vitamin D inhibits cellular proliferation and suppresses DNA damage (Pourgholami *et al*, 2000; Saha *et al*, 2001; Caputo *et al*, 2003; Ghous *et al*, 2008). Vitamin D also has been protective in several mouse models of liver cancer (Pourgholami *et al*, 2000; Saha *et al*, 2001; Ghous *et al*, 2008). In humans, several, but not all, studies have suggested that patients with fibrosis or cirrhosis have lower vitamin D levels than the general population (Masuda *et al*, 1989; Bonkovsky *et al*, 1990; Chen *et al*, 1996; Gallego-Rojo *et al*, 1998; Fisher and Fisher, 2007; Chiang *et al*, 2011; Putz-Bankuti *et al*, 2012; Kitson *et al*, 2013). Although previous epidemiologic studies have examined the association between vitamin D and cancers of the colorectum (Jenab *et al*, 2010), oesophagus (Chen *et al*, 2007; Abnet *et al*, 2010), stomach (Chen *et al*, 2007; Abnet *et al*, 2010), prostate (Li *et al*, 2007; Ahn *et al*, 2008) and pancreas (Stolzenberg-Solomon *et al*, 2010) among others, no studies have evaluated associations between serum vitamin D levels and subsequent risk of liver cancer.

To examine the association between serum 25(OH) vitamin D concentrations and subsequent risk of primary liver cancer incidence and chronic liver disease mortality, we performed a nested case–control study in the Linxian Nutrition Intervention Trial (NIT) cohorts in China.

MATERIALS AND METHODS

Study population and data collection. Subjects were participants in the Linxian NITs, including the Dysplasia Trial and the General Population Trial. The design and conduct of Linxian NITs have been described elsewhere (Blot *et al*, 1993; Li *et al*, 1993a, b). In brief, the Dysplasia Trial enrolled individuals between the ages of 40 and 69 years with cytologically diagnosed oesophageal dysplasia in three communes in northern Linxian. A total of 3318 residents were randomised and received either a multiple vitamin/mineral supplement (14 vitamins and 12 minerals), including 800IU vitamin D, or matching placebo for 6 years, beginning in May 1985.

The General Population Trial enrolled individuals between 40 and 69 years old from the general population of four communes in the high-risk Linxian cancer area. In all, a total of 29 584 healthy adults were randomised and received up to four daily vitamin/mineral supplement combinations for 5.25 years in a one-half replicate fractional factorial experimental design, beginning in March 1986; vitamin D was not among the supplemented vitamins in this trial. Individuals who had cancer, debilitating disease or required daily medications were excluded from both trials.

At the baseline exams, conducted between August 1984 and May 1985, the NIT subjects were interviewed providing data on age, alcohol, smoking and other variables, given a physical examination, and had a 10 ml blood sample drawn. Blood samples were drawn before either intervention started. Blood samples were stored on ice for 3–6 h during transport to the field station lab, where the serum was separated, frozen and stored at -85°C until 2011 when it was thawed for the current vitamin D measurements. Human subject protection procedures were approved by the Institutional Review Boards of US National Institutes of Health and the Chinese Academy of Medical Science, and all participants gave informed consent.

Follow-up for cancer and vital status. During the trial (1985–1991) and post-trial follow-up periods (after 1991), incident cancer cases and deaths were identified by several methods that ensured essentially complete ascertainment of events. During the trial period, village doctors visited all participants monthly, and trial staff monthly reviewed local and regional hospital records and the local cancer registry. New cancer diagnoses and all causes of death were confirmed by a panel of American and Chinese experts. During the post-trial follow-up period, village health workers continued to visit the participants monthly and a panel of Chinese experts verified new cancer diagnoses and all causes of death. Only six incident liver cancer cases were diagnosed by pathology; most were diagnosed by combined evidence from biochemical assays, clinical examination, ultrasound and computed tomography scan. Chronic liver disease deaths included cirrhosis and its complications, and were diagnosed by the symptoms (jaundice, ascites, bruising and bleeding, palmar erythema, gynaecomastia and hypogonadism), biochemical assays, and, in more recent years, computed tomography scan. No participants were diagnosed with incident liver cancer and then subsequently recorded as dying from chronic liver disease.

Nested case–control design and subject selection. A nested case–control design was used for this study. We identified a total of 255 incident primary liver cancer cases and 310 chronic liver disease deaths occurring from baseline through the end of 2007, and frequency-matched controls (2 : 1) to both case groups by age at baseline (± 3 years), sex and trial. Controls were NIT participants who were alive and free of cancer at time of case diagnosis. Vitamin D was then measured in all of the selected participants who had available serum (Dysplasia Trial: incident liver cancers: 29, chronic liver disease deaths: 29, controls: 131; General Population Trial: incident liver cancers: 197, chronic liver disease deaths: 253, controls: 932). For analyses, we used the entire set of controls (1063 subjects) for evaluation of each outcome.

Serum 25(OH) vitamin D and calcium measurements. Serum 25(OH) vitamin D was measured using a commercial enzyme immunoassay kit (IDS Inc., Fountain Hills, AZ, USA) at the laboratory of the Cancer Institute, Chinese Academy of Medical Sciences in 2011. Vitamin D measurements were performed in 42 batches. Each of the 42 batches included 39 samples (36 samples from cases or controls, and three quality control (QC) samples). Each sample was individually measured in duplicate. The three QC samples in each batch were aliquots of a single pooled sample. The overall mean coefficient of variation (CV) of these 126 blinded replicate QC samples was 8.0% (range 0.8–56.8%). In a sensitivity analysis, we excluded results from the eight batches with CVs $>10\%$. As this did not alter results, we present the full results from all all 42 batches here. For all analyses, the laboratory technicians were blinded to the case–control status of the samples. We calculated the vitamin D concentration from a standard curve based on the mean values of standard samples in the 42 batches. Total serum calcium was measured using electrochemiluminescence assayed on the Automatic Biochemistry Analyzer (Roche Cobas C501, Ichige, Hitachinaka-shi, Japan). The CV of 126 blinded QC samples for calcium measurements was 3.9%.

Statistical analysis. Medians and quartiles of serum 25(OH) vitamin D concentration were calculated by age at baseline (<50, $\geqslant 50$ to <60, $\geqslant 60$ sex, smoking status, drinking status, body

mass index (BMI), season of blood draw, and HBV seropositivity and hepatitis C virus (HCV) infection seropositivity. We tested the differences of serum 25(OH) vitamin D concentrations between groups using the nonparametric Kruskal–Wallis Test. Unconditional logistical regression models were used to estimate odds ratios (ORs) and 95% confidence intervals (CIs). We also used two different metrics of serum 25(OH) vitamin D to evaluate the association between serum 25(OH) vitamin D concentrations and risk of liver cancer incidence and chronic liver disease mortality: (1) as a continuous variable, scaled to one-half the interquartile range (7.9 nmol l^{-1}); and (2) as quartiles based on sex-specific cutoff values in the control population. Potential confounders included exposures associated with either vitamin D or the liver end points and included age at baseline (continuous variable), sex (male or female), tobacco smoking (yes or no), alcohol drinking (yes or no), BMI (continuous variable), season of blood draw (grouped by months with similar serum vitamin D levels in controls: summer (August–September) or winter (January–May)), HBV seropositivity (either HBsAg positive or HBcAg positive), and HCV seropositivity (HCsAg positive or negative). Finer adjustment for month of blood draw had no effect on our risk estimates. Stratified analyses were conducted by age at baseline (<55 and $\geqslant 55$, based on the median value in the controls), sex, season of blood draw (summer or winter), tertiles of follow-up (<7 years, $\geqslant 7$ to <14 years or $\geqslant 14$ years), HBV or HCV infection (HBV positive, HCV positive and both negative), trial, baseline self-reported cirrhosis and baseline serum calcium (<1.485 and $\geqslant 1.485$ mmol l^{-1}, based on the median value in the controls). Models restricted to women excluded the smoking variable because of the small number of women smokers. We also evaluated possible non-linear associations by comparing the fit of continuous models with and without vitamin D quadratic terms using a likelihood ratio test. We found a borderline significant quadratic association for chronic liver disease, but graphing the results showed only a modest deviation from linearity (data not shown). The quartile analysis adequately demonstrates the shape of the

Table 1. Baseline demographic characteristics by case and control groups in the Linxian Nutrition Intervention Trial cohort

		Case (*N* = 508)			
	Controls (*N* = 1063)	**Liver cancer incidence (*n* = 226)**	***P*-value**	**Chronic liver disease death (*n* = 282)**	***P*-value**
Age at baseline (years), median (IQR)	55.0 (50.0, 61.0)	55.0 (49.0, 61.0)	0.717	55.0 (49.0, 61.0)	0.710
Sex (%)					
Women	490 (46.1)	91 (40.3)	0.110	142 (50.4)	0.203
Men	573 (53.9)	135 (59.7)		140 (49.6)	
Smoking (%)					
No	683 (64.3)	138 (61.1)	0.356	186 (66.2)	0.558
Yes	379 (35.7)	88 (38.9)		95 (33.8)	
Alcohol drinking					
No	807 (76.0)	171 (75.7)		219 (77.9)	
A few times per year	223 (21.0)	46 (20.4)	0.625	57 (20.3)	0.338
A few times per month	24 (2.2)	6 (2.6)		3 (1.1)	
A few times per week	5 (0.5)	2 (0.9)		2 (0.7)	
Daily	3 (0.3)	1 (0.4)		0 (0.0)	
BMI (kg m^{-2}), median (IQR)	21.6 (20.2, 23.1)	21.8 (20.1, 23.1)	0.871	21.7 (20.5, 23.4)	0.107
Blood draw season(%)					
January–May	932 (87.8)	197 (87.2)	0.806	252 (89.7)	0.376
August–September	130 (12.2)	29 (12.8)		29 (10.3)	
HBsAg(%)					
No	1004 (94.5)	173 (76.6)	**<0.001**	198 (70.2)	**<0.001**
Yes	58 (5.4)	53 (23.4)		84 (29.8)	
HBcAg (%)					
No	441 (41.5)	69 (30.5)	**0.002**	84 (29.8)	**<0.001**
Yes	621 (58.5)	157(69.5)		198 (70.2)	
HCsAg (%)					
No	985 (92.8)	202 (89.4)	0.087	231 (81.9)	**<0.001**
Yes	77 (7.2)	24 (10.6)		51 (18.1)	
Calcium, median (IQR)	1.5 (1.0, 2.2)	1.5 (1.0, 2.5)	0.390	1.7 (1.1, 2.5)	**0.011**
Serum 25(OH) vitamin D (nmol l^{-1}), median (IQR)	20.1 (13.7, 30.3)	20.0 (14.0, 28.6)	0.973	15.3 (11.6, 25.4)	**<0.001**

Abbreviations: BMI = body mass index; HBcAg = hepatitis B core antigen; HBsAg = hepatitis B surface antigen; HCsAg = hepatitis C surface antigen; IQR = interquartile range. The bold entries are considered statistically significant.

association with chronic liver disease death. Tests of trend were conducted by assigning participants their vitamin D category and entering this variable into the regression models. All statistical analyses were conducted using SAS software (version 9.2, SAS Institute Inc., Cary, NC, USA). All tests were two sided and $P<0.05$ was considered statistically significant.

RESULTS

Demographic characteristics of the 226 incident liver cancer and 282 chronic liver disease deaths and controls are presented in Table 1. There were no statistically significant differences between the case and control groups for age at baseline, sex, smoking, drinking, BMI or season of blood draw. As expected, the prevalence of HBsAg, HBcAg and HCsAg positivity was significantly higher in the liver cancer and chronic liver disease groups than that in the control group. The median of serum 25(OH) vitamin D level was lower in those who later died from chronic liver disease than that in the control group, but was similar in the control and liver cancer groups (Table 1).

Table 2 summarises the serum 25(OH) vitamin D levels by baseline characteristics. Serum 25(OH) vitamin D levels varied significantly by age at baseline, sex, male smoking, BMI and season, but were unrelated to male drinking or HBsAg, HBcAg or HCsAg seropositivity.

Tables 3 and 4 present associations between serum 25(OH) vitamin D concentrations and risks of liver cancer incidence and chronic liver disease mortality. We found a significant association between higher serum 25(OH) vitamin D and lower risk of chronic liver disease mortality. Compared with the lowest quartile, subjects in the fourth quartile had a 66% lower risk of chronic liver disease deaths (OR = 0.34, 95% CI = 0.21–0.55), with evidence of a statistically significant monotonic trend ($P_{trend}<0.001$). This inverse association was present in both sexes, but was stronger among women (OR = 0.26; 95% CI = 0.12–0.52) than among men (OR = 0.41; 95% CI = 0.21–0.80), although this difference was not statistically significant. There were no significant associations between serum 25(OH) vitamin D and risk of liver cancer incidence. Although risk estimates decreased across increasing quartiles of vitamin D, (1.00 (ref); 0.91 (95% CI = 0.60–1.37); 0.87 (95% CI = 0.57–1.31); 0.74 (95% CI = 0.47–1.18), none of the risk estimates or the *P*-trend (0.208) were statistically significant.

In subgroup analyses, we observed comparable results in serum collected in the summer and the winter (Tables 3 and 4). Compared with the lowest quartile, subjects in the fourth quartile of serum 25(OH) vitamin D levels had ORs for chronic liver disease mortality of 0.40 (95% CI = 0.24–0.66) in winter and 0.10 (95% CI = 0.02–0.65) in summer (*P* for interaction = 0.124). We also examined the associations by duration of follow-up. In general, results were similar across the three strata of follow-up time, although associations were strongest in events occurring >7 years after baseline. No differences were observed by age at baseline. Results were also similar across strata of HBV and HCV seropositivity, although weaker in HCV-positive cases. Exclusion of eight cases with reported cirrhosis at baseline did not change any of our findings. When stratifying by the median serum calcium concentration among controls, the results for vitamin D appeared different across the strata for liver cancer incidence (*P* for interaction = 0.047), but similar for chronic liver disease mortality (*P* for interaction = 0.365). Serum 25(OH) vitamin D levels were significantly inversely associated with liver cancer incidence (Q4 *vs* Q1, OR = 0.43, 95% CI = 0.21–0.89) among those with baseline levels of serum calcium higher than $\geqslant 1.485$) mmol l^{-1}, but the association was not significant among those with lower levels (Q4 *vs* Q1, OR = 1.14, 95% CI = 0.61–2.13). The effect of serum 25(OH) vitamin D on chronic liver disease mortality was similar across these two strata of serum calcium (OR for Q4 *vs* Q1: 0.28 and 0.43, respectively). We also stratified our results by trial, finding similar results for each outcome in both (Tables 3 and 4). Within each trial, results were also similar across each stratification arm (data not shown).

Table 2. Serum 25(OH) vitamin D concentration percentiles in controls from the Linxian Nutrition Intervention Trial cohorts, overall and by baseline characteristics

	25th Percentile	50th Percentile	75th Percentile	*P*-value[a]
Overall	13.7	20.1	30.3	
Age at baseline				**0.021**
<50	14.7	22.2	33.0	
⩾50 to <60	13.1	18.9	28.1	
⩾60	13.3	20.6	32.3	
Sex				**<0.001**
Women	11.2	14.9	21.8	
Men	18.1	25.3	38.8	
Smoking (male)[b]				**0.001**
No	20.2	28.1	43.5	
Yes	17.1	24.2	36.4	
Alcohol drinking (male)[c]				0.071
No	17.3	24.8	39.2	
A few times per year	19.7	27.6	40.3	
A few times per month	20.7	25.0	34.2	
More than once per week	13.5	16.9	21.4	
BMI (kg m^{-2})				**0.011**
<25	13.7	20.3	31.0	
⩾25 to <30	12.9	18.3	25.1	
⩾30	7.9	8.3	11.2	
Blood draw season				**<0.001**
January–May	12.9	18.8	27.7	
August–September	24.7	41.2	65.3	
HBsAg				0.399
No	13.7	20.1	30.4	
Yes	12.5	18.2	28.8	
HBcAg				0.495
No	14.1	20.2	30.0	
Yes	13.3	20.0	30.4	
HCsAg				0.817
No	13.7	20.1	30.4	
Yes	13.0	19.9	29.9	

Abbreviations: BMI = body mass index. HBcAg = hepatitis B core antigen; HBsAg = hepatitis B surface antigen; HCsAg = hepatitis C surface antigen.
[a]We tested for serum 25(OH) D concentration differences between groups using the Kruskal–Wallis test.
[b]Smoking in males; only one smoker was female.
[c]Alcohol drinking in males; only 54 of 255 drinkers were female. The bold entries are considered statistically significant.

DISCUSSION

This is the first study to prospectively examine the association between serum 25(OH) vitamin D concentrations and risk of liver cancer incidence, and one of the first prospective studies of vitamin D and chronic liver disease mortality. In a population with low serum vitamin D levels, we observed a significant association between higher serum 25(OH) vitamin D concentrations and lower risk of chronic liver disease mortality. Compared with the lowest quartile, subjects in the fourth quartile of serum 25(OH) vitamin D

Table 3. Crude and adjusted OR and 95% CI for the associations between serum 25(OH) vitamin D concentration and risk of incident liver cancer in the case–control set nested in the Nutrition Intervention Trials cohort

	Continuous[a]		Quartile[b]												
			Q1 ($n=266$)[c]		Q2 ($n=265$)[c]			Q3 ($n=266$)[c]			Q4 ($n=265$)[c]				
	OR	95% CI	*n*	OR (Reference)	*n*	OR	95% CI	*n*	OR	95% CI	*n*	OR	95% CI	P_{trend}	$P_{interaction}$
Crude	1.00	0.94–1.06	63	1.00	55	0.88	0.59– 1.31	57	0.91	0.61–1.35	51	0.81	0.54–1.22	0.365	—
Age- and sex-adjusted	0.98	0.92–1.04	—	1.00	—	0.88	0.59–1.31	—	0.90	0.61–1.35	—	0.81	0.54–1.21	0.352	—
Fully adjusted[d]	0.95	0.88–1.03	—	1.00	—	0.91	0.60–1.37	—	0.87	0.57–1.31	—	0.74	0.47–1.18	0.208	—
Women	0.95	0.81–1.11	28	1.00	19	0.75	0.39–1.42	26	0.94	0.51–1.73	18	0.69	0.34–1.39	0.421	0.617
Men	0.95	0.87–1.05	35	1.00	36	1.02	0.59–1.77	31	0.81	0.46–1.44	33	0.79	0.42–1.48	0.338	
Age at baseline															
<55	0.94	0.84–1.05	31	1.00	23	0.77	0.41–1.43	32	0.96	0.54–1.72	24	0.73	0.37–1.44	0.538	0.997
⩾55	0.97	0.88–1.08	32	1.00	32	1.11	0.63–1.93	25	0.79	0.44–1.44	27	0.80	0.42–1.53	0.349	
Season of blood draw															
January–May	0.97	0.89–1.06	62	1.00	53	0.90	0.60–1.37	50	0.85	0.56–1.30	32	0.81	0.50–1.31	0.345	0.703
August–September	0.88	0.75–1.04	1	1.00	2	0.86	0.05–14.62	7	0.64	0.06–7.36	19	0.32	0.03–3.61	0.109	
Follow-up years															
<7	0.95	0.84–1.08	14	1.00	16	1.27	0.59–2.74	14	0.88	0.40–1.96	18	0.86	0.36–2.05	0.576	0.989
⩾7 to <14	0.96	0.85–1.09	22	1.00	18	0.90	0.47–1.75	16	0.78	0.39–1.54	15	0.79	0.37–1.67	0.447	
⩾14	0.95	0.84–1.08	27	1.00	21	0.77	0.42–1.43	27	0.93	0.52–1.67	18	0.64	0.32–1.28	0.327	
HBV/HCV seroposivity															
HBV+	0.97	0.89–1.05	43	1.00	37	0.85	0.53–1.36	40	0.90	0.56–1.44	37	0.79	0.47–1.32	0.425	0.442
HCV+	0.82	0.63–1.06	4	1.00	9	2.02	0.60–6.79	8	1.56	0.45–5.41	3	0.39	0.07–2.15	0.331	0.634
Neither	0.98	0.86–1.12	18	1.00	17	0.97	0.49–1.93	15	0.86	0.42–1.76	14	0.80	0.36–1.76	0.538	—
Calcium															
<1.485 mmol l^{-1}	1.02	0.93–1.13	26	1.00	24	1.10	0.60–2.04	32	1.28	0.71–2.28	25	1.14	0.61–2.13	0.561	**0.047**
⩾1.485 mmol l^{-1}	**0.88**	**0.78–0.98**	37	1.00	31	0.77	0.44–1.35	25	0.55	0.30–1.02	26	**0.43**	**0.21–0.89**	**0.014**	
Trial															
Dysplasia Trial	0.88	0.75–1.05	1	1.00	2	0.82	0.05–14.06	7	0.64	0.06–7.30	19	0.32	0.03–3.63	0.119	0.414
General Population Trial	0.95	0.86–1.04	62	1.00	53	0.83	0.54–1.26	50	0.75	0.52–1.10	32	0.69	0.42–1.15	0.127	

Abbreviations: BMI = body mass index; CI = confidence interval; HBcAg = hepatitis B core antigen; HBsAg = hepatitis B surface antigen; HBV = hepatitis B virus; HCsAg = hepatitis C surface antigen; HCV = hepatitis C virus; OR = odds ratio.

[a]ORs for continuous vitamin D were scaled to one-half the interquartile range (7.9 nmol l^{-1}).

[b]We used sex-specific cutoff values to calculate relative risks. Quartile for women: <11.17, ⩾11.17 to <14.86, ⩾14.86 to <21.80, ⩾21.80 (nmol l^{-1}); and quartile for men: <18.14, ⩾18.14 to <25.33, ⩾25.33 to <38.84, ⩾38.84 (nmol l^{-1}).

[c]Number of subjects in the control group.

[d]Adjusted for age at baseline, sex, smoking, drinking, BMI, season of blood draw, HBsAg, HBcAg and HCsAg. The bold entries are considered statistically significant.

had a lower risk of chronic liver disease deaths, with an OR of 0.34 (95% CI = 0.21–0.55). Stratification by season of blood collection did not alter the findings. In lag time analysis, we found a significant association after excluding cases that occurred during the first 7 years or the first 14 years of follow-up. Odd ratios for chronic liver disease deaths also decreased with higher serum 25(OH) vitamin D concentrations in participants with HBV seropositivity, but were weaker for participants with HCV seropositivity.

In contrast to results from chronic liver disease, we did not observe significant associations between serum 25(OH) vitamin D concentrations and the risk of liver cancer incidence. When we stratified by the median serum calcium concentrations in the controls, however, a significant association between serum 25(OH) vitamin D concentrations and risk of liver cancer incidence was observed in the higher serum calcium subgroup, although the *P*-value for interaction was only of borderline statistical significance. A number of studies suggest that vitamin D signalling is attenuated by low calcium levels in multiple organs (Peterlik and Cross, 2005). However, it is unclear why we might observe interactions with calcium for liver cancer but not for chronic liver disease mortality. As differences by calcium status for liver cancer could be due to chance, replication in future studies are needed to confirm these findings.

Although a number of studies have investigated associations between serum vitamin D levels and various cancers (Garland and Garland, 1980; Chen *et al*, 2007; Li *et al*, 2007; Abbas *et al*, 2008; Ahn *et al*, 2008; Abnet *et al*, 2010; Jenab *et al*, 2010; Stolzenberg-Solomon *et al*, 2010), little epidemiologic data for vitamin D and liver cancer are available, despite the important role of the liver in metabolising the circulating form of vitamin D. Supporting a possible association, vitamin D has been shown to inhibit liver carcinogenesis in cell lines and several animal models (Ghous *et al*, 2008), for example, vitamin D has been shown to reduce the number of chromosomal aberrations and double-strand breaks (Saha *et al*, 2001) as well as prevent cellular proliferation (Pourgholami *et al*, 2000; Caputo *et al*, 2003). For liver disease, results from existing epidemiologic studies, each modest in size, are mixed. A recent study nested in the HALT-C trial found no effect of baseline vitamin D levels on subsequent liver disease progression among HCV-positive participants with existing fibrosis (Corey *et al*, 2012). However, results from the two small European studies

Table 4. Crude and adjusted OR and 95% CI for the associations between serum 25(OH) vitamin D concentration and risk of death from chronic liver disease in the case–control set nested in the Nutrition Intervention Trials cohort

	Continuous[a]		Quartile[b]												
			Q1 ($n=266$)[c]		Q2 ($n=265$)[c]			Q3 ($n=266$)[c]			Q4 ($n=265$)[c]				
	OR	95% CI	*n*	OR (Reference)	*n*	OR	95% CI	*n*	OR	95% CI	*n*	OR	95% CI	P_{trend}	$P_{interaction}$
Crude	**0.88**	**0.82–0.94**	106	1.00	79	0.75	0.53–1.05	56	**0.53**	**0.37–0.76**	41	**0.39**	**0.26–0.58**	**<0.001**	—
Age- and sex-adjusted	**0.88**	**0.81–0.95**	—	1.00	—	0.75	0.54–1.05	—	**0.53**	**0.37–0.77**	—	**0.39**	**0.26–0.58**	**<0.001**	—
Fully adjusted[d]	**0.86**	**0.78–0.93**	—	1.00	—	0.79	0.55–1.14	—	**0.50**	**0.34–0.75**	—	**0.34**	**0.21–0.55**	**<0.001**	—
Women	**0.84**	**0.71–0.99**	54	1.00	47	0.95	0.58–1.57	24	**0.40**	**0.22–0.72**	17	**0.26**	**0.12–0.52**	**<0.001**	0.710
Men	**0.86**	**0.77–0.96**	52	1.00	32	0.64	0.37–1.10	32	0.58	0.33–1.01	24	**0.41**	**0.21–0.80**	**0.007**	
Age at baseline															
<55	0.89	0.78–1.00	50	1.00	37	0.84	0.49–1.45	27	**0.49**	**0.27–0.87**	23	**0.43**	**0.22–0.85**	**0.003**	0.459
⩾55	**0.84**	**0.74–0.96**	56	1.00	42	0.78	0.48–1.29	29	**0.55**	**0.32–0.94**	18	**0.29**	**0.15–0.58**	**<0.001**	
Season of blood draw															
January–May	**0.81**	**0.72–0.91**	103	1.00	75	0.79	0.55–1.15	49	**0.51**	**0.34–0.77**	25	**0.40**	**0.24–0.66**	**<0.001**	0.124
August–September	0.95	0.81–1.10	3	1.00	3	0.47	0.05–4.42	7	0.23	0.04–1.53	16	**0.10**	**0.02–0.65**	**0.004**	
Follow-up years															
<7	0.93	0.81–1.08	24	1.00	18	0.75	0.38–1.47	12	**0.46**	**0.22–0.98**	17	0.70	0.32–1.53	0.141	0.167
⩾7 to <14	**0.77**	**0.65–0.90**	42	1.00	29	0.77	0.45–1.30	25	**0.54**	**0.31–0.95**	8	**0.13**	**0.05–0.32**	**<0.001**	
⩾14	0.88	0.77–1.01	40	1.00	32	0.83	0.49–1.39	19	**0.43**	**0.24–0.79**	16	**0.35**	**0.17–0.70**	**<0.001**	
HBV/HCV seroposivity															
HBV+	**0.87**	**0.78–0.95**	74	1.00	53	0.68	0.46–1.02	44	**0.56**	**0.37–0.86**	30	**0.34**	**0.20–0.57**	**<0.001**	0.189
HCV+	0.98	0.85–1.13	16	1.00	16	1.09	0.52–2.26	7	0.44	0.17–1.09	12	0.80	0.34–1.89	0.240	0.140
Neither	0.84	0.70–1.01	31	1.00	19	0.64	0.35–1.18	11	**0.35**	**0.17–0.72**	10	**0.32**	**0.14–0.73**	**0.001**	—
Calcium															
<1.485 mmol l^{-1}	0.88	0.77–1.01	47	1.00	33	0.84	0.49–1.44	29	0.67	0.38–1.16	17	**0.43**	**0.22–0.83**	**0.009**	0.365
⩾1.485 mmol l^{-1}	**0.85**	**0.75–0.95**	59	1.00	46	0.74	0.45–1.21	27	**0.39**	**0.22–0.70**	24	**0.28**	**0.14–0.56**	**<0.001**	
Trial															
Dysplasia Trial	0.96	0.82–1.12	3	1.00	3	0.45	0.05–4.23	7	0.23	0.04–1.50	16	**0.10**	**0.02–0.65**	**0.004**	0.155
General Population Trial	**0.80**	**0.70–0.90**	103	1.00	76	0.76	0.52–1.10	49	**0.47**	**0.31–0.73**	25	**0.37**	**0.22–0.62**	**<0.001**	

Abbreviations: BMI = body mass index; CI = confidence interval; HBcAg = hepatitis B core antigen; HBsAg = hepatitis B surface antigen; HBV = hepatitis B virus; HCsAg = hepatitis C surface antigen; HCV = hepatitis C virus; OR = odds ratio.

[a]ORs for continuous vitamin D were scaled to one-half the interquartile range (7.9 nmol l^{-1}).

[b]We used sex-specific cutoff values to calculate relative risks. Quartile for women: <11.17, ⩾11.17 to <14.86, ⩾14.86 to <21.80, ⩾21.80 (nmol l^{-1}); and quartile for men: <18.14, ⩾18.14 to <25.33, ⩾25.33 to <38.84, ⩾38.84 (nmol l^{-1}).

[c]Number of subjects in the control group.

[d]Adjusted for age at baseline, sex, smoking, drinking, BMI, season of blood draw, HBsAg, HBcAg and HCsAg. The bold entries are considered statistically significant.

did observe evidence for an inverse association between serum vitamin D levels and liver disease progression (Baur et *al*, 2012; Putz-Bankuti *et al*, 2012). It is not clear why differences occurred across each of these studies, although chance and differences in study design and study population may have contributed. We could find no previous studies of vitamin D levels and liver cancer.

The association between sunlight exposure and vitamin D synthesis has been known since 1890. As such, a number of environmental factors influence vitamin D levels including season, length of day, smog, cloud cover and sunscreen use (Mavroeidi *et al*, 2010; Macdonald *et al*, 2011). In our study, season was a strong predictor of vitamin D levels. In this population, participants eat few vitamin D-rich foods (Zou *et al*, 2002), such that nearly all vitamin D is obtained by participants from sunlight. Therefore, potential confounding by season is a particular concern in such studies. However, adjustment for season had little effect on our risk estimates.

As mentioned earlier, vitamin D levels in our population were low relative to most previously studied populations, perhaps reflecting a diet poor in vitamin D-rich foods and a lack of vitamin D supplementation. Genetic differences may also be important, as previous studies have tended to observe lower vitamin D levels among Asian populations (McCullough *et al*, 2010). As our population had low vitamin D levels, it is not clear whether our results are generalisable to other populations with higher vitamin D levels. Future studies are needed to address this issue.

Our study was set within two nutritional intervention trials, which were conducted in parallel in Linxian, China. Although we measured vitamin D in baseline samples, which were collected before supplementation occurred, we were concerned that supplementation may have affected our results, particularly as the multivitamin used in one arm of the Dysplasia Trial included 800IU of vitamin D. However, we observed similar results in participants enrolled in the General Population Trial (which was not supplemented for vitamin D) and the

Dysplasia Trial (in which vitamin D was one of the included vitamins). In addition, we observed similar associations among the Dysplasia Trial among those who did and did not receive multivitamin supplementation.

Another potential concern is reverse causality, as several (Masuda *et al*, 1989; Bonkovsky *et al*, 1990; Chen *et al*, 1996; Gallego-Rojo *et al*, 1998; Fisher and Fisher, 2007; Chiang *et al*, 2011; Putz-Bankuti *et al*, 2012), but not all (Duarte *et al*, 2001; Kitson *et al*, 2013), studies have observed lower vitamin D levels among those with chronic liver disease, and have found inverse correlations between vitamin D levels and disease severity. Hence, we examined the association of vitamin D level and chronic liver disease death stratified by the number of follow-up years (<7, $\geqslant 7$ to <14 and $\geqslant 14$ years). A significant association between higher serum 25(OH) vitamin D concentrations and lower risk of chronic liver disease deaths persisted in cases occurring even after 14 years of follow-up. In addition, although several previous studies have observed lower vitamin D levels in HBV and HCV-positive participants (Gutierrez *et al*, 2011; Lange *et al*, 2011; Tang *et al*, 2011), we observed no such association in our study. HBV and HCV-positive individuals would be more likely to have fibrosis than unaffected individuals, such that a lack of association between HBV or HCV positivity and vitamin D levels argue against reverse causality as an explanation for our results. Furthermore, exclusion of eight cases who reported cirrhosis at study baseline did not alter our results. However, we did not have an assessment of fibrosis at baseline. Therefore, whether low baseline vitamin D levels simply reflected early pre-existing liver disease or instead actively contributed to the pathogenesis of chronic liver disease death or both cannot be determined from our data.

This study has several strengths, including its prospective design (serum vitamin D was measured in serum collected before the onset of disease) and its long follow-up time (over 22 years). We also adjusted for a number of important potential confounders in the multivariate model including age at baseline, sex, smoking, alcohol drinking, BMI, season of blood draw and HBV and HCV seropositivity. As mentioned previously, one limitation was that we lacked information about liver fibrosis at baseline. So we cannot determine for certain whether low baseline vitamin D levels simply reflected concurrent liver disease or contributed directly to the pathogenesis of chronic liver disease, or both. Also, we only had a single blood draw, possibly contributing to misclassification of vitamin D status. In addition, our sample size was limited for detecting modest associations and for examining stratifications, particularly among those with blood collected during the summer time. Another limitation was that most incident liver cancers and chronic liver disease deaths were diagnosed by combined evidence from biochemical assays, clinical examination, ultrasound and computed tomography scan, and only a few were diagnosed by pathology. Several lines of evidence argue that the methods used to diagnose liver cancer in our study were appropriate, even in the absence of pathology on every case. First, we observed the expected associations with the known risk factors of HBV and HCV. Second, we would expect that misclassification of the end point would not vary by baseline vitamin D status; thus any potential misclassification would bias our associations towards the null, and our observed associations would be attenuated relative to the true associations.

In summary, in a low vitamin D population, our study provides the first prospective evaluation between serum vitamin D concentrations and risk of liver cancer incidence and is one of the first prospective studies of vitamin D and chronic liver disease mortality. We found a significant association between higher serum 25(OH) vitamin D concentrations and lower risk of chronic liver disease mortality, even among cases occurring $\geqslant 14$ years after baseline blood collection. We observed only modest evidence for associations with incident liver cancer, which became statistically significant only among participants with higher baseline serum calcium. Our results suggest that vitamin D may be important in the aetiology of liver cancer and chronic liver disease. However, additional studies are needed to confirm these findings, particularly among populations with a wider range of vitamin D, particularly at higher levels.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

NDF, CCA, SMD, PRT and Y-LQ: designed and conducted the research; J-BW: analysed the data and wrote the manuscript; J-HF, WC, L-YY and JY: provided essential reagents or provided essential materials; JMM, CCA, NDF, SMD, PRT and Y-LQ: advised on the analysis and preparation of the report, after which every author participated in editing and finalisation; NDF and Y-LQ: had primary responsibility for final content. All authors read and approved the final manuscript.

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