

## Sex difference in liver-related mortality and transplantation associated with dietary cholesterol in chronic hepatitis C virus infection

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### Abstract

Dietary cholesterol induces hepatic inflammation and fibrosis in animals. We aimed to determine whether dietary cholesterol affects liver-related mortality in hepatitis C virus (HCV)-infected patients. We performed a retrospective cohort study using extended follow-up data from the Hepatitis C Antiviral Long-Term Treatment Against Cirrhosis Trial. The study included HCV patients with advanced fibrosis and compensated cirrhosis. The analysis included 657 patients who completed two FFQ. We assessed whether cholesterol intake, measured in mg/4184 kJ (mg/1000 kcal) of energy intake, was associated with liver-related death or transplantation. In 4.7 (SD 1.6) years, the incidence of liver-related death ( $n$  46) or transplantation ( $n$  52) was 31.8/1000 person-years. The relationship between cholesterol intake and liver-related death or transplantation was significantly different between men and women (test for interaction,  $P$  value = 0.01). Each higher quartile of cholesterol intake was associated with an increased risk for liver-related death or transplantation in women (adjusted hazard ratio (AHR) 1.83; 95% CI 1.12, 2.99;  $P_{\text{trend}}$  = 0.02), but not in men (AHR 0.96; 95% CI 0.76, 1.22;  $P_{\text{trend}}$  = 0.73). Compared with women whose cholesterol intake was within the recommended guidelines (300 mg/d with a 8368 kJ (2000 kcal) diet), women who consumed more cholesterol had significantly increased risk for liver-related death or transplantation (AHR 4.04; 95% CI 1.42, 11.5). High dietary cholesterol was associated with an increased risk for liver-related death and transplantation in HCV-infected women with advanced fibrosis or compensated cirrhosis. Future studies should assess whether reducing cholesterol intake, among women who consume an excessive amount, can decrease HCV-related mortality.

**Key words:** Diets: Cholesterol: Cirrhosis: Sex differences: Hepatitis C virus

Chronic hepatitis C virus (HCV) infection affects approximately 184 million people worldwide<sup>(1)</sup>. In the USA, its prevalence was estimated to have peaked in 2001 when 3.57 million individuals were chronically infected. Although the prevalence of HCV infection has been declining, the number of patients with HCV-related cirrhosis is continuing to increase and is projected to reach one million in 2020<sup>(2)</sup>.

Advanced fibrosis or cirrhosis develops at a variable rate in HCV-infected patients<sup>(3–5)</sup>, suggesting that factors beyond the presence of virus influence disease progression. In the absence of HCV infection, several lines of evidence suggest that dietary cholesterol adversely affects liver function. Rabbits fed a cholesterol-supplemented diet developed steatosis, sinusoidal fibrosis and ascites<sup>(6)</sup>. The authors hypothesised that activation of platelet-derived growth factors may be involved in dietary cholesterol-mediated fibrogenesis, because dipyridamole, a platelet aggregation inhibitor, was able to halve the amount of fibrosis developed in cholesterol-fed animals. More recently,

Teratani *et al.*<sup>(7)</sup> demonstrated that wild-type C57BL/6 mice fed a high-cholesterol diet developed significantly more fibrosis in the setting of bile duct ligation or carbon tetrachloride administration than did mice fed a diet without excess cholesterol. The authors suggested that free cholesterol accumulation within hepatic stellate cells was responsible for their activation and increased fibrogenesis.

There is also circumstantial evidence that liver infected with HCV may be especially susceptible to the toxic effect of dietary cholesterol. Compared with controls, liver homogenate from transgenic mice expressing HCV core protein had significantly decreased activity of microsomal triglyceride transfer protein (MTP)<sup>(8)</sup>, an intracellular protein in mammalian liver microsomes responsible for transferring cholesterol esters onto ApoB lipoproteins<sup>(9)</sup>. MTP is also critical in the assembly of VLDL<sup>(10)</sup>. MTP inhibition results in a decrease in hepatic esterified cholesterol and an increase in free cholesterol<sup>(9)</sup>, which is toxic to cells<sup>(11)</sup>. It is possible that excessive dietary cholesterol

**Abbreviations:** AHR, adjusted hazard ratio; HALT-C, Hepatitis C Antiviral Long-Term Treatment Against Cirrhosis; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; MTP, microsomal triglyceride transfer protein.

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exacerbates free cholesterol accumulation in HCV-infected hepatocytes, which may already have a higher concentration of free cholesterol because of viral-induced MTP inhibition compared with uninfected cells.

In a *post hoc* analysis of the original Hepatitis C Antiviral Long-Term Treatment Against Cirrhosis (HALT-C) Trial, a randomised controlled study that assessed the effect of low-dose peginterferon-alfa-2a on liver disease progression<sup>(12)</sup>, we previously demonstrated a significant association between high dietary cholesterol intake and incident liver disease progression in patients with advanced fibrosis or compensated cirrhosis<sup>(13)</sup>. In the current analysis using extended follow-up data from the HALT-C Trial<sup>(14)</sup>, which were not available to us previously, we aimed to investigate whether the adverse effect of dietary cholesterol translated into liver-related mortality and transplantation.

## Methods

### Study design

We performed a retrospective cohort study using extended long-term follow-up data from the HALT-C Trial (ClinicalTrials.gov no. NCT00006164). The trial included HCV-infected patients with histologically defined advanced fibrosis (Ishak fibrosis score<sup>(15)</sup> 3 or 4) or compensated cirrhosis (Ishak fibrosis score 5 or 6) who did not achieve a sustained virological response to prior interferon-based therapy. No patients had a history of Child-Turcotte-Pugh (CTP) score  $\geq 7$ , bleeding related to oesophageal varices, ascites, encephalopathy or hepatocellular carcinoma (HCC) at the time of entry into the study. After the randomised treatment phase of the trial, subjects were followed for an additional 3 years for clinical decompensation and death until October 2009<sup>(14)</sup>. Use of peginterferon-alfa-2a was not associated with improved outcomes in the HALT-C study. The data generated by the HALT-C study have been used to evaluate multiple risk factors for liver disease progression in HCV-infected patients<sup>(16–18)</sup>. Details on the design, patient population and study outcome have been published elsewhere<sup>(12,19)</sup>.

### Assessment of dietary cholesterol

Dietary intake was assessed using the well-validated Block 98.2 FFQ (Block Data Systems), which estimates nutrient intake over the past year based on self-reported frequency and portion of foods<sup>(20)</sup>. Out of 1050 patients who participated in the randomised phase of the study, 808 patients completed an FFQ at baseline, 822 patients completed a follow-up FFQ 661 (sd 269) d after enrolment, and 672 patients completed both FFQ. Because diet may change over time, the average nutrient intake, calculated using multiple assessments over time, can capture dietary intake more accurately than a single assessment<sup>(21)</sup>. We therefore defined cholesterol intake as the average daily cholesterol intake, obtained by dividing the sum of daily cholesterol intake estimated from the baseline and the follow-up FFQ by 2, among participants who completed both FFQ.

### Study population

Among 672 patients who completed both FFQ, we excluded one patient who reported an extremely high energy intake ( $>2$  interquartile ranges (IQR) from the median). We also excluded fourteen patients who underwent transplantation before the second FFQ ( $n$  2), whose date of completion of the second FFQ was unknown ( $n$  10) and who completed their follow-up FFQ on their last visit ( $n$  2), leaving 657 patients in the current analysis. The number of subjects in the current analysis is higher compared with our previous analysis (657 *v.* 608) because we included subjects who did not have paired liver biopsies<sup>(13)</sup>.

### Outcome definition

The outcome of the study was the time to development of either liver-related death or liver transplantation starting from the date of the follow-up FFQ. A seven-person, central review committee consisting of HALT-C Trial investigators reviewed all cases of liver-related death<sup>(14)</sup>.

### Statistical analysis

We used Cox's proportional hazards regression to determine whether cholesterol consumption was associated with liver-related death or transplantation<sup>(22)</sup>. As cholesterol intake was positively correlated with total energy intake (Pearson's correlation:  $r$  0.78;  $P < 0.0001$ ), quartiles of cholesterol intake were created after dividing cholesterol intake by total energy intake, according to the nutrient density model of total energy adjustment<sup>(23)</sup>. Total energy intake was calculated by dividing the sum of daily energy intake (kJ (kcal)) estimated from the baseline and the follow-up FFQ by 2, among participants who completed both FFQ. Details on the changes in cholesterol intake between the baseline and follow-up FFQ are presented in online Supplementary Table S1. Cholesterol intake quartiles were modelled in two ways: (1) as a 'dummy' categorical variable, where each higher quartile of cholesterol intake (2nd, 3rd and 4th) was compared with the lowest (1st) quartile; and (2) as an ordinal variable (1 through 4) to yield a 'test for trend' where each higher quartile of cholesterol intake was compared with the lower quartile. Patients were censored at their last clinic visit. We performed multiple Cox's regression analyses adjusting for the following variables that may influence disease progression in chronic liver diseases: age, sex, race, BMI, diabetes, lifetime alcohol consumption, smoking status, self-reported health status, coffee intake, duration of HCV infection, treatment with low-dose peginterferon, presence of cirrhosis and total energy intake. Normality for continuous variables was assessed using the Shapiro–Wilk's test. Variables were modelled linearly except for sex, race, diabetes status (yes or no, defined as a fasting glucose  $\geq 126$  mg/dl or having been told to have diabetes), self-reported health status (excellent, very good, good, fair and poor, based on a single question from the Block FFQ), low-dose peginterferon treatment group, current smoking status, cirrhosis status and coffee intake (non-drinker,  $<1$ , 1–2 and  $\geq 3$  cups/d). The assumption of

proportional hazards was tested and met using weighted residual methods. We specifically tested whether sex, underlying cirrhosis (cirrhosis *v.* advanced fibrosis), low-dose peginterferon treatment group (treatment *v.* no treatment) and menopause status (yes *v.* no) among women affected the relationship between dietary cholesterol and liver-related death or transplantation. Women were classified as post-menopausal if they were  $\geq 55$  years of age or, among those who were  $< 55$ , if they had a self-reported history of naturally stopping menses or history of bilateral oophorectomy. Each interaction term was entered into the regression model separately.

Because sex was found to be a significant effect modifier on the relationship between dietary cholesterol and liver-related death or transplantation, we performed the above analyses separately for men and women, using both non-sex-specific and sex-specific quartiles of cholesterol intake. Because diet may change after major clinical events, such as development of HCC, rise in CTP score, variceal bleeding, ascites, peritonitis and encephalopathy, we performed the same analyses after excluding an additional twenty-seven patients who developed such a clinical event before completion of the follow-up FFQ. Because it may be more practical and informative for patients, in addition to models using quartiles of cholesterol intake described above, we provided risk estimates for liver-related death or transplantation, also separately for men and women, comparing those whose cholesterol intake was above ( $> 150$  mg cholesterol/4184 kJ ( $> 150$  mg cholesterol/1000 kcal) of energy intake, or  $> 300$  mg/d based on a 8368 kJ (2000 kcal) diet) with those whose intake was within the Dietary Guidelines for Americans 2010 ( $\leq 150$  mg/4184 kJ ( $\leq 150$  mg/1000 kcal) of energy intake, [www.dietaryguidelines.gov](http://www.dietaryguidelines.gov)).

Finally, because methods of total energy adjustment affect the risk estimates of the nutrient–disease relationship, especially when the exposure nutrient is categorised (rather than continuous)<sup>(24)</sup>, we presented the results separately for men and women with three other models of total energy adjustment. (1) Nutrient residual model, in which quartiles of cholesterol intake were based on residuals derived from the linear regression model wherein the cholesterol intake was regressed on the total energy intake. Total energy intake was also included in the multivariate model; (2) standard model, in which quartiles of cholesterol intake were based on simply the absolute amount of cholesterol intake. Total energy intake was also included in the multivariate model; (3) energy-partition model, in which quartiles of cholesterol intake were also based on the absolute amount of cholesterol intake. However, instead of total energy intake, absolute intake of carbohydrate, protein, SFA and unsaturated fat was simultaneously included in the multivariate model. Average macronutrient intake was calculated by dividing the sum of the macronutrient intake estimated from the baseline and the follow-up FFQ by 2, among participants who completed both FFQ. Analyses were performed with Stata SE, version 11.0 (StataCorp LP).

## Results

Higher cholesterol intake was strongly associated with male sex, greater intake of macronutrients and higher lifetime alcohol

consumption. The proportion of subjects who consumed  $\geq 1$  alcoholic drink/d during the trial was low, and was similar among groups of different cholesterol intake. In terms of metabolic parameters, higher cholesterol intake was associated with higher BMI, fasting glucose, insulin, homeostatic model assessment (HOMA-IR) and prevalence of diabetes. In terms of liver disease parameters, higher cholesterol intake was associated with higher baseline alanine transaminase, but not with Ishak scores of inflammation, fibrosis or steatosis score. The proportion of patients with cirrhosis or oesophageal varices at baseline did not vary significantly among categories of cholesterol intake. Finally, higher cholesterol intake was not associated with higher serum cholesterol (Table 1).

During a mean follow-up of 4.7 (SD 1.6) years, the incidence of liver-related death ( $n$  46) or transplantation ( $n$  52) was 31.8/1000 person-years. The association between increasing cholesterol intake quartiles and liver-related death or transplantation was significantly different between men and women (test for interaction,  $P$  value = 0.01). After adjusting for potential confounders, each higher quartile of cholesterol intake was associated with a significant increase in the risk for liver-related death or transplantation in women (Table 2; adjusted hazard ratio (AHR) 1.83; 95% CI 1.12, 2.99;  $P_{\text{trend}}$  = 0.02), but not in men (Table 3; AHR 0.96; 95% CI 0.76, 1.22;  $P_{\text{trend}}$  = 0.73). These risk estimates were similar using sex-specific quartiles of cholesterol intake (online Supplementary Tables S2 and S3: in women, AHR 1.81; 95% CI 1.05, 3.10;  $P_{\text{trend}}$  = 0.02; in men, AHR 1.02; 95% CI 0.80, 1.28;  $P_{\text{trend}}$  = 0.91). Inclusion of baseline histological steatosis score as a continuous variable in the model did not change these results. Inclusion of physical activity level, in terms of total weekly recreational and non-recreational metabolic equivalents, yielded the same estimate for men (AHR 1.03; 95% CI 0.77, 1.36;  $P_{\text{trend}}$  = 0.8). However, for women, inclusion of physical activity in the model resulted in a slightly stronger risk (AHR 2.93; 95% CI 1.16, 7.35;  $P_{\text{trend}}$  = 0.02). Furthermore, the increased risk was only apparent among post-menopausal women ( $n$  113, AHR 5.51; 95% CI 1.42, 21.3;  $P_{\text{trend}}$  = 0.02), but not among pre-menopausal women ( $n$  80, AHR<sub>trend</sub> 1.14; 95% CI 0.51, 2.55;  $P_{\text{trend}}$  = 0.8). This difference was statistically significant (test for interaction 0.02). Compared with women whose cholesterol intake was within the Dietary Guidelines for Americans 2010 ( $\leq 300$  mg/d based on a 8368 kJ (2000 kcal) diet,  $n$  160), women whose cholesterol intake was above the recommended intake ( $> 300$  mg/d,  $n$  33) had a 4-fold increased risk for liver-related death or transplantation (AHR 4.04; 95% CI 1.42, 11.5; online Supplementary Table S4). This relationship was not observed in men (online Supplementary Table S5).

In both sexes, the association between increasing cholesterol intake quartiles and liver-related death or transplantation was not significantly different in the following subgroups: patients with cirrhosis *v.* those with advanced fibrosis (test for interaction in men:  $P$  value = 0.43; in women:  $P$  value = 0.91); and those who received low-dose peginterferon *v.* those who received no treatment (test for interaction in men:  $P$  value = 0.62; in women:  $P$  value = 0.94).

Liver-related death ( $n$  46) or transplantation ( $n$  52) was preceded by other major clinical events in the majority of patients

**Table 1.** Baseline characteristics of 657 hepatitis C virus (HCV)-infected patients according to quartiles of average dietary cholesterol intake (measured in mg/4184 kJ (mg/1000 kcal) of daily energy intake) (Medians and interquartile ranges (IQR); numbers, mean values and standard deviations)

	Quartiles of cholesterol intake										<i>P</i> <sub>trend</sub>
	All patients		1st		2nd		3rd		4th		
	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR	
<i>n</i>	657		165		164		164		164		
Average daily dietary intake											
Cholesterol (mg)	220	153–310	143	101–190	200	146–260	261	203–333	307	235–430	<0.001
Total energy (kJ)	7644	5623–9824	7698	5498–9201	7744	5657–10 330	8180	6293–10 544	6882	5159–9736	0.8
Total energy (kcal)	1827	1344–2348	1840	1314–2199	1851	1352–2469	1955	1504–2520	1645	1233–2327	0.8
Carbohydrate (g)	227	163–297	242	168–297	243	178–317	234	190–303	180	133–259	<0.001
Total fat (g)	75	54–104	69	48–93	72	52–104	80	60–110	75	55–107	0.01
Protein (g)	66	48–86	580	43–77	66	47–85	71	58–94	67	49–90	<0.001
Age at randomisation (years)	50	46–54	51	47–55	50	46–54	49	46–53	50	47–55	0.6
Female (%)	29		40		34		24		20		<0.001
Race (%)											0.02
White	75		76		80		77		68		
Black	15		12		11		16		22		
Hispanic	6.7		7.3		5.5		6.7		7.3		
Other	2.6		4.8		3.7		0		1.8		
BMI (kg/m <sup>2</sup> )	29	26–32	28	25–31	29	26–32	30	27–33	30	27–33	<0.001
Waist:hip ratio*	0.57	0.52–0.62	0.56	0.51–0.61	0.57	0.52–0.61	0.57	0.53–0.61	0.57	0.54–0.62	0.03
Self-reported health status(%) <sup>†</sup>											0.08
Excellent	3.5		5.4		4.3		1.2		3.1		
Very good	23		23.0		23.5		25.2		20.5		
Good	45		42.4		45.1		43.6		49.1		
Fair	24		22.4		21.6		26.4		24.2		
Poor	4.8		6.7		5.6		3.7		3.1		
Lifetime alcohol consumption											0.001
Number of drinks	7207	1075–20 628	3982	240–14 168	8123	1661–23 830	7484	1634–20 454	10 943	1278–33 142	
Consumes ≥1 alcoholic drink/d during the trial (%) <sup>‡</sup>	4.7		3.9		3.8		7.4		3.7		0.3
Daily coffee intake (%) <sup>§</sup>											0.8
Non-drinkers	17		19		20		12		18		
<1 cup	26		25		28		29		24		
1–2 cups	45		45		41		47		47		
≥3 cups	11		9.9		11		12		12		
Current smokers (%) <sup>  </sup>	27		24		32		25		28		0.02
Weekly recreational and non-recreational physical activity (metabolic equivalents) <sup>¶</sup>	116	77–160	114	79–163	118	82–160	119	64–168	110	66–150	0.4
Glucose (mmol/l)	5.3	4.9–6.3	5.3	4.8–6.0	5.2	4.8–5.9	5.3	4.9–6.2	5.7	4.9–7.1	0.01
Insulin (pmol/l) <sup>**</sup>	236	160–396	222	146–389	236	160–354	257	160–438	250	174–417	0.04
HOMA2-IR <sup>††</sup>	4.2	2.9–6.8	3.8	2.5–6.5	4.1	2.9–6.0	4.6	2.9–6.8	4.5	3.2–7.8	0.02
Diagnosis of diabetes (%) <sup>‡‡</sup>	20		16		15		21		31		0.001
Diagnosis of hypercholesterolaemia or hypertriglycerolaemia (%) <sup>§§</sup>	7.8		4.8		9.8		8.0		8.5		0.4
On medication for hypercholesterolaemia or hypertriglycerolaemia (%) <sup>   </sup>	44		57		44		38		43		0.9
Baseline serum TAG (mmol/l)	1.2	0.9–1.8	1.2	0.8–1.6	1.2	0.9–1.8	1.2	0.8–1.9	1.3	0.9–1.9	0.1
Baseline serum cholesterol (mmol/l) <sup>¶¶</sup>	4.4	3.9–5.0	4.4	3.9–5.0	4.4	3.8–5.0	4.5	4.0–4.9	4.3	3.8–5.2	0.9
Follow-up length (years since follow-up FFQ)	5.0	3.9–5.7	5.1	3.7–5.9	5.0	3.8–5.8	5.1	4.1–5.6	5.0	3.9–5.7	0.8
Randomised to peginterferon (%)	51		45		48		55		55		0.1
Duration of HCV infection (years) <sup>***</sup>	29	24–33	29	24–33	29	23–33	29	24–33	29	24–33	0.7
HCV genotype 1 (%)	93		92		94		94		92		0.7
HCV viral load (log IU/ml)	6.5	6.1–6.8	6.6	6.2–6.9	6.4	6.2–6.7	6.5	6.2–6.8	6.5	6.1–6.8	0.3
Cirrhosis (%)	41		38		44		39		43		0.6
Varices (%) <sup>†††</sup>	26		23		27		30		24		0.5
ALT (µkat/l)	1.4	1.0–2.2	1.4	0.9–2.0	1.4	1.0–2.2	1.5	1.1–2.3	1.5	0.9–2.4	0.04
AST (µkat/l)	1.2	0.8–1.8	1.1	0.8–1.6	1.2	0.9–1.9	1.2	0.8–1.8	1.2	0.8–2.1	0.08
Total bilirubin (mmol/l)	12.0	8.6–17.1	12.0	8.6–17.1	12.0	8.6–17.1	12.0	8.6–17.1	13.7	10.3–17.1	0.2
Albumin (g/l)	39	36–42	39	36–41	40	37–41	39	36–42	39	36–41	0.7
Platelets (10 <sup>9</sup> per l)	161	116–207	165	123–211	162	114–209	164	127–198	147	108–208	0.2
INR	1.0	1.0–1.1	1.0	1.0–1.1	1.0	1.0–1.1	1.0	1.0–1.1	1.0	1.0–1.1	0.3
Ishak inflammation score											0.1
Mean	7.6		7.5		7.5		7.5		7.9		
SD	2.1		2.1		2.1		2.0		2.1		
Ishak fibrosis score	4.0	3.0–5.0	4.0	3.0–5.0	4.0	3.0–5.0	4.0	3.0–5.0	4.0	3.0–5.0	0.4

Table 1. Continued

	Quartiles of cholesterol intake										<i>P</i> <sub>trend</sub>
	All patients		1st		2nd		3rd		4th		
	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR	
Steatosis score											0.5
Mean	1.3		1.3		1.4		1.3		1.4		
SD	0.9		0.9		0.9		1.0		1.0		

HOMA-IR, homeostatic model assessment-insulin resistance; ALT, alanine transaminase; AST, aspartate transaminase; INR, international normalised ratio.

\* 644 patients had known waist:hip ratio status.

† 651 patients had known self-reported health status.

‡ 635 patients had known alcohol intake estimated about month 18 after enrolment.

§ 621 patients had known coffee intake estimated from the baseline FFQ.

|| 636 patients had known smoking status.

¶ 500 patients had known weekly self-reported recreational and non-recreational physical activity.

\*\* 496 patients had baseline insulin measurement.

†† 481 had baseline HOMA-IR status.

‡‡ Diabetes was defined as baseline fasting glucose  $\geq$  126 mg/dl or having been diagnosed with diabetes.

§§ 656 patients had known status of whether the diagnosis of hypercholesterolaemia or hypertriglycerolaemia was present.

|||| Among fifty-one patients who carried the diagnosis of hypercholesterolaemia or hypertriglycerolaemia, fifty had known status of whether they were being treated with medications.

¶¶ 478 patients had known baseline serum cholesterol measurement.

\*\*\* 621 patients had known duration of HCV infection.

††† 651 patients had known baseline varices status.

(*n* 86) (Table 4). The most common events were the development of HCC (*n* 25) and CTP score  $\geq$  7 on two consecutive study visits (*n* 46). After excluding the twenty-seven patients who developed a major clinical event before the completion of their follow-up FFQ (two patients with HCC, eighteen with CTP score  $\geq$  7 on two consecutive visits, four with variceal haemorrhage, one with ascites and two with encephalopathy), the association between higher cholesterol intake quartiles and liver-related death or transplantation remained significantly different between men and women (test for interaction, *P* value = 0.01). Again, after adjusting for potential confounders, each higher quartile of cholesterol intake was associated with an increased risk for liver-related death or transplantation in women (AHR 2.59; 95% CI 1.37, 4.88; *P*<sub>trend</sub> = 0.003), but not in men (AHR 1.00; 95% CI 0.77, 1.30; *P*<sub>trend</sub> = 0.99).

In addition to the nutrient density model, which was our primary analysis, we also used three additional models for total energy adjustment: nutrient residual, standard and energy partition (Table 5). The associations between dietary cholesterol and liver-related mortality or transplantation in women were similar across all four models, but were only statistically significant in the nutrient density and nutrient residual models. The difference between men and women in the associations of dietary cholesterol with liver-related mortality or transplantation was evident with all four models, but statistically significant interaction was present only with the nutrient density and nutrient residual models.

## Discussion

Using carefully collected prospective data from the HALT-C Trial, we have demonstrated a dose-dependent and novel sex-specific association between high dietary cholesterol intake and liver-related mortality or transplantation among women with advanced hepatic fibrosis. This adverse effect was most

significant for post-menopausal women and for those whose cholesterol intake was above the recommended intake (300 mg/d in a 8368 kJ (2000 kcal) diet) based on Dietary Guidelines for Americans 2010.

Why the risk of liver-related death or transplantation associated with dietary cholesterol intake affected only women, an important yet unexpected finding of our study, is not known. The two models, nutrient density and nutrient residual, which demonstrated statistical significance for this sex difference, are considered more robust than other methods of total energy adjustment primarily because of less residual confounding<sup>(23,24)</sup>. We suspect that using the energy-partition method of total energy adjustment partly explains why we did not see a significant sex difference in our previous analysis<sup>(13)</sup>. It is possible that the sample size of the HALT-C study was insufficient to demonstrate a similar association in male patients. Male subjects, however, constituted the majority of HALT-C patients (71%), making insufficient sample size a less likely explanation for the observed sex difference.

Mechanistic data are currently lacking to explain this sex difference. In fact, most animal studies showing hepatotoxicity from dietary cholesterol all involved males<sup>(6,7,25,26)</sup>. Female mice, however, have been demonstrated to absorb cholesterol more efficiently than male mice, possibly owing to their larger bile acid pool<sup>(27)</sup>. In the setting of a cholesterol 'challenge', female mice developed significantly more hepatic accumulation of free cholesterol than did males<sup>(27)</sup>. To our knowledge, there is no direct evidence of a sex difference in humans in terms of cholesterol absorption or hepatic cholesterol accumulation in response to dietary cholesterol. It is important to note that, on the basis of the estimated median age of menopause in the general population, 51.4 years<sup>(28)</sup>, the majority of female subjects in the current analysis were post-menopausal (median age 52 years, IQR 46–60). We suspect that the fibrogenic effect of dietary cholesterol is especially pronounced in



**Table 2.** Risk of liver-related death or transplantation in women (*n* 193) according to non-sex-specific quartiles of cholesterol intake derived from average daily cholesterol intake (measured in mg/4184 kJ (mg/1000 kcal) of daily energy intake) (Hazard ratios (HR), adjusted hazard ratios (AHR) and 95% confidence intervals)

Categories	Range (mg/4184 kJ (mg/1000 kcal) of energy) of cholesterol intake	<i>n</i>	Person-years	Liver-related death		Transplant		Incidence of liver-related death or transplant (per 1000 person-years)		HR	95% CI	AHR*	95% CI	AHR†	95% CI	AHR‡	95% CI	AHR_trend‡	95% CI	<i>P</i> _trend
				death	Transplant	death	Transplant	death or transplant												
1	31–98	66	296	0	5	1	1	16.9	1	1	1	1	1	1	1	1	1	1	1	1
2	98–118	55	262	3	3	1	3	22.9	1.37	0.42, 4.50	1.35	0.41, 4.44	1.54	0.42, 5.61	1.48	0.32, 6.88				
3	118–151	39	184	4	1	1	1	27.2	1.64	0.47, 5.66	1.59	0.45, 5.61	1.22	0.27, 5.46	1.74	0.31, 9.58				
4	151–508	33	149	7	2	2	2	60.5	3.70	1.24, 11.06	3.75	1.25, 11.3	3.89	1.16, 13.0	5.69	1.27, 25.6	1.83	1.12, 2.99	0.02	

\* Adjusted for average daily energies and age.

† Adjusted for average daily energies, age, race, BMI, diabetes, lifetime alcohol intake, smoking status, coffee intake and self-reported health status. Only women with complete data (*n* 183) were included in this multiple Cox's regression model.

‡ Adjusted for average daily total energies, age, race, BMI, diabetes, lifetime alcohol intake, smoking status, coffee intake, self-reported health status, cirrhosis status, duration of infection and peginterferon treatment group. Only women with complete data (*n* 171) were included in this multiple Cox's regression model.

**Table 3.** Risk of liver-related death or transplantation in men (*n* 464) according to non-sex-specific quartiles of dietary cholesterol intake (measured in mg/4184 kJ (mg/1000 kcal) of daily energy intake) (Hazard ratios (HR), adjusted hazard ratios (AHR) and 95% confidence intervals)

Categories	Range (mg/4184 kJ (mg/1000 kcal) of energy) of cholesterol intake	<i>n</i>	Person-years	Liver-related death		Transplant		Incidence of liver-related death or transplant (per 1000 person-years)		HR	95% CI	AHR*	95% CI	AHR†	95% CI	AHR‡	95% CI	AHR_trend‡	95% CI	<i>P</i> _trend
				death	Transplant	death	Transplant	death or transplant												
1	31–98	99	465	5	9	1	1	30.1	1	1	1	1	1	1	1	1	1	1	1	1
2	98–118	109	514	6	12	1	12	35.0	1.17	0.58, 2.36	1.26	0.62, 2.56	1.06	0.51, 2.22	1.13	0.52, 2.45				
3	118–151	125	600	10	12	1	12	36.6	1.22	0.62, 2.38	1.27	0.65, 2.49	1.05	0.51, 2.17	1.11	0.52, 2.41				
4	151–508	131	613	11	8	1	8	31.0	1.04	0.52, 2.07	1.04	0.52, 2.09	0.92	0.44, 1.93	0.90	0.41, 1.98	0.96	0.76, 1.22	0.73	

\* Adjusted for average daily energies and age.

† Adjusted for average daily energies, age, race, BMI, diabetes, lifetime alcohol intake, smoking status, coffee intake and self-reported health status. Only men with complete data (*n* 435) were included in this multiple Cox's regression model.

‡ Adjusted for average daily energies, age, race, BMI, diabetes, lifetime alcohol intake, smoking status, coffee intake, self-reported health status, cirrhosis status, duration of infection and peginterferon treatment group. Only men with complete data (*n* 415) were included in this multiple Cox's regression model.



**Table 4.** First major clinical event among ninety-eight patients who experienced liver-related death or transplantation

Categories	Range (mg/4184 kJ (mg/1000 kcal) of energy) of cholesterol intake)	First clinical event									
		Liver-related death	HCC	CTP $\geq$ 7	Variceal haemorrhage	Ascites	Bacterial peritonitis	Encephalopathy	Liver transplantation	Total (n)	
1	31–98	3	4	8	0	2	0	0	0	0	7
2	98–118	1	9	10	1	0	0	2	1	1	4
3	118–151	1	7	11	2	3	0	1	2	7	
4	151–508	3	5	17	0	1	0	3	1	0	

HCC, hepatocellular carcinoma; CTP, Child-Turcotte-Pugh.

**Table 5.** Risk of liver-related death or transplantation in men v. women according to four different models of total energy adjustment (Adjusted hazard ratios (AHR) and 95 % confidence intervals)

Models for total energy adjustment	P for the interaction term between quartiles of cholesterol intake and sex in the full model*	Men					Women						
		Non-sex-specific quartiles*		Sex-specific quartiles*			Non-sex-specific quartiles*		Sex-specific quartiles*				
		AHR	95 % CI	P	AHR	95 % CI	P	AHR	95 % CI	P	AHR	95 % CI	P
Nutrient density	0.01	0.96	0.76, 1.22	0.7	1.02	0.80, 1.28	0.9	1.83	1.12, 2.99	0.02	1.81	1.05, 3.10	0.02
Nutrient residual	0.02	0.99	0.78, 1.26	1.0	1.03	0.81, 1.30	0.8	1.83	1.13, 2.99	0.02	1.88	1.08, 3.25	0.03
Standard	0.13	1.27	0.93, 1.74	0.1	1.20	0.88, 1.65	0.2	1.79	0.93, 3.46	0.08	1.71	0.87, 3.37	0.1
Energy partition	0.23	1.40	0.98, 1.99	0.06	1.30	0.92, 1.84	0.1	1.96	0.96, 4.00	0.07	1.91	0.88, 4.13	0.1

\* All models were adjusted for age, race, BMI, diabetes, lifetime alcohol intake, smoking status, coffee intake, self-reported health status, cirrhosis status, duration of infection and peginterferon treatment group. For total energy adjustment, the first three (nutrient density, nutrient residual and standard) models included total energy intake in the multivariate model, whereas the energy-partition model included carbohydrate, protein, SFA and unsaturated fat in the multivariate model. Only men (n 415) and women (n 171) with complete data were included in this multiple Cox's regression model.

post-menopausal women as they are no longer protected by the effect of oestrogen, which promotes biliary cholesterol excretion<sup>(29)</sup>. Although we did demonstrate that the adverse effect of dietary cholesterol was restricted to women who were post-menopausal, given the small number of patients and the multiple levels of effect modification, these results require confirmation from larger studies.

The first limitation of our study is that cholesterol intake estimated from a self-reported FFQ may not accurately reflect actual cholesterol intake. Although no studies have examined misreporting of cholesterol intake using specific biomarkers, total energy intake, which correlates with cholesterol intake in our study, has been found to be under-reported in both men and women employing doubly labelled water, the gold standard for estimating total energy expenditure<sup>(30)</sup>. To our knowledge, there is no statistical method that can account for this misreporting. If, on the other hand, the pattern of misreporting of cholesterol was random in the HALT-C cohort, the association we have demonstrated is likely real because random or non-differential misclassification of exposure typically reduces true associations towards the null<sup>(31)</sup>. Future research on the relationship between diet and HCV infection should characterise the pattern of misreporting by incorporating validation and calibration studies that compare the FFQ with other dietary assessment tools – for example, multiple dietary recalls, or, preferably, biomarkers such as doubly labelled water and urinary N excretion<sup>(32)</sup>.

The second limitation of our study is that the relationship between cholesterol intake and liver-related mortality or transplantation may be confounded by other factors, despite our extensive adjustments. The most obvious potential confounders are other dietary factors. Although we adjusted for total energy intake, to what extent other nutrients confound the observed association between categorised cholesterol intake and liver-related mortality is unknown. One known example is dietary fructose, which has also been implicated as a cofactor in HCV pathogenesis<sup>(33)</sup>. The HALT-C database unfortunately did not contain an estimate on fructose intake. The issue of residual confounding can only be minimised with a randomised controlled study. Finally, we cannot exclude the possibility that our results represent a chance finding, an important consideration for most non-experimental studies.

In conclusion, we hope that the results of our study will generate awareness and interest in the clinical significance of dietary cholesterol not only in HCV infection but in liver disease in general. Although new antiviral treatments have significantly improved the HCV clearance rate<sup>(34,35)</sup>, we believe that recognising the potential adverse effect of excessive dietary cholesterol will be important for the following reasons: (1) the fibrogenic effect of dietary cholesterol does not require the presence of HCV<sup>(7,25,26)</sup>; (2) HCV eradication does not eliminate the risk of HCC development<sup>(36)</sup>; (3) the cost of delivering state-of-the-art antiviral therapy is limiting the number of patients who receive treatment<sup>(37)</sup>; (4) the majority of HCV-infected individuals have not received treatment and are unlikely to be cured for years<sup>(38)</sup>. It is tempting to speculate that reducing dietary cholesterol intake, a low-risk and low-cost intervention, could reduce liver-related mortality or transplantation in

HCV-infected women whose cholesterol intake is above the current recommendation (300 mg/d in a 8368 kJ (2000 kcal) diet). Confirmation of this strategy will require investigation of the sex-specific mechanisms of cholesterol-associated hepatotoxicity with and without HCV infection, as well as well-designed diet intervention trials.

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## Supplementary material

For supplementary material/s referred to in this article, please visit <http://dx.doi.org/doi:10.1017/S0007114515004158>

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