Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with iron status in persons with type 2 diabetes mellitus

Miguel Arredondo, Denisse Jorquera, Elena Carrasco, Cecilia Albala, and Eva Hertrampf

ABSTRACT

Background: High iron stores are known to cause type 2 diabetes mellitus in persons with hemochromatosis. However, it is not clear whether moderately elevated iron stores predict the risk of type 2 diabetes in healthy persons. Heme oxygenase (HO) 1 expression is increased when intracellular iron increases. Furthermore, HO shows a microsatellite polymorphism in its gene promoter that could be related to its expression and activity.

Objectives: We aimed to determine the length of (GT)n repeats in the HO1 gene promoter by using capillary electrophoresis and HO enzymatic activity in mononuclear cells (MNCs) from adult diabetes patients. We also aimed to assess the relation between these results and iron stores.

Design: We studied 99 patients with type 2 diabetes mellitus and 90 nondiabetic (control) subjects. We determined iron status (serum iron, ferritin, and transferrin receptor), HO activity, and microsatellite polymorphism.

Results: One diabetes patient and 5 control subjects had iron deficiency anemia. No iron overload was detected in either group. Diabetes patients had significantly greater iron stores (P < 0.0001), total body iron (P < 0.001), and HO activity (P < 0.001) than did control subjects. A positive association between serum iron and HO activity was seen in the diabetes patients (P < 0.0001). Allelic frequency did not differ significantly between diabetes patients and control subjects; however, the frequency of the SM genotype was significantly higher and that of the SS and MM genotypes was significantly lower in the diabetes patients than in control subjects (P < 0.001 for all).

Conclusions: Type 2 diabetes patients carrying short (GT)n repeats may have higher ferritin values and greater HO enzymatic activity and may have greater susceptibility to diabetes than may those with long (GT)n repeats. Am J Clin Nutr 2007;86:1347–53.

KEY WORDS: Type 2 diabetes mellitus, iron, heme oxygenase, microsatellite polymorphism

INTRODUCTION

Susceptibility to type 2 diabetes mellitus is determined by multiple genetic and environmental factors and their interactions

1 Noninsulin-dependent diabetes mellitus is a common complication of iron overload diseases such as hemochromatosis; 53%–80% of persons with hemochromatosis develop diabetes. It has been observed that the development of diabetes in persons with hemochromatosis is related to the magnitude of iron excess (2). However, it is not clear whether moderately elevated iron stores are associated with the risk of type 2 diabetes in healthy persons (3). The mechanisms that determine whether iron promotes the development of diabetes are unknown. A study by Barbieri et al (4) showed in vivo evidence of a relation between hyperinsulinemia or insulin resistance (or both)—the main variables of insulin resistance syndrome—and erythropoiesis. The evidence supporting a role for oxidation in the pathogenesis of diabetes is scant, although some data suggest that free radical formation may play a role by disrupting insulin action and total-body glucose disposal (5). The facts that iron is a powerful prooxidant and that oxidative stress is increased in glucose-intolerant persons suggest possible mechanisms for a role of iron (6–8), and chronically high concentrations of glycemia produce a greater expression of stress genes (ie, HO1, glutathione peroxidase, Zn/Cu-SOD, Mn-SOD, Catalase, HSP70, BCL-2, and NF-kB) than is seen without high concentrations of glycemia. These facts suggest that the expression of stress genes is part of the process by which β cells adapt to chronic hyperglycemia (9).

Heme oxygenase (HO) is a rate-limiting enzyme in heme degradation, whose action results in the generation of free iron, biliverdin, and carbon monoxide. Biliverdin is subsequently reduced to bilirubin by the enzyme biliverdin reductase. There are 3 HO isoforms; of these, HO-1 is a stress-responsive protein than can be induced by oxidative agents (including iron), heme, or hemoglobin, inflammatory mediators, and ultraviolet light (10). Considerable evidence shows that the induction of HO-1 represents an important cytoprotective defense mechanism against oxidative insults in vivo (11). The human HO-1 gene has a (GT)n dinucleotide repeat in the proximal promoter region (12). The (GT)n repeat is the most frequent of the simple repeats scattered throughout the human genome, and many of these exhibit length

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polymorphism (13) and may negatively affect transcription activity. A (GT)n repeat in the 5′flanking region of the human HO-1 gene is indeed highly polymorphic (14) and may modulate gene transcription in the face of an oxidant challenge (15). Therefore, it could be hypothesized that, if the expression of the HO-1 gene is increased, according to the number of (GT)n repeats, the microsatellite polymorphism may be associated with the development of oxidative stress-induced diseases. Thus, persons with diabetes who had a short polymorphism in the HO-1 gene promoter may have increased HO activity, which in turn could be related to iron metabolism, expressed as an increase in iron stores.

In the present study, we screened allelic frequencies of the (GT)n repeat in the HO-1 gene promoter of Chilean subjects with and without type 2 diabetes mellitus and examined the association among HO enzymatic activity, iron stores, and the length of the (GT)n repeats.

SUBJECTS AND METHODS

Subjects and study design

Ninety-nine persons with type 2 diabetes mellitus who were ≥45 y old and had experienced ≥2 y of diabetes evolution, as diagnosed and controlled by the Diabetes Program in the Nutrition Unit of Juan de Dios Hospital (Santiago, Chile), and 90 nondiabetic unrelated volunteers with no apparent medical or family history of diabetes and without metabolic syndrome according to Adult Treatment Panel III classification (16) participated in the study. The clinical characteristics of the diabetes patients and control subjects are shown in Table 1. A complete review of the medical history of all subjects, including arterial hypertension, body mass index, and waist circumference, was carried out before the beginning of the study.

Venous blood samples (20 mL) were collected from all subjects after a 48-h low-fat diet and a 12-h overnight fast. Plasmatic biochemical characterization included glycemia, lipid profile (total, LDL, and HDL cholesterol and triacylglycerol), glutamate oxaloacetic acid transaminase (GOT), glutamate pyruvate transaminase (GPT), total bilirubin (TB), alkaline phosphatase (AP), and creatinine. All of these variables were measured enzymatically (DiaLab, Wiener Neudorf, Austria). High-sensitivity C-reactive protein (hsCRP) Química Clínica Aplicada SA, Amposta, Spain] was assessed to discard inflammatory events. A cutoff of 3 mg/L was used as the upper limit. Hematic variables included hemoglobin, measured by using a counter (Cell Dyn 1700; Abbott Laboratories, Abbott Park, IL); total serum iron, measured by using graphite furnace atomic absorption spectrometry (Simaa 6100; Perkin-Elmer, Shelton, CT); free erythrocyte protoporphyrin (FEP), measured by using a hematofluorimeter (model 206D; AVIV Biomed, Lakewood, NJ); transferrin receptors (Ramco Laboratories Inc, Houston, TX); and serum ferritin, measured by using an enzyme-linked immunosorbent assay (Dako Corp, Carpinteria, CA). To evaluate iron status, the following cutoffs were used: 120 g/L and 135 g/dL as the lower-normal limit for hemoglobin in women and men, respectively; 70 μg/dL as the upper-normal limit for FEP in red blood cells; and 70 μg/dL as the lower-normal limit for serum iron. Depleted iron stores were defined as serum ferritin concentrations <12 μg/L, iron deficiency without anemia was defined as normal hemoglobin and ≥2 other abnormal laboratory results, and iron deficiency anemia was defined as below-normal hemoglobin and ≥2 other abnormal laboratory results. Iron overload
was defined as a serum ferritin concentration > 200 μg/L in women and > 300 μg/L in men (17). Total body iron was calculated from the ratio of transferring receptors to serum ferritin according to the following equation (18):

\[
\text{Body iron (mg/kg)} = -\left[\log(\text{TfR/SF ratio})\right]/0.1207 \quad (1)
\]

Written informed consent was obtained from all subjects. The study protocol was approved by the ethics committees of the Instituto de Nutrición de los Alimentos, the University of Chile, and the Nutrition Unit of the Juan de Dios Hospital.

**Isolation of mononuclear leukocytes**

Mononuclear leukocytes (MNCs) were obtained by following the protocol described by Muñoz et al (19). Briefly, blood was diluted in a 1:1 ratio with sterile phosphate-buffered saline (PBS) (137 mmol NaCl/L, 2.7 mmol KCl/L, 8.1 mmol Na2HPO4/L, and 1.5 mmol KH2PO4/L; pH 7.4), layered onto a Histopaque gradient with a density of 1.119 g/mL (Histopaque 1077; Sigma Diagnostics, St Louis, MO) and centrifuged at 400 × g for 35 min at room temperature. The mononuclear layer (buffy coat) was then removed and washed twice for 10 min in PBS at 180 g. Immediately afterward, the same wash protocol was performed with RPMI 1640 medium (Invitrogen Life Technology, Carlsbad, CA). Finally, the cells were resuspended in 500 μL PBS.

**Genomic DNA and amplification of HO-1 gene**

Genomic DNA was obtained from MNCs by using Chomczinsky reagent and quantified at 260 nm (Gene Quant; Pharmacia Biotech, Cambridge, United Kingdom). The amplification of the HO-1 gene promoter was conducted by using polymerase chain reaction with the primers HO-1s cccaaagcttgcagctttcttagat-NED and HO-1a gggaagaatgctgcatagac in a thermal cycler (model #2720; Applied Bio-Systems, Foster City, CA) using 30 cycles of 20 s at 94 °C, 20 s at 60 °C, and 20 s at 72 °C (20, 21). The polymerase chain reaction product (3 μL) was loaded into a 2.5% agarose gel to check the amplification. Finally, capillary electrophoresis was performed to detect the (GT)n length.

**Heme oxygenase activity**

MNCs (1 × 10⁶) were suspended in 1 mL RPMI-1640 (Invitrogen Life Technology) and 100 μL of 900 μmol H2O2/L and incubated in 5% CO₂ for 18 h at 37 °C. Cells were centrifuged for 10 min at 1500 × g at 4 °C, and the resulting pellet was homogenized in 100 μL of non-denaturing lysis buffer (20 mmol KH2PO4/L, 135 mmol KCl/L, and 0.1 mmol EDTA/L; pH 7.4) and centrifuged for 20 min at 10 000 × g at 4 °C. The supernatant (100 μL) was incubated at 1 h at 37 °C in the dark with 100 μL of 15 μmol hemin/L (Sigma-Aldrich), 100 μL of 10 μg biliverdin reductase/mL isolated from rat liver (see the next paragraph), and 600 μL resuspension buffer (100 mmol KH2PO4/L; pH 7.4). The reaction was started with 100 μL of 1 mmol NADPH/L (Sigma-Aldrich). We extracted bilirubin with 1 mL chloroform by stirring it for 1 h and then centrifuging it for 5 min at 200 × g and 4 °C. Bilirubin concentrations were measured at 530 nm (model UV-1601; Shimadzu, Tokyo, Japan). HO activity was expressed as nmol bilirubin/mg protein −1·h−1 (bilirubin mole extinction coefficient: ε = 43.5 mmol/mL−1·cm−1) (21–23).

**Bilirubin reductase isolation**

The livers of rats (Rattus norvegicus) were perfused in situ with saline (0.9% NaCl, pH 7.2) until they reached complete discoloration; then the livers were dissected and homogenized in lysis buffer A (0.1 mol sodium citrate/L; pH 5.0; 10% glycerol) and centrifuged first for 20 min at 10 000 × g at 4 °C and then for 1 h at 105 000 × g at 4 °C. The supernatant was diluted with 20 mmol KH2PO4/L, 135 mmol KCl/L, and 0.1 mmol EDTA/L (pH 7.4). Protein concentrations were determined by BCA assay (BioRad Laboratories, Hercules, CA). The extract was divided into aliquots, which were stored at −20 °C.

**Statistical analysis**

We used a chi-square test to compare the allelic frequencies and genotypes. A 2-factor repeated-measures analysis of variance was used to evaluate the effect of group (sex × diagnosis) and possible interactions between groups regarding either iron status or the micropolymorphism. Because ferritin concentrations and HO activity had a skewed distribution, their results

### Table 2

Hematologic characteristics of the total population

<table>
<thead>
<tr>
<th></th>
<th>Control subjects</th>
<th>Diabetes patients</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/L)</td>
<td>138 ± 15⁴</td>
<td>140 ± 14</td>
<td>NS</td>
</tr>
<tr>
<td>Serum iron (mg/dL)</td>
<td>101.5 ± 32.5</td>
<td>115.1 ± 43.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Serum ferritin (μg/L)</td>
<td>38.6 (20.0–74.8)⁴</td>
<td>53.8 (32.2–89.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>&gt; 50 μg/L</td>
<td>30/90</td>
<td>63/99</td>
<td></td>
</tr>
<tr>
<td>&gt; 100 μg/L</td>
<td>0/90</td>
<td>6/99</td>
<td></td>
</tr>
<tr>
<td>&gt; 200 μg/L</td>
<td>0/0</td>
<td>0/0</td>
<td></td>
</tr>
<tr>
<td>Transferrin receptor (μg/mL)</td>
<td>6.8 ± 2.6</td>
<td>5.5 ± 2.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total body iron (mg/kg)</td>
<td>5.0 ± 3.3</td>
<td>7.0 ± 2.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ZnPP (μg red blood cells/mL)</td>
<td>62.3 ± 29.0</td>
<td>59.8 ± 11.0</td>
<td>NS</td>
</tr>
<tr>
<td>HO (nmol bilirubin · mg protein¹−1 · h−1)</td>
<td>0.22 (0.07–0.66)</td>
<td>0.71 (0.23–2.18)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

¹ ZnPP, zinc protoporphyrin; HO, heme oxygenase (enzymatic activity). Iron concentrations and HO activity differed significantly by diagnosis, P < 0.001; serum ferritin, transferrin receptors, and total body iron differed significantly by sex and diagnosis, P < 0.001 (repeated-measures ANOVA for all). There were no significant group × variable interactions.

² Subject groups were compared with the use of a t test.

³ x ± SD (all such values).

⁴ Geometric x; range (± 1 SE) in parentheses (all such values).
were retransformed into anti-logarithms to recover the original units and were expressed as geometric means ± SDs. Differences were considered significant at P < 0.05 with the use of STATISTICA for WINDOWS software (release 5.1; Statsoft, Tulsa, OK). Logistic regression techniques (stepwise, forward estimation) were used to assess gene-disease associations after adjustment for confounding variables (STATA software; version 9.2; Statacorp LP, College Station, TX).

RESULTS

As expected, patients with diabetes mellitus had a greater number of conventional risk factors for cardiovascular events than did the control subjects. The 2 groups did not differ significantly in either age or body mass index (in kg/m²), despite the significant differences in weight and height (P = 0.02 for both) between the 2 groups. There were significant differences in waist circumference, systolic pressure, and diastolic pressure (P < 0.001 for all) between diabetes patients and control subjects, whether analyzed by group or by sex. Liver enzyme activities (ie, GOT, GPT, and AP), cholesterol, HDL, LDL, creatinine, and glycemia also differed significantly between the 2 groups. Although the mean hsCRP did not differ significantly between diabetes patients and control subjects, 51.5% of diabetes patients had values higher than 3 mg/L, whereas only 31.4% of control subjects did so.

Only 1 of the diabetes patients had iron deficiency anemia. However, of the 90 control subjects, 6 had depleted iron stores and 5 had iron deficiency anemia. Mean hemoglobin concentrations did not differ significantly between control subjects and diabetes patients. Furthermore, mean total serum iron was significantly (P < 0.05) higher in subjects with diabetes than in control subjects. No subjects in either group had iron overload. Serum ferritin was significantly higher in diabetes patients than in control subjects (53.8 and 38.8 µg/L, respectively; P < 0.001). Furthermore, 63 of the diabetes patients and 30 of the control subjects had serum ferritin concentrations > 50 µg/L. On the other hand, only 6 of the diabetes patients had concentrations > 100 µg/L, and none had concentrations > 200 µg/L. None of the control subjects had serum ferritin concentrations > 100 µg/L. Transferrin receptor values were significantly lower in diabetes patients than in control subjects (5.5 and 6.8 µg/mL, respectively; P < 0.001). Finally, the total-body iron calculation showed that diabetes patients had significantly more body iron.

FIGURE 1. Top) Relation between serum iron and serum ferritin in diabetes patients (right) and control subjects (left). Serum iron (mg/dL) was measured by using graphite furnace atomic absorption spectrometry, and serum ferritin (µg/L) was measured with an enzyme-linked immunosorbent assay. Bottom) Relation between serum iron and hemo oxygenase (HO) activity in diabetes patients (right) and control subjects (left). Serum iron was measured as described above, and HO activity was measured (nmol bilirubin/mg protein⁻¹·h⁻¹) in mononuclear leukocytes. n = 99 diabetes patients and 90 control subjects.

TABLE 3

<table>
<thead>
<tr>
<th>Allele class</th>
<th>Control subjects</th>
<th>Diabetes patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>78 (45)</td>
<td>89 (43)</td>
</tr>
<tr>
<td>M</td>
<td>90 (49)</td>
<td>97 (50)</td>
</tr>
<tr>
<td>L</td>
<td>12 (6)</td>
<td>12 (7)</td>
</tr>
</tbody>
</table>

Chi-square test found no significant differences.
TABLE 4
Distribution of genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>SS</th>
<th>SM</th>
<th>SL</th>
<th>MM</th>
<th>ML</th>
<th>LL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control subjects [n (%)]</td>
<td>12 (13.3)</td>
<td>49 (54.4)</td>
<td>5 (5.6)</td>
<td>18 (20.0)</td>
<td>5 (5.6)</td>
<td>1 (1.1)</td>
</tr>
<tr>
<td>Diabetes patients [n (%)]</td>
<td>2 (2.0)</td>
<td>78 (87.8)</td>
<td>7 (7.1)</td>
<td>8 (8.1)</td>
<td>3 (3.0)</td>
<td>1 (1.0)</td>
</tr>
</tbody>
</table>

*P < 0.001 (chi-square test).

than did control subjects (7.0 and 5.0 mg/kg, respectively; *P < 0.001) (Table 2).

The HO activity in MNCs was significantly higher in subjects with diabetes than in control subjects [0.22 (0.07–0.66) and 0.71 (0.23–2.18) nmol bilirubin–mg protein−1·h−1, respectively; *P < 0.001] (Table 2). There was a positive association between serum iron and serum ferritin in both control subjects (*r² = 0.57) and diabetes patients (*r² = 0.47) (Figure 1). The subjects with diabetes had a more positive relation between serum iron and HO activity (*r² = 0.53) than did the control subjects (*r² = 0.01) (Figure 1).

The distribution of (GT)ₙ repeats in the HO1 gene was trimodal; it ranged between 16 and 40 (GT)ₙ repeats. We divided the alleles into 3 subclasses, according to the number of (GT)ₙ repeats: the lower component (S) with ≤26 GT repeats, the middle components (M) with GT repeats between 27 and 32, and the upper components (L) with ≥33 GT repeats. The most frequently distributed numbers of (GT)ₙ were (GT)₁₉/(GT)₂₆ and (GT)₂₁/(GT)₂₈ in subjects with diabetes and control subjects, respectively. When the proportions of allele frequency (S, M, and L) (Table 3) and genotypes (SS, SM, SL, MM, ML, and LL) (Table 4) were analyzed, the distribution of allelic frequency did not differ significantly between the diabetes patients and the control subjects. However, the proportion of allelic frequencies of the S and M classes were significantly higher than that of the L class in both groups. In contrast, the genotypes differed significantly (*P < 0.001; chi-square test: 75.187) between diabetes patients and control subjects, although all subjects had normal iron stores. Because control subjects do not show high values of HO activity or serum iron and ferritin, we cannot rule out the possibility that persons without diabetes but with high serum iron or ferritin could present with high HO activity.

Iron stores usually are quantified by the measurement of transferrin receptors and serum ferritin concentrations. However, although serum ferritin is considered a good indicator of body iron stores, it is not the gold standard. Given that serum ferritin is an acute phase protein and may reflect inflammatory activity, its
concentration can be overestimated during an inflammatory process (24). Jiang et al (3) showed that women with elevated ferritin concentrations and a lower ratio of transferrin receptor to ferritin had a greater risk of diabetes, which was independent of known diabetes risk factors and of inflammation as measured by C-reactive protein, than did women with low ferritin concentrations and a high ratio of transferring receptor to ferritin. The population in the present study had signs of inflammatory processes. To refine the evaluation of iron stores, we measured transferrin receptors, which enabled us to calculate the ratio of transferrin receptor to ferritin as a means of estimating iron stores. These measurements differed significantly between the 2 groups, which showed that the diabetes patients had more total body iron than did the control subjects.

Our data support the theory that high iron stores, even in a range not considered to be associated with hemochromatosis, contribute to the development of insulin-nondependent diabetes. Furthermore, the diabetes patients had higher concentrations of serum iron and ferritin and greater HO activity than did the control subjects. The association between serum ferritin and diabetes was described previously (3, 24–26); in one of those studies, men with high stores of iron were 2.4 times as likely to develop diabetes than were men with lower stores of iron, as assessed by using a logistic regression model (25). Similarly, the results of the present study confirm that even a modest elevation in the ferritin concentration predicts incident diabetes independently of known risk factors and confounders (27).

We show that the poly-(GT) sequence present in the HO1 gene promoter is highly polymorphic in the population in the present study. The (GT)\textsubscript{R} repeat is located between the putative heat-shock element and the cis-regulatory element (28). A specific allele for this polymorphism may affect the transcription of the HO1 gene. Greater expression of the HO1 gene may not be beneficial for the host, given the potential cell toxicity of the heme degradation product (ie, iron). There is extensive literature about the role of iron in the production of oxidative stress, and some studies showed that increased oxidative stress in diabetic rats leads to the induction of HO1 (29, 30). It was suggested that the 5’flanking polymorphism in the HO1 gene is associated with the development of oxidative stress–mediated diseases, in which subjects possessing $S/S$ had higher expression of HO1 mRNA and HO activities than did those without $S/S$ (21). These results are very similar to those reported here—ie, the diabetes patients have higher expression of $S$ and $S$ HO1 polymorphism and higher HO activities than do the controls subjects. Therefore, it is particularly important that HO1 gene expression be properly regulated in humans.

Most HO1 polymorphism studies had been performed in Asian populations (14, 15, 20). These studies showed a higher frequency of $S$ (46%) and $M$ (44%) alleles in control subjects than in diabetes patients (15) or similar proportions of the $M$ allele in control subjects (77%) and diabetes patients (81%) (20). The results in the present study showed no difference in allele frequencies between control subjects and diabetes patients (Table 3). Chen et al (20) showed a similar frequency of HO1 promoter genotypes between control subjects and subjects with diabetes; however, we observed a marked difference between the 2 groups, especially with respect to SS, SM, and MM HO1 promoter genotypes (Table 4). It is necessary to remark that HO activity in control subjects with the SM polymorphism also could be influenced by serum iron and ferritin. However, in the present study, subjects with these characteristics were not represented. Furthermore, a logistic regression analysis showed that the results of the present study confirm that diabetes patients with the SM polymorphism have a higher relative risk ratio when presenting with high ferritin concentrations and high HO activity than do those patients without the SM polymorphism.

In summary, our results show an association between iron metabolism and type 2 diabetes mellitus, even in the normal ranges of iron status variables. Iron is a first-line prooxidant element that contributes to the development of several systemic diseases, including diabetes and atherosclerosis. Thus, higher concentrations of iron could determine an amplification of the oxidative stress produced by free radicals. The participation of iron in oxidative stress could explain in part the association of iron to abnormalities regarding insulin sensibility.

We thank the staff of the Diabetes Program, Nutrition Unit, Juan de Dios Hospital, for their teamwork and persistent efforts in this project.

The authors’ responsibilities were as follows—MA: study design; management of laboratory work, data collection, analysis, and interpretation; and writing of the manuscript; DJ: laboratory analysis, data collection, and analysis and interpretation; EH: study design, management of hemochromatosis patients, and revision of the manuscript; EC: management of the field site, sample collection, and revision of the manuscript; and CA: study design, management of diabetes patients, and revision of the manuscript. None of the authors had a personal or financial conflict of interest.

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