Analysis of TP53 aflatoxin signature mutation in hepatocellular carcinomas from Guatemala: A cross-sectional study (2016-2017)

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Abstract

**Background and aims:** Guatemala has the highest incidence of hepatocellular carcinoma (HCC) in the Western hemisphere. The major risk factors in Guatemala are not well characterized, but the prevalence of hepatitis B virus (HBV) and hepatitis C virus (HCV) appears to be low, while the prevalence of aflatoxin (AFB1) exposure appears to be high. To examine whether AFB1 may contribute to the elevated incidence of HCC in Guatemala, this study examined the frequency of the AFB1-signature mutation in the TP53 gene (R249S) as well as other somatic mutations. In addition, we assessed whether the frequency of the TP53 mutation differed by sex.

**Methods:** Formalin-fixed, paraffin-embedded (FFPE) HCC tissues were obtained from three hospitals in Guatemala City between 2016 and 2017. In addition, tumor tissues preserved in RNAlater were also obtained. Sociodemographic and clinical information including HBV and HCV status were collected. Targeted sequencing of TP53 was performed in the FFPE samples, and a panel of 253 cancer-related genes was sequenced in the RNAlater samples.

**Results:** Ninety-one FFPE tissues were examined, from 52 men and 39 women. Median (IQR) age at diagnosis was 62 (51-70). Among those with known HBV and HCV status, two were HBV+ and three were HCV+. Overall, 47% of the HCCs had a TP53 mutation. The AFB1-signature R249S mutation was present in 24%. No overlap between the R249S mutation and HBV+ was observed in this cohort. Among 18 RNAlater samples examined, 44% had any TP53 mutation and 33% had the R249S mutation. Other somatic mutations were identified in known HCC driver genes.

**Conclusions:** The presence of the TP53 R249S mutation in the samples studied suggests that AFB1 may contribute to the high incidence of HCC in Guatemala.
Liver cancer is the seventh most common cancer and the second leading cause of cancer mortality globally. The most common histological subtype is hepatocellular carcinoma (HCC), which accounts for 80% of all liver cancers. Sex and geographic variation in HCC incidence has been reported across regions worldwide, as has the variability in the prevalence of known risk factors. Major risk factors for HCC, such as hepatitis B virus (HBV), hepatitis C virus (HCV), excessive alcohol consumption, and aflatoxin B1 (AFB1) exposure vary between high-rate and low-rate areas. In many high-rate areas (eg, regions in Asia and Africa), HBV and AFB1 exposure are dominant factors, while in low-rate areas, HCV and alcohol consumption are more common. In addition, non-alcoholic fatty liver disease (NAFLD) is becoming recognized as an important risk factor for HCC in both high and low incidence regions.

In Guatemala, the estimated incidence and mortality rates of liver cancer are the highest in the Western hemisphere (age-standardized rates [ASRs]: 14.9 cases per 100 000 person-years and 14.5 deaths per 100 000 person-years), with 1787 new cases of liver cancer estimated to have occurred in 2018. Unlike most other countries, where men have a two- to three-fold higher rate of liver cancer than women, Guatemala has a unique 1:1 ratio of liver cancer rate between the sexes. A recent cross-sectional study that assessed risk factors for liver cancer in Guatemala found a very low prevalence of both HBV and HCV infection (<1%). In contrast, the study found high serum levels of AFB1-albumin adducts among the participants, with significantly higher geometric mean levels among men (10.93 pg/mg albumin) than women (7.92 pg/mg albumin). The results were consistent with previous evidence of high AFB1 levels in maize samples across the country. In addition, the study demonstrated that NAFLD (60.1%), obesity (30.9%), and metabolic syndrome (64.2%) are highly prevalent in Guatemala.

AFB1 forms DNA adducts at the N7 position of guanine, inducing primarily G → T transversions. One particular G → T mutation in codon 249 (AGG to ATT, arginine to serine, R249S) of TP53 is a molecular signature of AFB1 exposure in HCC. Studies in AFB1 endemic regions in Asia and Africa have reported a wide range in the prevalence of the R249S mutation in HCC, from 4.8% to 67%. In the Americas, the R249S mutation prevalence reportedly ranges between 1.3% and 28%. In the U.S., a recent study reported that 7% of the HCCs among Hispanic patients in southern Texas had the R249S mutation. No studies have previously been conducted in northern Central America, a region characterized by high AFB1 exposure but low prevalence of chronic HBV infection.

To evaluate the possible role of AFB1 in the elevated incidence of HCC in Guatemala, tumor samples were retrieved from three major hospitals in Guatemala City, and targeted sequencing analysis of TP53 was performed. Among a smaller set of RNA later-preserved samples, 253 cancer-related genes were sequenced. In addition, the frequency of TP53 mutations by sex was assessed.

METHODS

2.1 Study population

Formalin-fixed, paraffin-embedded (FFPE) HCC tissue slides and blocks were obtained from the pathology departments of hospitals in Guatemala City between 2016 and 2017. The three hospitals that provided tissue were: Hospital General San Juan de Dios, a large public hospital; Hospital Militar, a hospital serving military personnel and their families; and the Instituto de Cancerología (INCAN), an adult cancer hospital. The HCC diagnoses were histologically confirmed by Dr. David Kleiner, at the U.S. National Cancer Institute (NCI). In addition, HCC tissues stored in RNAlater were obtained from INCAN.

Socio-demographic information, such as age, sex, residence (Guatemala and contiguous departments vs other departments), as well as HBV and HCV status were abstracted, when available, from medical records.

2.2 DNA extraction

One half of the tissue from each pathology slide was scraped from the slide and extracted by a phenol-chloroform procedure. FFPE blocks were sectioned in a microtome, and curls (10 μm sections) were collected for DNA extraction. Tissue stored in RNAlater (ThermoFisher Scientific, Catalog #AM7020) was stored at −20°C until DNA was extracted using the AllPrep DNA/RNA Micro kit (Qiagen, Catalog # 80284). DNA was quantified using the PicoGreen dsDNA assay method (ThermoFisher Scientific, Catalog #P7581).

2.3 DNA sequencing

Targeted sequencing of TP53 was performed on the HCC FFPE samples using a primer panel provided by Ion Torrent (Ampliseq) using the manufacturer's protocol. Positive PCR products were quantified using Kapa's
related genes is presented in Table S2. In addition, the list of the 253 cancer-related genes in the 18 samples with fresh tumor DNA is presented as well as the somatic variants and targeted sequence of the analysis. SAS software v 9.4 (SAS Institute, Cary, NC) was used for Fisher's exact test. A \( p \)-value <0.05 was considered statistically significant.

2.5 | Ethical considerations

The study was approved by the ethical review board at INCAN, the Hospital General San Juan de Dios, and the Hospital Militar. Cases provided written informed consent at INCAN, and a waiver of consent was approved by the other two hospitals. In addition, there was an exemption by the NIH Office of Human Subjects Research (OHSR) to receive the samples without personal identifiers at NCI.

3 | RESULTS

In total, 91 FFPE HCC tissues and 18 additional tumor tissues in RNAlater were collected. Of the 91 HCC samples examined, 52 were from men and 39 were from women. The median age at diagnosis among men was 62 years (Interquartile range [IQR]: 48-73) and among women, 61 years (IQR: 52-68). Among the persons for whom information on residence was recorded (34.0%, 31/91), more than 70% lived in the department of Guatemala or in contiguous departments. Among the persons for whom HBV (27.5%, 25/91) and HCV status (28.6%, 26/91) was recorded, only two were HBV positive (defined as being positive for HBsAg) and only three were HCV positive (defined as being positive for anti-HCV) (Table 1).

3.1 | Prevalence of TP53 mutations in the FFPE samples

Of the 91 FFPE samples, two yielded insufficient DNA; thus, a total of 89 samples were successfully sequenced. Overall, 47% of the FFPE HCCs (42/89) had any TP53 mutation (Table 2). The mutation prevalence was somewhat higher in the tumors from women (58%) than in the tumors from men (39%), but the difference was not statistically significant \( (P = .09, \text{ Fisher's exact test}) \). The prevalence of the R249S mutation was 24%, with no major difference observed in the prevalence of the mutation in the tumors from men (22%) and women (26%) \( (P = .62, \text{ Fisher's exact test}) \). The prevalence of any G -> T transversion, including the R249S mutation, was 27%, and the prevalence of any C -> T transition at CpG was 32%; we observed no statistically significant difference in the prevalence by sex \( (P = .47 \text{ and } P = .17, \text{ respectively}) \).

3.2 | Cancer gene sequence analysis of the tumor samples preserved in RNAlater

Table 3 shows the results of the gene sequence analysis for the 18 tumor tissues in RNAlater that were analyzed for targeted capture of the exons of 253 known cancer-related genes, compared to the genome sequence analysis results of HCCs in other populations. Among the 18 cases, 6 tumors (33%) had the TP53 R249S mutation. The proportion with any TP53 mutation was 44%, while the prevalence in other studies ranged between 21%
TABLE 1  Demographic characteristics of the study sample by sex, Guatemala 2016–2017

| Characteristics                        | Total (N = 91) | Men n = 52 (57.1%) | Women n = 39 (42.9%) |
|----------------------------------------|---------------|-------------------|---------------------|
| Age, y, median (IQR)                   | 62 (51, 70)   | 62 (48, 73)       | 61 (52, 68)         |
| Hospital, n (%)                        |               |                   |                     |
| INCAN                                   | 52 (57.1)     | 29 (55.8)         | 23 (59.0)           |
| Hospital General San Juan de Dios      | 26 (28.6)     | 13 (25.0)         | 13 (33.3)           |
| Hospital Militar                       | 13 (14.3)     | 10 (19.2)         | 3 (7.7)             |
| Residence†, n (%)                      |               |                   |                     |
| Guatemala and contiguous departments   | 22 (71.0)     | 13 (61.9)         | 9 (90.0)            |
| Other departments                      | 9 (29.0)      | 8 (38.1)          | 1 (10.0)            |
| HBV status‡, n (%)                     |               |                   |                     |
| Positive                               | 2 (8.0)       | 2 (11.8)          | 0 (0.0)             |
| Negative                               | 23 (92.0)     | 15 (88.2)         | 8 (100.0)           |
| HCV status‡‡, n (%)                    |               |                   |                     |
| Positive                               | 3 (12.0)      | 3 (16.7)          | 0 (0.0)             |
| Negative                               | 23 (88.0)     | 15 (83.3)         | 8 (100.0)           |

†Missing for residence: 60; Missing for HBV status: 66; Missing for HCV status: 65.
‡Defined as being positive for HBsAg.
‡‡Defined as being positive for anti-HCV.

and 82%.28-35 Mutations were also observed in known HCC driver genes such as AT-rich interaction domain 2 (ARID2) (28%), AT-rich interaction domain 1 (ARID1) (17%), adenomatous polyposis coli (APC) (17%), and catenin beta-1 (CTNNB1) genes (17%). In addition, mutations were observed in axis inhibition protein 1 (AXIN1), SWI/SNF related, matrix associated, actin-dependent regulators of chromatin, subfamily a, member 4, (SMARCA4), guanine nucleotide- binding protein (GNAS), retinoblastoma (RB1), Fms-like tyrosine kinase 3 (FLT3), DNA (cytosine-5)-methyltransferase 3A (DNMT3A), cyclin-dependent kinase inhibitor 2A (CDKN2A), albumin (ALB), ribosomal protein S6 kinase, 90 kDa, polypeptide 3 (RPS6KA3), ataxia-telangiectasia mutated (ATM), and fibroblast growth factor receptor 3 (FGFR3).

Table S1 shows the mutation spectrum of the TP53 and the 253 cancer-related genes grouped into the six possible classes of base substitutions. The analysis found an excess of C>A, G>T mutations in the TP53 genes (57%) compared to 22% in all 253 genes. A deficit in the T>A, T>C and T>G classes is also seen in the TP53 mutations. The mutation profile of all 253 cancer-related genes grouped into 16 possible 3 base pairs (bp) sequence context is displayed in Figure S1.

4  | DISCUSSION

This is the first report of TP53 mutations in HCCs from northern Central America. Although the region is one of the three high-rate HCC regions in the world, it is characterized by a low prevalence of chronic HBV infection. The study found an overall TP53 mutation rate of nearly 50% in both FFPE and RNAlater samples. In addition, among all the samples analyzed, the proportion with the AFB1-signature mutation (R249S) was 25%.

Prior studies conducted in high-rate HCC areas have reported a TP53 R249S mutation prevalence as high as 67%.14,15,20,36 In Western Africa, a study in the Gambia found the R249S mutation in 36% (19/53) of HCCs, while a study in Senegal reported a prevalence of 67% among 15 HCCs.14,18 In China, studies from high-rate areas have reported the R249S mutation in 36% (18/50) of HCCs from Guangxi, and 54% (97/181) of HCCs from Qidong in the early 2000s.15,16 More recently, a study in Thailand found the R249S mutation in 34% of HCCs.37 The majority of HCCs in sub-Saharan Africa and eastern Asia, however, are HBV positive (>50%), and it has been suggested that HBV sensitizes hepatocytes to the effects of AFB1.15

The proportion of tumors with the R249S mutation found in the current study is similar to that reported in other regions in the Americas. For example, a study in Brazil found the mutation in 28% (21/74) of HCCs, with 16% (13/74) being HBV positive.29 In Colombia, the R249S mutation was found in only four of the 38 HCC cases (11%), with 25 of the cases being positive for HBV infection.22 A study in Peru found the aflatoxin signature mutation in only one tumor out of 80 HCCs.23 In Mexico, 18% of HCC samples (3/16) had the AFB1-signature mutation, with only three cases being positive for HBV.21 As maize is commonly consumed in a number of Latin American countries, exposure to AFB1 is likely. The high levels of AFB1...
TABLE 3  Prevalence of genetic mutations in HCCs reported by different studies

| Population | Zhang et al\textsuperscript{29} | Ahn et al\textsuperscript{31} | Fujimoto et al\textsuperscript{32} | Guichard et al\textsuperscript{33} | Li et al\textsuperscript{34} | Huang et al\textsuperscript{28} | Schulze et al\textsuperscript{80} | TCGA cBioPortal\textsuperscript{35} | Current study 2020 |
|------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| N          | China                         | Korea                         | Japan                         | Not provided                  | China                         | Europe                        | US                            | Guatemala                    |
|            | 49                            | 231                           | 300                           | 125                           | 139                           | 110                           | 243                           | 366                           | 18                            |
| TP53       | 82%                           | 32%                           | 28%                           | 21%                           | 28%                           | 59%                           | 25%                           | 33%                           | 44%                           |
| TP53 R249S |                               |                               |                               |                               |                               |                               |                               |                               | 33%                           |
| AXIN1      | 20%                           | 7%                            | 5%                            | 15%                           | 20%                           | 12%                           | 2%                            | 6%                           |
| CTNNB1     | 20%                           | 23%                           | 26%                           | 33%                           |                               | 38%                           | 17%                           |
| KIT        | 12%                           |                               |                               |                               |                               |                               |                               |                               |
| SMARCA4    | 8%                            |                               |                               |                               |                               |                               |                               | 6%                           |
| JAK3       | 8%                            |                               |                               |                               |                               |                               |                               |                               |
| PBRM1      | 8%                            |                               |                               |                               |                               |                               |                               |                               |
| GNAS       | 8%                            |                               |                               |                               |                               |                               |                               |                               |
| MED12      | 8%                            |                               |                               |                               |                               |                               |                               |                               |
| RB1        | 8%                            | 8%                            | 6%                            |                               | 7%                            | 11%                           | 6%                            |
| RET        | 8%                            |                               |                               |                               |                               |                               |                               |                               |
| ARID1A     | 6%                            | 12%                           | 17%                           | 33%                           | 12%                           | 10%                           | 17%                           |
| ARID2      | 6%                            | 9%                            | 6%                            | 6%                            | 4%                            | 9%                            | 28%                           |
| DNMT1      | 6%                            |                               |                               |                               |                               |                               |                               |                               |
| DNMT3A     | 6%                            |                               |                               |                               |                               |                               |                               |                               |
| FLT3       | 6%                            |                               |                               |                               |                               |                               |                               |                               |
| ABL1       | 6%                            |                               |                               |                               |                               |                               |                               |                               |
| FGFR2      | 6%                            |                               |                               |                               |                               |                               |                               |                               |
| MAP3K1     | 6%                            |                               |                               |                               |                               |                               |                               |                               |
| SETD2      | 6%                            |                               |                               |                               |                               |                               |                               |                               |
| ARID1B     | 6%                            |                               |                               |                               |                               |                               |                               |                               |
| CDKN2A     | 4%                            | 6%                            | 6%                            | 7%                            | 10%                           | 8%                            | 6%                            |
| ALB        | 4%                            | 14%                           |                               |                               | 12%                           | 14%                           | 6%                            |
| RPS6KA3    | 4%                            | 6%                            | 10%                           |                               | 9%                            |                               |                               |
| CDKN1B     | 4%                            |                               |                               |                               |                               |                               |                               |                               |
| MYC        | 4%                            |                               |                               |                               |                               |                               |                               |                               |
| APC        | 2%                            | 2%                            |                               |                               |                               |                               |                               | 17%                           |
| ATM        | 2%                            | 44%                           |                               |                               | 8%                            |                               |                               | 11%                           |
| NFE2L2     |                               |                               |                               |                               | 6%                            |                               |                               |                               |
| IRF2       |                               |                               |                               |                               |                               |                               |                               | 5%                            |
| IL6ST      |                               |                               |                               |                               |                               |                               |                               | 2%                            |
| PIK3CA     |                               |                               |                               |                               |                               |                               |                               | 4%                            |
| DMXL1      |                               |                               |                               |                               |                               |                               |                               | 4%                            |
| KRAS       |                               |                               |                               |                               |                               |                               |                               | 2%                            |
| PTEN       |                               |                               |                               |                               |                               |                               |                               | 7%                            |
| CDKN1A     |                               |                               |                               |                               |                               |                               |                               | 4%                            |
| FGFR3      |                               |                               |                               |                               |                               |                               |                               | 11%                           |
| CASP8      |                               |                               |                               |                               |                               |                               |                               | 0%                            |

In the current study, we observed no overlap between the R249S mutation and HBV infection among those individuals with known HBV status. As AFB\textsubscript{1} exposure appears to be high in Guatemala, these

reported in Guatemala\textsuperscript{8,9} are comparable to the levels found in some high-rate parts of China before the transition there from a maize-based to a rice-based diet.\textsuperscript{38}
results suggest that the R249S mutation in HCC may be less common in regions where HBV is not endemic than in HBV-endemic regions. However, results from a previous meta-analysis reported only a 6% mean difference (95%CI: -1, 13%, P = .11) in the proportion of the R249S mutation between HBV positive and HBV negative cases with similar AFB1 exposures. 

In total, there were 24 mutations with a G -> T transversion. The majority of the G -> T transversions occurred in codon 249, and the rest were in codon 157 (n = 2) and codon 248 (n = 1). These somatic mutations have been reported in HCCs previously. Furthermore, one third of the HCCs in the current study has a C -> T transition at a CpG, suggesting that DNA methylation changes could play a role in these tumors. For example, a study that characterized genome-wide DNA methylation patterns in HCC identified a large subset of CpG sites associated with HCV infection, liver cirrhosis, or HCC.

The p53 protein plays an important role in growth regulation as well as in tumor suppression and DNA repair. Somatic mutations in TP53 are common alterations in the majority of human cancers, including HCC. The most common site for TP53 mutations is in the DNA-binding domains, which decreases the binding affinity to responsive elements, leading to reduced activity of the p53 protein. Etiological factors, in addition to AFB1, such as HBV, HCV, and alcohol have been implicated in generating TP53 mutations in HCC. Inactivation of p53 by core proteins produced by HBV (eg, HBx and CT-HBx) and HCV (eg, NS3 and NS5A) may lead to the development and progression of HCCs. Furthermore, in animal studies, p53 has been implicated in the progression of steatosis to non-alcoholic steatohepatitis (NASH), involving mechanisms such as upregulation of TP53 activity with increased mRNA levels of p21 and p66ShC, which are associated with fibrosis severity.

The current study also identified somatic mutations in other known HCC driver genes, including CTNNB1, ARID1A, ARID2, AXIN1, among others. A recent review of 20 studies with HCC genome sequence data reported recurrent mutations in 12 genes, including TP53, CTNNB1, AXIN1, ALB, ARID2, ARID1A, RPS6KA3, APOB, RB1, CDKN2A, LRP1B, and PTEN. Mutations in CTNNB1 have commonly been reported in HCCs, with prevalences ranging from 20% to 38%. The results of the current study are in line with prior findings, as CTNNB1 mutations were identified in 17% of the HCCs. In addition, mutations in ARID1A and ARID2, which are involved in WNT (cell-differentiation) pathway activation, have also been reported. Deregulation of ARID1/2 signaling appears to affect 6%-18% of HCC tumors, similar to the mutation prevalence found in the current study (17% ARID1A and 28% in ARID2). Furthermore, inactivating mutations in ARID2 have been found in HCCs of various etiologies. A European study that performed exome sequencing analysis of 243 HCCs reported associations between some known risk factors and mutational patterns. The study reported that alcohol-related HCCs were more likely to have mutations in CTNNB1, TERT, CDKN2A, SMARCA2, and HGF, while HBV-related HCCs were more likely to have TP53 mutations. In contrast, no mutations were identified in either HCV- or NAFLD-related HCCs. Overall, nearly 100 somatic mutations have been determined as HCC driver mutations, and approximately five to six driver mutations are considered necessary to cause cancer within a particular patient.

The current study provides evidence that the elevated incidence and mortality of HCC in Guatemala could at least partially be explained by AFB1. However, other factors, such as other mycotoxin exposure, metabolic disorders (eg, obesity, diabetes, and NAFLD), among others, may also be contributors. For example, our previous work reported high prevalences of NAFLD (60.1%), diabetes (21.6%), and obesity (30.9%) among Guatemalan adults (≥40 years old). In addition, a study found high levels of fumonisin B1 (FB1) in maize samples across Guatemala. No studies have examined the role of these risk factors for liver cancer in either Guatemala or other Central American countries where there is also an unusual 1:1 ratio of liver cancer incidence between men and women.

To our knowledge, this study is the first to examine mutations in HCC from Guatemala. Other strengths include the sizable number of HCCs included and the histologic confirmation of all diagnoses by a liver cancer pathologist. Limitations of the study include that the tumors were not collected as part of a systematic protocol, so they may not be representative of all HCCs seen at the study hospitals, or in the country. In Guatemala, it is estimated that approximately 40% of HCCs are biopsied. Another limitation is that there was incomplete information available on risk factors, so it was not possible to determine the extent to which the R249S mutation corresponded to AFB1 exposure. It was also not possible to determine HBV or HCV status of all cases. In addition, there was incomplete clinical information available on the tumors, so the number of somatic mutations could not be correlated with extent of disease. Furthermore, mutations in the TERT gene, the most commonly mutated gene in HCC, could not be examined because it was not included in the panel of 253 cancer-related genes that were sequenced in the RNAlater samples.

In conclusion, the presence of the TP53 AFB1-signature mutation suggests that AFB1 may play a role in the high incidence of HCC in Guatemala. As the prevalence of HBV was low among those with known HBV status, the current results suggest that AFB1 is associated with HCC in the absence of concomitant HBV infection. These results suggest that further investigation of AFB1 and other risk factors for HCC in Guatemala is warranted.

**CONFLICT OF INTEREST**

The authors declare no potential conflicts of interest.

**AUTHOR CONTRIBUTIONS**

Conceptualization: Michael Dean, Katherine McGlynn, Eduardo Gharzouzi. Resources: Jeremy Ortiz, Giovanna Bendfeldt-Avila, Roberto Orozco, Eduardo Gharzouzi, Joaquin Barnoya. Investigation: Michael Dean, Yi Xie, Mingyi Wang, Dongjing Wu, Herbert Higson, Elisa Lee, Kedest Teshome, David Kleiner. Methodology: Michael Dean, Yi Xie, Mingyi Wang, Dongjing Wu, Herbert Higson, Elisa Lee, Kedest Teshome. Validation: David Kleiner. Formal analysis: Christian S. Alvarez, Michael Dean.
Visualisation: Christian S. Alvarez, Katherine McGlynn, Michael Dean. Writing - original Draft Preparation: Christian S. Alvarez. Writing - review & editing: Christian S. Alvarez, Katherine McGlynn, Michael Dean, Eduardo Gharzouzi, Roberto Orozco, Joaquin Barnoya, John Groopman.

All authors have read and approved the final version of the manuscript.

Christian S. Alvarez, the corresponding author, had full access to all the data in this study and takes complete responsibility for the integrity of the data and the accuracy of the data analysis.

TRANSPARENCY STATEMENT

The lead author, Christian S. Alvarez, affirms that this manuscript is honest, accurate, and transparent account of the study being reported; no aspects of the study have been omitted; any discrepancies from the study as planned have been explained.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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