

Endotoxemia Is Associated With an Increased Risk of Incident Diabetes

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OBJECTIVE—Diabetes is accompanied with a chronic low-grade inflammation, which may in part be mediated by endotoxins derived from Gram-negative bacteria.

RESEARCH DESIGN AND METHODS—We investigated in a population-based cohort whether endotoxemia is associated with clinically incident diabetes. The serum endotoxin activity was measured by limulus assay from the FINRISK97 cohort comprising 7,169 subjects aged 25–74 years and followed up for 10 years.

RESULTS—Both the subjects with prevalent diabetes ($n = 537$) and those with incident diabetes ($n = 462$) had higher endotoxin activity than the nondiabetic individuals ($P < 0.001$). The endotoxin activity was significantly associated with increased risk for incident diabetes with a hazard ratio 1.004 (95% CI 1.001–1.007; $P = 0.019$) per unit increase resulting in a 52% increased risk ($P = 0.013$) in the highest quartile compared with the lowest one. The association was independent of diabetes risk factors: serum lipids, γ -glutamyl transferase, C-reactive protein, BMI, and blood glucose. Furthermore, the association of endotoxemia with an increased risk of incident diabetes was independent of the metabolic syndrome as defined either by the National Cholesterol Educational Program-Adult Treatment Panel III or the International Diabetes Federation. Endotoxin activity was linearly related ($P < 0.001$) to the number of components of the metabolic syndrome.

CONCLUSIONS—Both prevalent and incident diabetes were associated with endotoxemia, which may link metabolic disorders to inflammation. The results suggest that microbes play a role in the pathogenesis of diabetes.

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Endotoxin (lipopolysaccharide [LPS]) is one of the potent virulence factors of Gram-negative bacterial species and has a major role in both acute and chronic infections. This unique glycolipid is located at the outer membrane of the bacteria, but in the circulation system 80–97% of it is bound to the lipoproteins (1). LPS may be cleared from the circulation system mainly by HDLs, which also neutralize its activity effectively.

Circulating endotoxin may derive from bacteria causing either overt acute infections or common chronic conditions. Additionally, endotoxin is believed to translocate from microbiota in the gut.

Experiments in animal models kept in germ-free environments have shown that endotoxin is associated with cardiometabolic abnormalities including obesity, insulin resistance, and diabetes (2). Endotoxin activates both adaptive and innate immune systems characterized by a release of antibodies, cytokines, and other inflammatory mediators, which may promote hepatic insulin resistance. Treatment of rats with an antibiotic specifically targeted against Gram-negative bacteria reduces macrophage tumor necrosis factor- α expression and hepatic steatosis (3).

In humans, energy-enriched diets increasing weight gain and insulin resis-

tance associate with absorption of endotoxin from the gastrointestinal track (4–6). This “metabolic endotoxemia” (7) resulting from the increased intestinal permeability/motility may lead to low-grade inflammation. Severity of inflammation may depend on a complex interplay between specific proteins, receptors, and lipoproteins that mediate the endotoxin bioactivity and metabolic fate.

In two small case-control studies, circulating LPS was higher in both type 1 and type 2 diabetic subjects compared with nondiabetic subjects (8,9). High serum LPS activity also associates with the development of diabetic nephropathy in patients with type 1 diabetes (10). It has been hypothesized that bacterial endotoxins can act as triggers, linking inflammation to metabolic syndrome and thereby diabetes (4,11,12). Studies of this association in humans, however, are scarce as a result of the methodological challenges. Therefore, we investigated in a large population-based cohort followed up for 10 years, if endotoxemia is associated with clinically incident diabetes.

RESEARCH DESIGN AND METHODS

The FINRISK97 involved a population-based sample ($n = 7,169$) of 25- to 74-year-old participants of the survey, which was conducted in five geographical areas in Finland (13). The survey included a self-administered questionnaire and a clinical examination with weight, height, and blood pressure measurements as well as blood drawing. The study was approved by the Ethics Committee of the National Public Health Institute and conducted according to the Helsinki Declaration. All subjects gave written informed consent.

Laboratory analyses

Before blood sampling, the participants were asked to fast for 4 h and to avoid heavy meals earlier during the day. The median fasting time was 5 (interquartile range 3–7) hours. All laboratory measurements, except serum glucose (14), were carried out at the Disease Risk Unit in the National Institute for Health and Welfare, Helsinki. Lipids and

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γ -glutamyltransferase (GGT) measurements were performed from fresh serum samples on clinical chemistry analyzers: Olli-C (Thermo Scientific, Vantaa, Finland) in 1997 and ultrasensitive C-reactive protein (CRP) from frozen samples (-70°C) on Architect c8000 analyzer (Abbott Laboratories, Abbott Park, IL) in 2005. Total cholesterol, triglyceride, and GGT concentrations were determined enzymatically using commercial reagents (Thermo Scientific). HDL cholesterol concentration was measured after precipitation of apolipoprotein B-containing lipoproteins with dextran sulfate and magnesium chloride. CRP was measured using a latex immunoassay (Sentinel diagnostics, Milan, Italy). LDL cholesterol concentrations were calculated according to the Friedewald formula. For standardizing the measurements, the laboratory has taken part in the World Health Organization Lipid Reference Program (Prague) and External Quality Assessment Schemes organized by Labquality (Helsinki, Finland).

Serum endotoxin activities were determined by kinetic Limulus Amebocyte Lysate (LAL) test kit with a chromogenic substrate (HyCult biotechnology b.v., Uden, the Netherlands) on diluted (1:5, vol/vol in endotoxin-free water) samples. The interassay coefficient of variation was 9.2% ($n = 75$).

Diabetes at baseline and follow-up

Baseline diabetes was defined either from 1) the questionnaire as a doctor-diagnosed disease or impaired glucose tolerance, 2) the drug reimbursement records of purchases of hypoglycemic drugs (ATC-class A10) from the Social Insurance Institution of Finland (SII), 3) the SII records of patients entitled to reimbursed diabetes medication, or 4) the National Hospital Discharge Register for hospitalizations with diabetes (E10-E14/ICD-10; 250/ICD-9) as the main or additional diagnosis.

The follow-up of the cohort was until the end of the year 2007. The cases with incident diabetes were identified from 1) the drug reimbursement records of hypoglycemic drugs (ATC-class A10) from SII, 2) the SII records of patients entitled to reimbursed diabetes medication, 3) the National Hospital Discharge Register for hospitalizations with diabetes (ICD-codes as above), and 4) the National Causes-of-Death Register with diabetes (ICD-codes as above) as the underlying, direct or contributing cause of death.

Table 1—Baseline risk factors of the subjects with and without incident diabetes

	No diabetes ($n = 6,170$)	Incident diabetes ($n = 462$)	P^*
	Mean (SD)		
Age (years)	53.2 (11.0)	57.3 (9.4)	<0.001
Education (years)	10.8 (4.0)	9.4 (3.5)	<0.001
Cholesterol (mmol/L)	5.7 (1.0)	5.9 (1.1)	<0.001
HDL cholesterol (mmol/L)	1.40 (0.36)	1.21 (0.33)	<0.001
Triglycerides (mmol/L)†			
Determined	1.48 (0.95)	2.19 (1.30)	<0.001
Corrected for fasting time	1.34 (0.85)	2.01 (1.18)	<0.001
LDL cholesterol (mmol/L)	3.61 (0.91)	3.81 (0.94)	<0.001
G-GT (U/L)†	36.4 (58.6)	58.1 (112)	<0.001
CRP (mg/L)†	2.47 (5.17)	4.57 (8.02)	<0.001
Glucose (mmol/L)			
Determined ($n = 5,691$)	5.06 (0.63)	5.84 (1.92)	<0.001
Missing values replaced ($n = 6,632$)	5.08 (0.51)	5.59 (1.58)	<0.001
Endotoxin (EU/mL)†	61.06 (36.11)	77.03 (42.03)	<0.001
BMI (kg/m^2)	26.7 (4.1)	31.6 (5.2)	<0.001
Waist (cm)	89.0 (12.7)	102.9 (13.3)	<0.001
Waist-to-hip ratio	0.87 (0.09)	0.94 (0.09)	<0.001
Systolic blood pressure (mmHg)‡	138.3 (20.4)	148.3 (20.0)	<0.001
Diastolic blood pressure (mmHg)‡	83.8 (10.8)	88.7 (11.5)	<0.001
	n (%)		$P\&$
Male	2,962 (50.5)	261 (60.1)	<0.001
Female	2,900 (49.5)	173 (39.9)	
Smoking			
Never	3,321 (53.8)	234 (50.6)	0.374
Former	1,507 (24.4)	117 (25.3)	
Current	1,342 (21.8)	111 (24.0)	
Hypertensive	3,080 (50.0)	341 (73.8)	<0.001
Family history of diabetes	1,278 (20.7)	129 (27.9)	<0.001
Free-time physical inactivity	1,271 (20.6)	169 (36.6)	<0.001
Metabolic syndrome (ATP-III)	1,175 (20.1)	269 (62.0)	<0.001
Metabolic syndrome (IDF)	1,455 (24.9)	287 (66.1)	<0.001

* t test. †Log transformation before comparisons. ‡Mean value of two measurements. § χ^2 test.

Definitions of metabolic syndrome

The nondiabetic subjects were classified into those with and without metabolic syndrome using two definitions: 1) the National Cholesterol Educational Program-Adult Treatment Panel III (ATP III) (15) and 2) the International Diabetes Federation (16). Missing glucose concentrations ($n = 941$, 14%) were substituted with the mean observed value, 5.12 mmol/L, and used in the calculations of the prevalence of metabolic syndrome.

Statistics

The statistical significance of the differences between the subjects with and without diabetes was tested with t test or χ^2 test. The values with skewed distribution, serum triglyceride and CRP concentrations, and endotoxin activities, were log-transformed before comparisons.

Bivariate correlations were examined by a two-tailed Pearson correlation analyses. Smoking habit was used in three categories: never, former, and current. Hypertension was defined according to the American Heart Association definition, if the systolic or diastolic blood pressure exceeded 140 and 90 mmHg, respectively, or if the subject was on medication for hypertension. Years of education was used to describe the socioeconomic status. Family history of diabetes (either the mother or the father) and free-time physical inactivity (level 1 on a scale of 1–4) were obtained from the questionnaires. The association of serum endotoxin activity with prevalent diabetes was analyzed using a logistic regression model adjusted for age and sex.

The association of endotoxin activity with incident diabetes was analyzed using

Cox regression model adjusted first for age and sex, then for diabetes risk factors (education, smoking, hypertension, family history of diabetes, free-time physical inactivity, and BMI, as well as serum cholesterol, triglyceride, HDL cholesterol, GGT, and CRP concentrations), and further for serum glucose concentrations (determined values only, $n = 5,691$). Both semifasting serum triglyceride concentrations determined and calculated fasting triglyceride concentrations (17) were accounted for in separate models. In another Cox model, metabolic syndrome defined as described above was included as a covariate together with age, sex, smoking habit, education, family history of diabetes, free-time physical inactivity, and GGT and CRP concentration. Linear trend of endotoxin activity versus number of positive metabolic syndrome parameters was tested using ANOVA with linear contrasts and assuming equal variances. The statistical analyses were performed using SPSS 15.0.

RESULTS—The FINRISK97 cohort included 537 (7.9%) subjects with prevalent diabetes at baseline. Their mean (SD) serum endotoxin activity was higher than that in nondiabetic subjects, 70.73 (42.62) vs. 62.18 (36.77) EU/mL, $P < 0.001$, respectively. This produced odds ratios (95% CI) of 1.005 (1.003–1.008, $P < 0.001$)/unit increase, or 1.043 (0.759–1.433, $P = 0.79$), 1.717 (1.290–2.284, $P < 0.001$), and 2.232 (1.689–2.949, $P < 0.001$) for prevalent diabetes in the 2nd, 3rd, and 4th quartile of serum endotoxin activity compared with the 1st quartile when adjusted for age and sex. These subjects were excluded from further analyses.

Serum endotoxin activity had a significant positive correlation with CRP, cholesterol, and triglyceride concentration, and a negative correlation with HDL cholesterol concentration. It did not correlate with the fasting time. Characteristics of those with ($n = 462$) and without ($n = 6,170$) incident diabetes during the median follow-up time of 10.8 years are summarized in Table 1. The risk factors associated with diabetes and cardiovascular diseases accumulated in those with incident diabetes: all differences were highly significant except that of the smoking habit. Also serum endotoxin activity was higher ($P < 0.001$) in those who developed diabetes compared with those who did not. It was significantly associated with increased risk for diabetes with a hazard ratio (HR) (95% CI) of

1.008 (1.006–1.010, $P < 0.001$)/unit, when adjusted for age and sex, and 1.004 (1.001–1.007, $P = 0.019$)/unit, when adjusted for diabetes risk factors. The regression models for endotoxin quartiles are shown in the table (Table 2, Fig. 1). Replacing determined semifasting triglyceride concentrations with calculated fasting triglyceride concentrations or adding glucose concentrations into the model did not notably change the results.

According to ATP III and IDF definitions, 1,444 (23.0%) and 1,742 (27.7%) had metabolic syndrome, which was more prevalent in those with incident diabetes than in those without (Table 1). Serum endotoxin activity increased linearly ($P < 0.001$) in the categories of positive metabolic syndrome parameters (Fig. 2). In a Cox regression model, it remained significantly associated with an increased risk for incident diabetes, when the

presence of metabolic syndrome was introduced as a covariate: the HRs in the highest endotoxin quartiles were 1.650 (95% CI 1.210–2.250, $P = 0.002$) (Table 3) and 1.701 (1.249–2.316, $P = 0.001$) depending on the metabolic syndrome definition, ATP III or IDF, respectively.

CONCLUSIONS—In this large prospective cohort we show that endotoxemia is associated with increased risk for clinically incident diabetes. Importantly, the risk was independent of established diabetes risk factors, i.e., glucose, lipid, and CRP levels, as well as BMI. It was also independent of factors known to affect serum endotoxin activity, i.e., cholesterol, triglyceride, and HDL cholesterol concentrations, as well as smoking (10,18). Interestingly, despite the linear relation between endotoxemia and the

Table 2—Associations between risk factors and incident diabetes

	HR	95% CI		Significance
		Lower bound	Upper bound	
Model 1				
Age (years)	1.046	1.036	1.056	<0.001
Male	1.382	1.139	1.678	0.001
Endotoxin quartiles*	1.000			<0.001†
Q1				
Q2	1.073	0.768	1.498	0.681
Q3	1.703	1.255	2.309	0.001
Q4	2.751	2.071	3.654	<0.001
Model 2				
Age (years)	1.040	1.028	1.053	<0.001
Male	1.194	0.938	1.520	0.150
Education (years)	0.989	0.958	1.022	0.512
Family history of diabetes	1.481	1.187	1.848	0.001
Free-time physical inactivity	1.370	1.094	1.715	0.006
Cholesterol (mmol/L)	1.098	0.983	1.226	0.097
HDL (mmol/L)	0.438	0.292	0.659	<0.001
Smoking				
Never	1.000			0.062†
Former	0.994	0.768	1.288	0.965
Current	1.335	1.028	1.734	0.030
Triglycerides (mmol/L)	1.090	0.994	1.194	0.066
GGT (U/L)	1.001	1.000	1.002	0.001
BMI (kg/m ²)	1.149	1.128	1.171	<0.001
Hypertensive	0.869	0.708	1.067	0.179
CRP (mg/L)	1.017	1.005	1.030	0.007
Endotoxin quartiles*				
Q1	1.000			0.012†
Q2	0.939	0.652	1.351	0.733
Q3	1.233	0.885	1.718	0.216
Q4	1.518	1.090	2.114	0.013

*Endotoxin quartiles: Q1 2.40–38.10 EU/mL, Q2 38.20–54.10 EU/mL, Q3 54.20–77.0 EU/mL, Q4 77.10–475.8 EU/mL. †P for trend.

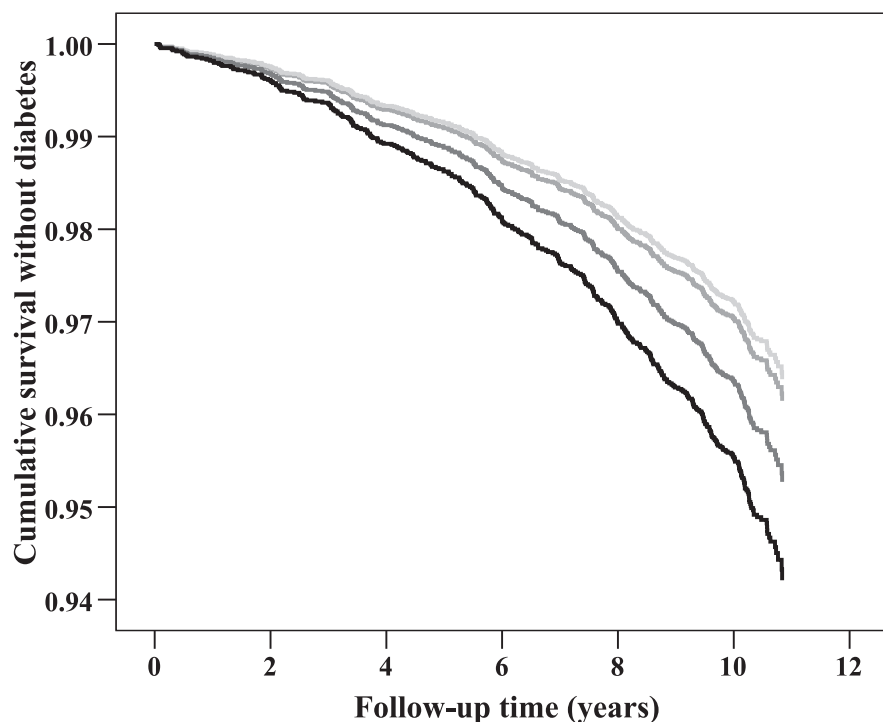


Figure 1—Cumulative survival without incident diabetes. The cumulative survival in LPS quartiles was analyzed by Cox regression model adjusted for age, sex, education, family diabetes history, free-time physical inactivity, smoking, hypertension, BMI, serum cholesterol, triglyceride, HDL cholesterol, GGT and CRP concentrations. The HR and 95% CI for the quartiles are shown in Table 2.

number of parameters defining the metabolic syndrome, high serum endotoxin activity was still associated with an increased diabetes risk.

Endotoxins can be found in the serum of apparently healthy subjects (19) because in common chronic infections and subclinical conditions the

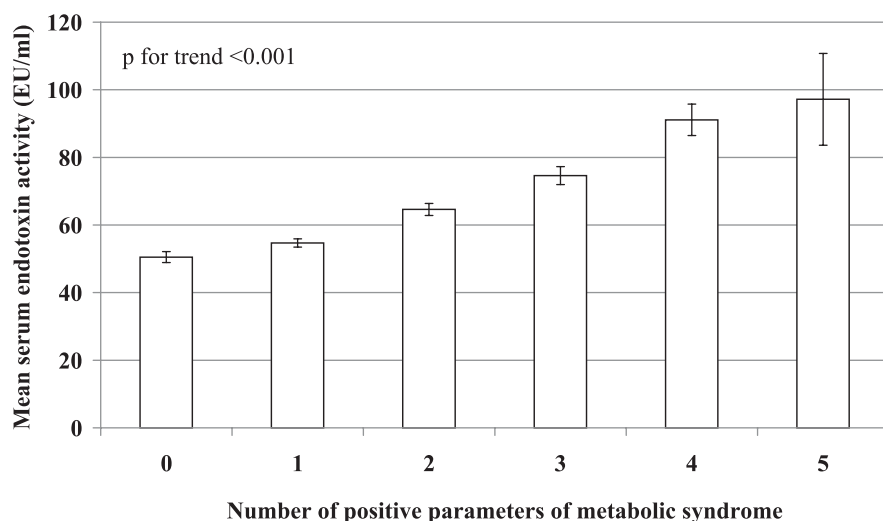


Figure 2—Serum endotoxin activities according to number of parameters of metabolic syndrome. Metabolic syndrome was defined according to the ATP III criteria ($n = 6,273$), and mean endotoxin activities with 95% CI are shown. The number of subjects in the categories is 0 = 1,203, 1 = 2,118, 2 = 1,507, 3 = 934, 4 = 459, and 5 = 52. Linear trend of endotoxin activity vs. number of positive metabolic syndrome parameters was tested using ANOVA with linear contrasts and assuming equal variances.

Gram-negative organisms colonize the human respiratory, genitourinary, and especially the gastrointestinal tract, including the oral cavity and the gut. For example, endotoxemia may arise from the most common chronic oral infection, periodontitis, which has an association with incident cardiovascular disease events and type 2 diabetes (18,20). The limulus assay used presently does not reveal the source of the endotoxin activity which varies greatly between bacterial clones. The assay is not specific to any bacterial species, but responds to the initial phase of the complement activation by LPS. Latest evidence suggests, however, that bacterial LPS deriving from the gut microbiota may trigger inflammation and oxidative stress in response to diets (4,6,21–23). High intake of fat or carbohydrates does not promote only endotoxemia, but also production of LPS transporting proteins and receptors (23). This “metabolic endotoxemia” has been shown to initiate or promote obesity, insulin resistance, metabolic syndrome, and finally diabetes (5). Whatever the sources, the current study clearly indicates for the first time an association between endotoxemia and incident diabetes.

The metabolic fate of endotoxins is highly dependent on serum lipoprotein balance, although it is bound by all lipoprotein classes (1). LPS mainly binds to HDL in health but to VLDL in sepsis, thereby increasing its affinity to lipoproteins with lower density during an infection (1,24). Dietary fat is incorporated from the gut into triglyceride-enriched lipoproteins, chylomicrons, whose formation promotes LPS absorption (23,25). This is in accordance with our present observation that endotoxin activity has a strong positive correlation with serum triglyceride concentration, as also reported earlier (10,18). More basic research, however, is needed to define the role of chylomicron-associated LPS in inflammation because the cellular responses also depend on the equilibrium of multiple endotoxin receptors and transfer proteins.

A clear limitation of the current study is the use of semifasting serum samples in the triglyceride, glucose, and endotoxin determinations. The mean relative change in triglyceride concentration per fasting hour is -3.7% (95% CI -4.2 to -3.1), and the semifasting results can be converted into fasting ones using a specific factor for the fasting time (17). In the current study, both semifasting and the

Table 3—Associations of serum endotoxin activity with incident diabetes when metabolic syndrome is included as a covariate

	HR	95% CI		Significance
		Lower bound	Upper bound	
Age (years)	1.028	1.016	1.039	<0.001
Male	1.243	1.003	1.541	0.047
Education (years)	0.959	0.929	0.990	0.010
Family history of diabetes	1.397	1.122	1.738	0.003
Free time physical inactivity	1.744	1.414	2.151	<0.001
Smoking				
Never	1.000			0.309*
Former	1.047	0.817	1.342	0.718
Current	1.218	0.944	1.571	0.130
CRP (mg/L)	1.023	1.014	1.033	<0.001
GGT (U/L)	1.001	1.000	1.001	0.002
Metabolic syndrome†	4.228	3.402	5.255	<0.001
Endotoxin quartiles‡				
Q1	1.000			<0.001*
Q2	0.909	0.636	1.297	0.598
Q3	1.329	0.960	1.841	0.086
Q4	1.650	1.210	2.250	0.002

*P for trend. †According to ATP III definition. ‡Endotoxin quartiles: Q1 2.40–38.10 EU/mL, Q2 38.20–54.10 EU/mL, Q3 54.20–77.0 EU/mL, Q4 77.10–475.8 EU/mL.

corrected results were applied, and they did not have an effect on the main result. Glucose measurements were used in an additional Cox model as a covariate, and they did not affect the association between endotoxemia and incident diabetes, either. In the absence of fasting blood glucose values, however, we did not have a chance to identify the clinically mild cases of incident diabetes, i.e., those treated with diet only, which may have caused an underestimation in the results. In addition, it may have resulted in some misclassifications of subjects with metabolic syndrome. Fasting time may also affect plasma endotoxin concentration (23), although in the current study no such correlation was found. The effect, however, is dependent on the composition of the meal before (23)—information which was not within the scope of our study.

The expanding epidemic of diabetes in industrialized countries requires more research on its predictors. Our results indicate for the first time that endotoxemia is a key player in the pathogenesis of diabetes and that microbes may have a central role.

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P.J.P. performed endotoxin laboratory measurements and statistical analyses, interpreted the data, and wrote the manuscript. A.S.H. performed statistical analyses and collected data. M.L. interpreted data. J.S. made laboratory determinations. V.S. designed the study, collected and interpreted data, and wrote the manuscript.

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