



# Endotoxemia contributes to steatosis, insulin resistance and atherosclerosis in chronic hepatitis C: the role of pro-inflammatory cytokines and oxidative stress

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## Abstract

**Purpose** Endotoxin is a component of the outer membrane of gram-negative bacteria that live in the intestine. Endotoxemia is reported in non-alcoholic fatty liver disease and in cirrhotic patients, causing various biological and clinical effects in the host. It is not known whether endotoxemia occurs in chronic hepatitis C patients (CHC), therefore we evaluated the occurrence of endotoxemia and its effect on inflammation, liver damage, insulin resistance (IR) and atherosclerosis.

**Methods** Consecutive CHC patients assessed by liver biopsy were enrolled. Endotoxemia was evaluated by LAL test. IR was estimated by HOMA-IR. Serum TNF- $\alpha$ , IL-8, adiponectin and MCP-1 were measured with ELISA tests. Oxidative stress was estimated by circulating IgG against malondialdehyde adducts with human serum albumin (MDA-HAS). Carotid atherosclerosis was assessed by ultrasonography.

**Results** Endotoxemia was found in 60% of the 126 patients enrolled. A serum level-dependent association between endotoxemia, steatosis ( $p < 0.001$ ) and HOMA-IR ( $p < 0.006$ ) was observed. Patients with endotoxemia showed significant increase in TNF- $\alpha$  and IL8 levels. TNF- $\alpha$  correlated with steatosis ( $p < 0.001$ ) and HOMA-IR ( $p < 0.03$ ), whereas IL8 correlated with steatosis ( $p = < 0.001$ ), TNF- $\alpha$  ( $p < 0.04$ ) and atherosclerosis ( $p < 0.01$ ). The highest levels of endotoxemia were associated with oxidative stress and a higher prevalence of carotid atherosclerosis. Multivariate logistic regression analysis showed that the independent factors associated with endotoxemia were hepatic steatosis, HOMA-IR, IL8 and MDA-HAS.

**Conclusions** Endotoxemia occurs with high frequency in CHC patients and contributes to the development of hepatic steatosis, IR and atherosclerosis through increased pro-inflammatory cytokines and oxidative stress. Anti-endotoxin treatment could be of clinical relevance.

**Keywords** Endotoxin · Chronic hepatitis C · Steatosis · Insulin resistance

## Introduction

Endotoxin or lipopolysaccharides (LPS) is a component of the outer membrane of gram-negative bacteria and the intestine is a large pool of LPS in the body. A small amount

of LPS can escape from the intestine and through the portal vein reach the liver where it is cleared from the reticuloendothelial system (RES), avoiding a spillover into the systemic circulation. However, endotoxemia can occur in various clinical conditions such as chronic hepatitis, cirrhosis, non-alcoholic fatty liver disease (NAFLD) and obesity. Endotoxemia is known to induce various biological effects in the host involving the liver, cardiovascular, metabolic and neurologic systems [1].

In cirrhotic patients, endotoxemia may be the result of a decrease in the phagocytic capacity of the liver, a leakage through portosystemic shunts and an increased adsorption of endotoxin due to amplified intestinal permeability [2, 3]. However, irrespective of cirrhosis, endotoxemia may be a

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consequence of a high-fat diet intake either through direct diffusion via intestinal paracellular permeability or through absorption by enterocytes during the secretion of chylomicron [4], as well as the result of excessive intestinal production by an altered microbiota.

Human studies have shown that endotoxemia occurs in both adult and pediatric patients with NALFD [5, 6], promoting an increase in the serum TNF- $\alpha$  level that aggravates insulin resistance (IR) and liver steatosis [7], and it is claimed that this condition can promote weight gain, the development of NASH, type 2 diabetes mellitus (T2DM) and atherosclerosis [8].

Liver steatosis and IR are features of chronic hepatitis C infection [9] and are of clinical relevance both in the progression of liver injury and in the development of extrahepatic manifestations [10] and, in particular, T2DM and atherosclerosis [11]. Therefore, it is of clinical and therapeutic interest to know whether endotoxemia is a condition that occurs in chronic hepatitis C and whether it may have a role in the development of HCV-associated conditions such as steatosis, IR, chronic inflammation and atherosclerosis.

Accordingly, the aim of this study was to assess in a series of consecutive patients with liver biopsy proven chronic hepatitis C the prevalence and effects of endotoxemia and its role in: (a) the progression of hepatic histological damage (steatosis, necroinflammatory activity, fibrosis), (b) the development of IR, (c) the production of pro-inflammatory cytokines, oxidative stress and chemokines and, (d) the development of atherosclerosis.

## Materials and methods

### Patients

Consecutive treatment-naïve chronic hepatitis C patients who underwent liver biopsy for diagnostic purposes were enrolled in this study. Patients with the following conditions were excluded from the study: co-infection with HBV or HIV, alcohol intake greater than 20 g/day, active drug user and with severe comorbidities such as cardiac, pulmonary, renal, rheumatologic, autoimmune and inflammatory gastrointestinal diseases. None of the patients received steatogenic drugs, antibiotics or probiotics. Patients with urinary tract infection or sepsis as evaluated by urine and blood culture and in general subjects with fever were not included in the study.

At the time of the liver biopsy, a liver ultrasound scan was performed, epidemiological and demographic data were obtained and BMI ( $\text{kg}/\text{m}^2$ ) was calculated for all patients. At that time, serum samples were obtained and stored at  $-70\text{ }^\circ\text{C}$  in pyrogen-free vials and in all patients, hepatic

and renal biochemical tests and blood cells count were determined.

Patients were assessed for carotid intima-media thickness (IMT) using high-resolution B-mode ultrasonography (Esaote Techos, Genova, Italy) equipped with a 7.5 MHz linear-array transducer as previously described [10]. IMT  $> 1$  mm was regarded as a cut-off value for carotid atherosclerosis (CA). Plaque was defined as a protrusion in the lumen of the vessel of at least 1.5 mm, as measured from the border between the adventitia and median layers.

All patients signed an informed consent and the study protocol in accordance with the ethical guidelines of the 1975 Helsinki Declaration was approved by the Local Ethics Committee.

### Viral markers

The anti-HCV antibodies were tested by ELISA (Abbott Laboratories, Chicago, IL) and HCV RNA was tested by PCR assay (Amplicor HCV; Roche Diagnostic SpA). HCV RNA serum levels were measured by real-time PCR (Taqman, Applied BioSystems). HCV genotyping was performed using a reverse hybridization line probe assay (INNO LiPA HCV assay, 2nd generation, Innogenetics, Zwijndrecht, Belgium) following the manufacturer's protocol.

Hepatitis B markers and HIV antibodies were assayed using commercially available ELISA tests.

### Histology evaluation

Liver histology was evaluated on liver tissue  $> 1.5$  cm through formalin-fixation, followed by paraffin embedment and staining with hematoxylin–eosin, trichrome and Prussia blue. “Grading” and “staging” were assessed separately, according to Ishak et al [12]. Different scores were assigned to different levels of steatosis: 0,  $< 5\%$  of steatosis; 1, 5–10% of hepatocytes with steatosis; 2, 11–30%; 3, 31–60%; 4,  $> 60\%$  of hepatocytes with steatosis.

### Endotoxemia

Quantification of serum LPS was obtained by Limulus Amebocyte Lysate (LAL) with end-point chromogenic method (LAL test, Cape Cod Incorporated/PBI). The cut-off of the test for positivity was calculated using a mean value of 30 healthy controls plus 2 times the standard deviation and was fixed at 0.78 UE/ml.

### Insulin resistance

Serum insulin levels were assayed with a radioimmunoassay method (Insulin RIA DSL-1600, Diagnostic System Laboratories, USA). The intra- and inter-assay coefficients of

variation ranged from 4.5 to 8.3% and from 4.7 to 12.2%, respectively. The percentages of insulin recovery in analyzed samples ranged from 92 to 119%. In each subject, the degree of insulin resistance was estimated by HOMA-IR using the following formula: [fasting plasma glucose (mmol/L) x fasting serum insulin (mIU/L)]/22.5.

### Cytokines and chemokines

Serum TNF- $\alpha$  and IL-8 were measured by ELISA test, using the Human ELISA kit (Pierce Biotechnology, Rockford, IL); serum adiponectin levels using the Human Adiponectin ELISA kit (B-Bridge International, Osaka, Japan); MCP-1 was measured using a Human MCP-1 ELISA Kit (Thermo Scientific™ Pierce™).

### Measurement of the antibody titers against lipid peroxidation-derived antigens

Oxidative stress was measured by circulating IgG against malondialdehyde adducts with human serum albumin (MDA-HSA) in HCV-infected patients and in 70 healthy controls. Serum human albumin (HSA) adducts with malondialdehyde (MDA) were prepared as previously described [13]. The enzyme-linked immunosorbent assay (ELISA) was performed with patient's serum as previously reported [13]. The results were expressed by subtracting the background reactivity observed with unmodified HSA. Immunological evidence of oxidative stress was considered when the value of anti-MDA-HSA IgG levels exceeded the 95th percentile of the control group (median value, o.d. 490 nm,  $0.126 \pm 0.052$ ).

### Statistical analysis

Values are expressed as the mean  $\pm$  standard deviation (SD) or median and range. The mean differences between the cases and controls were evaluated by the Student's *t* test for parametric data. The Mann–Whitney U test was used to compare non-parametric data between groups. The Chi square test was used to test differences between proportions. Spearman correlation was used to evaluate the association between endotoxin and other variables such as age, sex, BMI, visceral obesity, glycaemia, HOMA-IR, HCV genotype, serum HCV RNA levels, TNF- $\alpha$ , IL8, adiponectin, MCP-1, hepatic steatosis, HAI, fibrosis and atherosclerosis. The variables significantly associated at univariate analysis were evaluated in the multivariate analysis using a logistic regression analysis model. Statistical analysis was performed using the SPSS software for Windows (Version 17.0) (Chicago, IL, USA) and a *p* value  $< 0.05$  was considered significant.

## Results

### Patient's characteristics and prevalence of endotoxemia

One hundred and twenty-six consecutive chronic hepatitis C patients who underwent liver biopsy were enrolled in this study. Eight patients (6% of the entire eligible population) were not included in the study for one of the aforementioned causes. The median age was 55 years (range 18–68 years), males were 56%, steatosis was present in 54%, cirrhosis in 10% and type 2 diabetes in 10.3% of cases. Carotid atherosclerosis was present in 49.2% of patients. Endotoxemia was found in 76 (60%) patients. Serum anti-MDA-HSA IgG levels, as a marker of oxidative stress-mediated responses, were significantly higher in CHC patients than in healthy controls (o.d 490 nm:  $0.228 \pm 0.10$  vs  $0.126 \pm 0.052$ ;  $p < 0.001$ , respectively) and the overall prevalence of CHC patients with immunological evidence of oxidative stress (anti-MDA-HSA IgG levels above the 95th percentile of the control group) was 59%. Table 1 shows the characteristics of patients in relation to the presence or absence of serum endotoxin.

### Endotoxemia, steatosis and IR

Data showed that patients with endotoxemia had significantly higher levels of steatosis ( $p < 0.01$ ; Table 1). Similarly, a significant difference between the two groups was present for HOMA-IR ( $p = 0.05$ ). Such differences were irrespective of type 2 diabetes and/or cirrhosis (data not shown). The correlation test showed a significant association between LAL and steatosis ( $r = 0.475$ ;  $p < 0.001$ ) and HOMA-IR ( $r = 0.025$ ;  $p < 0.006$ ).

Considering that the endotoxin effects are reported to be dose-dependent [2], an analysis based on the different serum endotoxin levels was performed. Table 2 shows the analysis of quartile-based data of endotoxemia and shows a statistically significant difference between the first and fourth quartile in steatosis score ( $p = 0.001$ ), and HOMA-IR ( $p = 0.001$ ).

### Endotoxemia, inflammatory cytokines and oxidative stress

Endotoxemia was associated with increased systemic inflammation as indicated by higher levels of TNF- $\alpha$  and IL8, whereas it was not correlated with MCP-1 levels (Table 1). Table 2 shows the levels of cytokines in relation to quartile endotoxemia levels. The data showed an increase in serum TNF- $\alpha$  and IL8 levels with the progressive increase in endotoxin levels. Circulating IL8

**Table 1** Characteristics of the patients according to the presence or absence of endotoxemia

Variables	Endotoxemia		
	Absent < 0.78 EU/ml	Present > 0.78 EU/ml	<i>p</i>
No. of patients	50 (40%)	76 (60%)	
Age, median (range)	53 (20–68)	55 (18–64)	Ns
Male (%)	25 (50%)	46 (60%)	Ns
ALT U/L, median (range)	88 (46–461)	93.5 (40–433)	Ns
HCV RNA, UI/ml × 10 <sup>5</sup> median (range)	4.43 (0.8–56)	7.42 (0.1–32)	Ns
Cholesterol mg/dl, mean ± SD	173 ± 36	175 ± 39	Ns
Triglycerides mg/dl, mean ± SD	115 ± 107	103 ± 40	Ns
Glucose mg/dl, median (range)	93 (68–159)	93 (71–201)	Ns
Insulin μU/ml, median (range)	20.9 (6.5–47.8)	22.9 (6.4–96.4)	Ns
HOMA-IR, mean ± SD	4.3 ± 2.6	5.5 ± 2.9	0.05*
Type 2 diabetes	10%	11.4%	Ns
TNFα pg/ml, median (range)	12.0 (2.5–25)	51 (6.25–736)	0.016**
ADP, U/L median (range)	9.94 (3–12)	8.24 (2.6–26)	Ns
Serum IL8, (pg/ml), mean ± s.d	10.46 ± 3.7	24.02 ± 19.8	0.001**
Serum MCP-1, (pg/ml), mean ± s.d	142.34 ± 39	149.15 ± 42	Ns
MDA, mean ± SD	0.21 ± 0.08	0.24 ± 0.12	Ns
Steatosis score, mean ± s.d	1.2 ± 1.2	2.1 ± 1.4	0.01
HAI score, median (range)	6.0 (1–11)	6.2 (1–13)	Ns
Fibrosis score, mean ± s.d	2.6 ± 1.1	2.6 ± 1.2	Ns
Cirrhosis	12%	10%	Ns
Prevalence of carotid atherosclerosis+	48%	54.2%	Ns

+IMT increased plus plaque; \*Student's *t* test; \*\*Mann–Whitney *U* test

**Table 2** Distribution of variables according to the quartiles of endotoxemia values as evaluated by the LAL test

Quartiles	1st	2nd	3rd	4th	<i>p</i>
Serum LPS concentration	< 0.60 EU/l	0.61–1.20 EU/l	1.21–2.20 EU/l	> 2.20 EU/l	
Steatosis score mean ± SD	0.8 ± 1.1	1.6 ± 1.5	2.0 ± 1.4	2.4 ± 1.2*	0.001
HOMA-IR mean ± SD	3.9 ± 2.3	4.38 ± 3.3	4.76 ± 1.93	6.37 ± 3.2*	0.005
TNF—pg/ml median (range)	10.9 (2.5–25)	13 (2.6–525)	25.5 (6.2–497)	90.7 (6–736)*	0.03
Serum IL8 (pg/ml) mean ± SD	10.7 ± 3.7	M 26.1 ± 19.1	M 24.3 ± 8.1	29.5 ± 22.6	0.001+
Serum MCP-1 (pg/ml) mean ± SD	130.9 ± 37	M 153.4 ± 59.7	M 144 ± 47.7	157 ± 42.2	Ns
MDA mean ± SD	0.20 ± 0.77	0.19 ± 0.07	0.20 ± 0.07	0.26 ± 0.12*	0.025
HAI score median (range)	5 (1–11)	5 (1–10)	6 (1–13)	6 (1–13)	Ns
Fibrosis score mean ± SD	2.3 ± 1.1	2.4 ± 1.2	2.4 ± 1.1	2.5 ± 1.3	Ns

\*Quartile 4 vs quartile 1

+Quartiles 2–4 vs 1

correlated with steatosis ( $r = 0.632$ ,  $p = 0.001$ ), LAL ( $r = 0.681$ ,  $p = 0.001$ ) and TNF- $\alpha$  ( $r = 531$ ,  $p = 0.042$ ), whereas serum TNF- $\alpha$  levels correlated also with steatosis ( $r = 0.291$ ;  $p < 0.001$ ) and HOMA-IR ( $r = 0.129$ ;  $p < 0.029$ ). On the contrary, MCP-1 was not associated with any of the parameters evaluated, regardless of the levels of endotoxemia. The highest levels of endotoxemia were also associated with a significant increase in anti-MDA IgG levels (Table 2), indicating that high levels of endotoxemia are associated with oxidative stress.

### Endotoxemia as a risk factor of atherosclerosis

Chronic HCV infection is known to be associated with atherosclerosis [11]. In this context, a pro-inflammatory environment and oxidative stress was proposed to play an important role in explaining this association [14, 15]. Therefore, based on the aforementioned data, we investigated the possible role of endotoxemia in modulating the risk of atherosclerosis among HCV patients. The overall prevalence of atherosclerosis (increased IMT plus plaque) was no different

**Table 3** Prevalence of atherosclerosis (increased IMT or plaque) according to the median value of endotoxemia

	Endotoxineamia		<i>p</i>
	< 1.21 UE/l (%)	> 1.21 UE/l (%)	
Increased IMT	56.5	70.0	
Plaque	17.4	40.0	0.002

**Table 4** Independent factors associated with all endotoxemia using all values\* or using the highest values of serum endotoxemia levels (4th quartile) \*\*

	Coefficient B std. error		95% CI		<i>p</i>
			Lower	Higher	
Steatosis*	0.243	0.082	0.080	0.406	0.004
IL8*	0.124	0.064	0.042	0.628	0.022
Homa-IR**	0.030	0.015	0.001	0.059	0.046
MDA**	0.272	0.102	0.069	0.475	0.001

between patients with and without endotoxemia (Table 1). However, as shown in Table 3, when the data were analyzed using the median value of endotoxemia as cut-off, a higher prevalence of both increased IMT and plaque were observed in the third and fourth quartiles compared to that observed in the first and second quartiles ( $p < 0.002$ ). A correlation was also found between IL8 and the presence of atherosclerosis ( $r = 0.34$ ,  $p < 0.01$ ).

### Factors independently associated with endotoxemia

An overall analysis of the variable independently associated to the serum endotoxin was evaluated in the multivariate logistic regression model and showed that hepatic steatosis and IL8 were independently associated with endotoxemia. However, as seen in Table 4, when the analysis was performed using the highest serum endotoxin levels (4th quartiles), HOMA-IR and MDA also became independent variables.

### Discussion

In this study we have shown that, regardless of liver cirrhosis, endotoxemia is a condition that occurs with high frequency (60%) in our series of chronic hepatitis C patients and that patients with endotoxemia, especially those with the highest serum LPS concentrations showed significantly higher levels of pro-inflammatory cytokines and oxidative stress and a significant association with

higher levels of hepatic steatosis, IR and prevalence of atherosclerosis. These data support the concept that by increasing the levels of pro-inflammatory cytokines and oxidative stress, endotoxemia contributes to the development of hepatic and extrahepatic manifestations associated with HCV such as fatty liver, IR and atherosclerosis.

In experimental models, LPS has been shown to play a role in the increase of IR, hepatic steatosis and inflammation [16]. Most of the biological effects of LPS have been related to the release of pro-inflammatory cytokines, such as TNF- $\alpha$ , interleukins (IL-1, IL-6, IL-8 and IL-12), chemokines (MPC-1) and oxidative stress [17]. Kupffer's liver cells, through their receptors for LPS, toll-like receptors (TLRs), are the primary cells involved in the LPS response producing inflammatory cytokines [18]. TNF- $\alpha$ , which is the main cytokine induced by LPS-TLR4, is recognized as the main mediator of hepatotoxicity, inflammation and development of IR and steatosis [19].

Endotoxemia has been reported in patients with NAFLD [5, 6], promoting the development of NASH and a worsening of IR [8]. These effects are at least partly related to the increase in intestinal permeability [20], with subsequent translocation of LPS into the portal vein, activating the inflammatory phenotype of Kupffer cells [21] and promoting an inflammatory response [22, 23]. In particular, among the mediators of inflammation (adipocytokines), TNF $\alpha$  plays an important role in the pathogenesis of NAFLD/NASH and IR [24, 25].

The role of the imbalance between TNF- $\alpha$  and adiponectin is known in the pathogenesis of IR in chronic hepatitis C [26, 27], and the release of free fatty acids from peripheral sites to the liver could be a fascinating hypothetical mechanism contributing to steatosis and IR development in chronic hepatitis C. Furthermore, it has been shown that endotoxin, associated with HCV core protein, is able to promote inflammation in chronic hepatitis C by activating monocyte/macrophage [28].

Our study showed that endotoxemia occurs with high frequency in chronic hepatitis C patients and is level-dependent associated with increased levels of TNF- $\alpha$ , which, in turn, contribute to the development of IR and fatty liver deposition. In this study, we were unable to demonstrate an increase in necroinflammatory damage and hepatic fibrosis associated with endotoxemia. However, in previous experimental and clinical studies, the presence of endotoxemia has been associated with an increase in necroinflammatory hepatic activity and at an advanced stage of liver disease [2, 21, 29]. Moreover, considering that the role of hepatic steatosis and IR is well-known in the progression of liver disease leading to fibrosis [9], it is therefore possible that endotoxin, modulating steatosis and IR, may contribute to the development of liver fibrosis and type 2 diabetes in the long term.



Among the biological responses of endotoxemia, oxidative stress is one of the significant effects implicated in hepatic, cardiovascular and metabolic damage [30]. Oxidative stress is a condition observed with high frequency in chronic hepatitis C and contributes to the development and progression of liver injury as well as liver in the fat deposition, IR and cardiovascular damage [9, 13, 31]. Data from this study showed that HCV patients with the highest levels of endotoxemia showed an increase in levels of MDA-HAS as an expression of chronic oxidative stress. Therefore, by causing oxidative stress, endotoxemia contributes to the hepatic and metabolic damage associated with chronic hepatitis C and in particular steatosis, IR and cardiovascular damage.

Atherosclerosis is known to be associated with chronic HCV infection [11] and both direct and indirect mechanisms have been proposed [32]. Among the indirect mechanisms, a pro-inflammatory environment has been reported in which pro-inflammatory cytokines and oxidative stress play an important role [15]. LPS through TLR4 has been reported as one of the triggers of the inflammatory pathway in the generation of atherosclerosis [33]. Furthermore, oxidative stress contributes to the inflammatory and metabolic processes that generate and maintain atherosclerosis [34, 35]. In this study, patients with a significant amount of endotoxemia showed a higher prevalence of carotid atherosclerosis in association with pro-atherogenic conditions such as hepatic steatosis, IR, oxidative stress and pro-inflammatory cytokines. Among the latter, in addition to TNF- $\alpha$ , we demonstrated a significant increase in IL-8 but not MPC-1. IL-8 is mainly monocyte and macrophage secreted and its main effect is the recruitment and activation of monocytes and neutrophils in an acute site of inflammation [36]. Furthermore, IL-8 is involved in the regulation of the oxidant gene expression and is highly sensitive to oxidants [37]. The role of oxidants in the regulation of IL-8 and other chemokines has relevance in the field of cardiovascular disease [38]. There is a body of evidence that IL-8 plays an important role in atherosclerosis [38]. IL-8 is involved in the adhesion phase of monocytes in the early stages of atherosclerosis [39], but it plays a role also in the advanced phase of atherosclerosis [40]. Furthermore, LPS is known to release the pro-inflammatory cytokine IL8 from monocytes. Data from our study indicate that in chronic hepatitis C patients, significant levels of endotoxemia contribute to the development of atherosclerosis through increased IL8 secretion as well as by oxidative stress, steatosis and IR.

Treatment with direct antiviral agents (DAAs) against HCV is now able to achieve a sustained virological response (SVR) in more than 95% of patients treated; however, histological liver damage and systemic involvement remain and will probably diminish only over the years. Under experimental and clinical conditions, a decrease in endotoxemia

levels by antibiotic or probiotic treatment has been shown to lead to improvement of the associated conditions [41]. Thus, anti-endotoxin treatment in chronic hepatitis C patients with endotoxemia could be valuable for improving steatosis, IR and atherosclerosis.

In conclusion, the data from this study indicate that endotoxemia in chronic hepatitis C occurs with high frequency and is of clinical relevance, influencing, with cross-reaction mechanisms, IR, systemic inflammation, oxidative stress, hepatic steatosis and atherosclerosis. Control of serum endotoxin levels could be useful also in patients with SVR by DAA, slowing the progression of hepatic and extrahepatic manifestations and perhaps improving their long-term prognosis.

## Compliance with ethical standards

**Conflict of interest** There are none to declare.

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