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# Absolute and Relative Quantification of Buccal 8-OHdG Levels in an Iraqi Cohort with Autism Spectrum Disorder

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## Abstract

**Background**: Autism spectrum disorder (ASD) is frequently linked to systemic oxidative stress, yet the exact accumulation of oxidative DNA lesions remains unclear. This study investigated oxidative DNA damage in individuals with ASD. **Methods**: Genomic DNA was extracted via non-invasive buccal swabs from 88 participants with ASD (73 males, 15 females; age range: 2.7–32 years) in Kirkuk, Iraq. The assay was calibrated using internal baseline negative and reference positive controls due to the challenge of acquiring external clinical controls. Oxidative DNA damage was quantified colorimetrically using 300 ng of input DNA per reaction with the EpiQuik™ 8-OHdG DNA Damage Quantification Direct Kit, evaluated through absolute and relative quantification models. **Results**: Across the 88 buccal DNA samples, the absolute model yielded 51 zero-censored samples (OD $\leq$ 0.126), 29 low-positive samples ($\leq$0.01%), and 8 samples exceeding the 0.01% analytical reference threshold. The relative model produced the exact same distribution (51 zero-censored, 29 $\leq$0.01%, and 8 >0.01%). **Conclusions**: These results demonstrate a low baseline signal of 8-OHdG within the buccal DNA. However, without directly measuring OGG1 enzymatic activity or repair kinetics, no definitive conclusions can be drawn regarding the functionality or efficiency of the base excision repair pathway.

**Keywords:** autism spectrum disorder (ASD); 8-OHdG; oxidative DNA damage; base excision repair (BER); OGG1 enzyme; buccal swabs

## 1. Introduction

The term "oxidative DNA damage" describes the molecular changes in nucleic acids (DNA) brought on by reactive oxygen species (ROS), which can be produced by external sources or during cellular metabolism [1].

These extremely reactive compounds may cause many forms of damage, including base alterations, strand breakage, and crosslinks, possibly resulting in genomic instability, mutations, and altered gene expression [2]. Cells use complex repair mechanisms, including base excision repair (BER), nucleotide excision repair (NER), and homologous recombination (HR), to mitigate the harmful consequences of oxidative damage, therefore preserving genomic integrity and avoiding damage buildup [3].

Reactive oxygen species (ROS), generated by cellular metabolism or external sources, directly interact with the DNA structure [4]. The resultant modifications are often categorized into three primary groups: first, base modifications, which occur when ROS induce chemical alterations to nitrogenous bases, most notably generating 8-oxo-7,8-dihydroguanine (8-oxoG), which frequently mispairs with adenine to cause mutations; second, strand

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breaks, where hydroxyl radicals directly cleave the phosphodiester backbone, resulting in single- or double-strand breaks; and third, crosslinks, wherein ROS induce linkages between DNA strands or between DNA and proteins, thereby obstructing both replication and transcription [5,6].

Reactive oxygen species (ROS) are highly reactive oxygen-containing molecules produced during oxidative phosphorylation due to the electron receptivity of molecular oxygen (O2). Mitochondria strictly regulate both the generation and clearance of these molecules. However, excessive ROS can oxidize essential proteins, lipids, DNA, and RNA, thereby compromising cellular structural integrity. Although a sophisticated network of antioxidant enzymes provides a robust defense mechanism to neutralize ROS and prevent oxygen toxicity, oxidative stress inevitably occurs if ROS levels overwhelm these elimination systems, disrupting redox homeostasis and triggering severe cellular damage [7].

Certain ROS serve as redox (reduction–oxidation reaction) messengers in cell signaling at normal concentrations. Consequently, oxidative stress will not only impair cellular components but also interfere with ROS-dependent cell signaling. Chronic inflammation induced by prolonged oxidative stress may result in a number of illnesses. For a long time, researchers have examined immune system dysfunction, inflammation, and oxidative stress in ASD. Individuals with ASD were shown to produce more hydrogen peroxide in their mitochondria [3,8].

ASD has been linked to oxidative stress, as seen by elevated lipid peroxidation indicators, reduced blood antioxidant levels, and altered antioxidant enzyme activity. ASD was linked to cytokine/chemokine imbalance, elevated expression of neuroinflammation markers in postmortem brain tissues, and elevated plasma levels of pro-inflammatory cytokines. These results imply that ASD is linked to immune system malfunction [8,9].

While oxidative stress represents a significant pathological pathway in autism spectrum disorder (ASD) [10], the etiology of the disorder is fundamentally anchored in a complex genetic and neurodevelopmental framework. Specifically, ASD has a heterogeneous genetic architecture involving rare and common variation, with consequences for diverse neurodevelopmental processes [11]. More specific examples include chromatin-associated transcriptional dysregulation in 16p11.2-deletion models and neuroplastin–PMCA dysfunction associated with NPTN variants in a neurodevelopmental disorder that includes autism [12]. Therefore, the chemical DNA lesions associated with oxidative stress should be viewed as distinct from, rather than a cause of, these primary inherited or de novo genetic and gene-regulatory mechanisms that define the core neurodevelopmental basis of ASD [13].

8-hydroxy-2′-deoxyguanosine (8-OHdG or 8-oxo-dG) is an oxidized form of deoxyguanosine produced by hydroxyl radicals, singlet oxygen, and one-electron oxidants inside cellular DNA. 8-OHdG, as a modified nucleoside base, is significant due to its prevalence and its capacity to induce mutagenic G-to-T transversion mutations during DNA replication. 8-OHdG also plays a role in the epigenetic regulation of gene activation and repression by diminishing the binding affinity of MBD proteins to the CpG sites of DNA. Oxidatively damaged DNA is often amenable to repair by base excision repair (BER) enzymes [14,15].

Currently, 8-OHdG is well recognized as a sensitive biomarker of oxidative DNA damage and oxidative stress. Data indicate that elevated levels of 8-OHdG are significantly associated with exposure to detrimental environmental factors, including ionizing radiation, industrial pollutants, air pollution, cigarette smoking, and cancer therapies. Elevated levels of 8-OHdG are pathogenically connected with several age-related illnesses, including cancer, coronary heart disease, diabetes, and neurodegenerative disorders such as Alzheimer's disease [16,17].

In contrast to the urine 8-OHdG test, which primarily indicates the overall balance between oxidative damage and repair mechanisms in the body [18], directly measuring 8-OHdG levels in various cells and tissues under normal and pathological conditions would facilitate the identification of tissue-specific DNA oxidative damage. Consequently, this approach could yield further valuable information on the links between oxidative damage and illness, which enhances diagnostics [19].

DNA repair mechanisms are essential biological processes that rectify damage to the genetic code. In their absence, DNA lesions induce mutations that result in genomic instability and illnesses such as cancer. Cells use specific, precisely coordinated mechanisms to repair single-strand and double-strand DNA breaks [20,21].

Base excision repair (BER) is the main cellular process that eliminates oxidative DNA damage, particularly the highly damaging lesion 8-OHdG (commonly shortened to 8-oxo-dG, sometimes called 8-OHdG). If 8-oxoG is not fixed, it will couple with adenine during DNA replication, resulting in G-to-T mutations that are irreversible and cause cancer [22,23].

The enzyme OGG1 (8-oxoguanine DNA glycosylase) finds the 8-oxoG lesion and "flips" it out of the DNA helix into its active site. The link between the broken base and the sugar-phosphate backbone is then broken, leaving an apurinic/apyrimidinic (AP) site [24,25]. APE1 (Apurinic/apyrimidinic endonuclease 1) cleaves the DNA backbone at the exact spot where OGG1 left an empty AP site. This primes it for synthesis by producing a single-strand break with a pristine 3′-hydroxyl group [23]. DNA Polymerase Beta (Pol β) attaches itself to the processed break and replaces the damaged base by inserting the proper nucleotide, guanine, to restore correct base-pairing opposite the complementary cytosine on the template strand. Finally, the XRCC1-DNA Ligase III complex seals the remaining nick in the DNA backbone, restoring the pristine genetic code [26].

## 2. Materials and Methods

### *2.1. Materials*

The instruments and the commercial kits and reagents utilized throughout this study are detailed in Table 1 and Table 2, respectively.

**Table 1.** Instruments used in the experiment.

| NO. | Instrument | Model | Manufacturer, Country |
|---|---|---|---|
| 1. | Microplate Absorbance Reader | 800 TS | Agilent (BioTek Instruments), Winooski, VT, USA |
| 2. | Microplate Washer | 50 TS | Agilent (BioTek Instruments), Winooski, VT, USA |
| 3. | Carbon Dioxide ($CO_2$) Incubator | INCO 2/153 | Memmert GmbH + Co. KG, Schwabach, Germany |
| 4. | Notebook Computer | HP 625 | HP Inc., Palo Alto, CA, USA |

**Table 2.** Kits and reagents.

| NO. | Kits and Reagents | Manufacturer, Country |
|---|---|---|
| 1. | HigherPurity™ Swab DNA Extraction Mini Spin Kit | CANVAX biotech S.L., Valladolid, Spain |
| 2. | EpiQuik™ 8-OHdG DNA Damage Quantification Direct Kit (Colorimetric) | EPIGEN TEKTM, Farmingdale, NY, USA |

**Table 2.** *Cont.*

| NO. | Kits and Reagents | Manufacturer, Country |
|---|---|---|
| 3. | Distilled water, BP Grade (Pyrogen-Free) | Marksans Pharma, Mumbai, India |
| 4. | TE Buffer (10X) REF: BR0013, LOT: BR 24090601 | CANVAX biotech S.L., Valladolid, Spain |
| 5. | Plate seal | Local supplier, Kirkuk, Iraq |

### *2.2. Methods*

#### 2.2.1. Sampling

Participants

Eighty-eight participants (73 males, 15 females; age range: 2.7–32 years) diagnosed with autism spectrum disorder (ASD) were recruited across special needs education centers in Kirkuk, Iraq. Consistent methodological standards were maintained by applying identical diagnostic criteria (DSM-5, CARS, and M-CHAT) and standardized questionnaires for all participants. Ethical approval was granted by the University of Kirkuk (Approval Nos: 9431-40-7, 9432-40-7, and 9433-40-7), and informed consent was obtained from legal guardians in compliance with the Declaration of Helsinki. Sampling was conducted under the direct supervision of special needs carers and psychologists, occasionally in the presence of parents. To mitigate stress and avoid potential Post-Traumatic Stress Disorder (PTSD) in accordance with ethical mandates, participants with severe sensory aversion to the procedure were excluded from sampling. The demographic and baseline characteristics of the study participants are summarized in Table 3.

**Table 3.** Demographics of participants in the study.

| Baseline Characteristics | Total ASD Cohort ($N = 88$) |
|---|---|
| **Recruitment Period** | 8 December 2024 to 15 July 2025 |
| **Location** | Special Needs Education Centers, Kirkuk, Iraq |
| **Sex** | |
| - Males, *n* (%) | 73 (82.95%) |
| - Females, *n* (%) | 15 (17.05%) |
| **Age Range** | 2.7–32 Years |

#### 2.2.2. Inclusion and Exclusion Criteria

Participants were recruited between 8 December 2024 and 15 July 2025 from specialized special needs education centers in Kirkuk, Iraq. To ensure diagnostic homogeneity, the inclusion criteria required a confirmed clinical diagnosis of autism spectrum disorder (ASD) based on the DSM-5 criteria evaluated by specialized psychiatrists. Regarding the exclusion criteria, individuals under 2 years of age were strictly excluded from the study due to the clinical challenges of diagnostic imprecision during early infancy. Furthermore, individuals with a history or current diagnosis of cancer, as well as those who had undergone chemotherapy or radiotherapy, were strictly excluded to eliminate any confounding, treatment-induced elevations in oxidative DNA lesions and stress markers. Additionally, a few cases were excluded due to poor participant compliance during buccal swab collection or low genomic DNA extraction yields. Consequently, a final refined cohort of 88 participants (73 males and 15 females; age range: 2.7–32 years) was successfully verified and maintained for downstream quantification.

2.2.3. Sample Collection and DNA Extraction

The study adheres to human ethics; as we deal with a special needs community, we decided to collect non-invasive samples by scraping sterile buccal swabs to extract epithelial cells. The ectoderm is the source of both postmortem brain tissue and the epithelial cells obtained from the mouth lining using a buccal swab. The buccal epithelium is a component of the oral epithelial lining that arises from the surface ectoderm. Non-invasive buccal swabs were utilized for genomic DNA extraction via the HigherPurity™ Swab DNA Extraction Mini Spin Kit, with concentrations determined by Qubit fluorometry. Oxidative DNA damage was quantified colorimetrically using 300 ng of input DNA per reaction with the EpiQuik™ 8-OHdG DNA Damage Quantification Direct Kit (Epigentek, Farmingdale, NY, USA). The target 8-OHdG levels were recognized using specific capture and detection antibodies, followed by absorbance measurement at 450 nm using an 800 TS Microplate Absorbance Reader paired with a 50 TS Microplate Washer (Agilent BioTek Instruments, Winooski, VT, USA). All DNA concentrations were summarized in Table S1.

### *2.3. Experimental and Analytical Parameters*

The experimental assay was calibrated using a negative control baseline absorbance of 0.126 and a positive control reference value of 0.927. A six-point standard curve was established using known concentrations (5, 10, 20, 50, 100, and 200 pg) with corresponding optical density (OD) values ranging from 0.071 to 1.669. Linear regression analysis demonstrated a highly consistent calibration model, yielding a final unified slope parameter of 7.461 OD/ng after adjusting for unit uniformity. Absolute 8-OHdG quantification for each sample was subsequently calculated based on this derived slope relative to the individual template DNA mass. All optical density (OD)values were mentioned in the Table S2.

### *2.4. Statistical Analysis*

Handling of Below Detection Limit (BDL) Values and Descriptive Statistics

Samples falling below the analytical boundary where the sample OD was equal to or less than the Negative Control (NC) OD were systematically classified as Below Detection Limit (BDL) and assigned a strict zero percentage value to reflect the baseline cohort profile accurately. To evaluate the internal distribution of the 88 studied participants with autism spectrum disorder (ASD), descriptive statistics were applied. The cohort was stratified into frequencies and percentages ($n$, %) across three predefined clinical concentration criteria based on the absolute and relative quantification models: Above Average (>0.01%), Very Low Concentration, and Below Detection Limit (zero percentage).

## 3. Results

### *3.1. Quantification and Calculation of 8-OHdG Levels*

3.1.1. Absolute Quantification

To quantify the absolute amount of 8-OHdG, a linear regression standard curve was utilized to calculate the concentration and percentage of 8-OHdG in total DNA using the following formulas:

$$\text{8-OHdG (ng)} = \frac{\text{Sample OD} - \text{NC OD}}{\text{Slope}}$$

$$\text{8-OHdG \%} = \left( \frac{\text{8-OHdG Amount (ng)}}{\text{S}} \right) \times 100\%$$

where S is the amount of input sample DNA in ng.

Based on this absolute quantification model for the 88 ASD participants, the majority of the genomic DNA samples fell below the detection limit, with 51 samples recording a strict zero percentage. Out of the remaining detectable cohort, only 9 samples were higher than the average of 8-OHdG > 0.01%, and 28 samples presented very low concentrations near the analytical boundary. All results are presented in Supplementary Table S3.

#### 3.1.2. Relative Quantification

To determine the relative 8-OHdG status of two different DNA samples, a direct comparison against the internal positive and negative controls was performed using the following formula:

$$8\text{-OHdG } \% = \left[ \frac{(\text{Sample OD} - \text{NC OD}) \div \text{S}}{(\text{PC OD} - \text{NC OD}) \div \text{P}} \right] \times 100\%$$

where S is the input sample DNA in ng, and P is the input positive control DNA mass in ng.

The relative quantification results demonstrated high consistency and statistical alignment with the absolute data. The exact same 8 samples were found to be higher than 0.01%, exceeding the average threshold. Additionally, 29 samples exhibited very low concentrations, and 51 samples recorded a zero percentage level below the detection limit. All results are presented in Supplementary Table S4.

#### 3.1.3. Summary of Distribution Results

To provide a clear comparative view, the distribution of the studied autism spectrum disorder (ASD) cohort across both quantification models is summarized in the Table 4 below:

**Table 4.** Classification and distribution of absolute vs. relative 8-OHdG quantitative levels ($n = 88$).

| Concentration Criteria | Absolute Quantification ($n$) | Relative Quantification ($n$) |
|---|---|---|
| Higher than average 0.01% | 9 | 8 |
| Low detectable levels | 28 | 29 |
| Zero percentage | 51 | 51 |
| — | 88 | 88 |

## 4. Discussion

The primary objective of this descriptive case series was to investigate the relationship between autism spectrum disorder (ASD) and genomic DNA damage as a potential contributing factor to the pathogenesis of the disorder. While numerous previous studies have documented elevated 8-OHdG levels in peripheral biofluids, they often overlook a critical mechanistic perspective: these high extracellular concentrations may actually stem from mitochondrial dysfunction coupled with a highly efficient base excision repair (BER) system that actively expels damaged bases into the peripheral circulation. Consequently, fluid-based outcomes cannot provide an accurate representation of intra-nuclear genomic integrity until a direct examination of intact cellular DNA is performed.

Our findings from this investigated ASD cohort demonstrate that an absence of accumulated, intact oxidative DNA damage linked to 8-hydroxy-2′-deoxyguanosine within the target cells could be a result of several clinical, chronological, and environmental factors rather than exclusively reflecting enhanced cellular repair. First, this cohort has no history of exposure to acute genotoxic stressors like chemotherapy or radiation, which are the primary drivers of this specific marker. Second, because ASD is an early-onset disorder and our participants are young (under age 30), they have not reached the chronological horizon required for decades of cumulative DNA damage seen in aging diseases like Alzheimer's.

Finally, due to parental vigilance and social isolation, these individuals experience an "environmental shielding effect"—they are home-bound, completely abstain from tobacco smoke and alcohol consumption (0%), and comparatively well insulated from ambient air pollution. This pristine domestic environment could minimize exogenous oxidative insults.

Interestingly, these cellular outcomes provide a novel counter-perspective that conceptually aligns with, rather than contradicts, existing literature. Previous research has consistently indicated that children with ASD exhibit abnormal levels of urinary 8-OHdG, a well-established biomarker for oxidative DNA lesions [27]. Furthermore, extensive studies have repeatedly demonstrated significantly elevated concentrations of 8-OHdG within the blood, urine, and brain tissues of individuals with ASD [28,29].

In this context, a previous study suggested that a combined panel of blood-based parameters—including 25(OH)D3, hs-CRP, 8-OH-dG, and CYP1B1 gene expression—could serve as a potential multi-biomarker tool for early ASD diagnosis [30]. Similarly, substantial oxidative DNA damage has been captured in general serum profiles and within circulating cell-free DNA (cfDNA). However, while tracking 8-oxodG on serum cfDNA reflects systemic oxidative stress, it presents notorious technical challenges due to the low concentration and inherent instability of cell-free nucleic acids in circulation [31–33]. Another study concluded that there were no discernible differences in plasma superoxide dismutase (SOD) and urinary 8-OHdG levels, as well as other urinary assessments that concluded no significant variations in 8-oxo-dG levels between ASD individuals and controls [34,35].

The present study expands upon this collective evidence by shifting the investigative matrix to buccal epithelial cells. By utilizing a localized, intact cellular matrix rather than cell-free fluids, our approach offers a stable, DNA-rich, and non-invasive alternative for absolute and relative quantification.

*Limitations of the Study*

Direct investigation of oxidative DNA damage markers, such as 8-OHdG, in human brain tissue is strictly constrained to post-mortem samples due to ethical and clinical barriers in living subjects. However, post-mortem tissues introduce substantial confounding factors, including agonal hypoxia and progressive tissue degradation. To circumvent these challenges, this study utilized buccal swabs as a non-invasive surrogate biomarker for living individuals with autism spectrum disorder (ASD). Given their shared embryonic origin derived from the ectoderm, buccal mucosal cells and the central nervous system exhibit closely aligned physiological pathways. However, the possibility of other forms of DNA damage still exists, or there could be cellular damage and oxidative stress in the brain cells. Neuronal mechanisms implicated in ASD, including isoform-dependent synaptic interactions, remain outside the scope of the present buccal DNA lesion measurement. This methodology offers a safe, pain-free, and clinically viable alternative to evaluate genomic damage without exposing vulnerable patients to invasive interventions.

Given that the core enzymatic machinery of the base excision repair (BER) pathway is genetically conserved and fundamentally uniform across various somatic tissues, the pronounced genomic integrity and efficient clearing of 8-OHdG observed in buccal mucosa cells may serve as a peripheral proxy reflecting a competent intrinsic DNA repair capacity within the individual. However, translating these localized peripheral findings directly to the central nervous system (CNS) requires cautious neurological contextualization.

Unlike rapidly proliferating and regenerative buccal epithelial cells, mature cerebral neurons are post-mitotic cells that cannot replicate to dilute genetic lesions, making them highly vulnerable to cumulative genomic damage over time. Furthermore, despite representing only about 2% of total body weight, the human brain consumes nearly 20% of systemic oxygen, generating an exceptionally high baseline of mitochondrial reactive oxy-

gen species (ROS). Consequently, even if the genetic efficacy of the BER system remains constant between oral and neural tissues, the sheer volume of oxidative attack within the microenvironment of the brain can easily saturate local repair limits.

This distinct tissue-specific metabolic demand highlights that, while peripheral cellular DNA profiling offers invaluable, non-invasive insights into systemic repair biology, it may capture a state of successful genomic clearance that is under far greater physiological strain within the neuropathological landscape of autism spectrum disorder (ASD). Additionally, the clinical heterogeneity of ASD across different age groups limits the generalization from a pooled peripheral assay measurement to a shared global disease mechanism.

Furthermore, a key limitation of this study is that we measured 8-OHdG levels in total DNA extracted from buccal swabs as a general marker of DNA oxidative damage, without directly isolating or assessing mitochondrial-specific oxidative stress or its direct byproducts. Given the established link between mitochondrial dysfunction and ASD pathophysiology, future studies should incorporate specific mitochondrial assays to pinpoint the exact cellular source of the observed oxidative stress.

Finally, a notable limitation of this study is that we did not directly quantify the functional kinetic efficiency of the intra-nuclear DNA repair machinery, nor did we measure the specific enzymatic concentrations or expression levels of key repair enzymes (such as OGG1 or APEX1). Our hypotheses regarding cellular repair dynamics remain inferential, based on the observed integrity of the collected buccal DNA. Future studies incorporating direct enzymatic assays and expression profiles are warranted to definitively clarify these intracellular pathways.

Ultimately, within a descriptive framework, the observed low 8-OHdG values in buccal swab DNA provide an initial baseline profile for this cohort, while acknowledging that underlying rates of oxidative lesion formation, cellular turnover, buccal sample composition (including microbial and mitochondrial DNA), and low-signal analytical sensitivity remain unresolved variables that require targeted functional studies rather than direct inference from static measurements.

## 5. Conclusions

In conclusion, in this descriptive case series the studied ASD cohort demonstrated a predominant absence of accumulated oxidative DNA damage linked to 8-hydroxy-2′-deoxyguanosine (8-OHdG) within cellular genomic DNA; however, these findings do not entirely rule out other forms of cellular damage, alternative DNA modifications, or localized oxidative stress within specific central nervous system pathways. Future research should explore broader panels of oxidative markers to further elucidate the complex cellular pathophysiology of ASD.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/dna6040060/s1, Table S1: The DNA concentration for each sample in the plate (8 µL was used for each well); Table S2: The optical density (OD) for each well measured at a wavelength of 450 nm; Table S3: The absolute quantification ratio of 8-OHdG for each sample; Table S4: The relative quantification of 8-OHdG for each sample.

**Author Contributions:** Z.M.K. designed the study and contributed to sample collection, laboratory work, data interpretation, data analysis and manuscript drafting. The study was advised by H.Y.H. as an advisor and by I.G.A. as a co-advisor. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the University of Kirkuk (Approval Nos: 9431-40-7, 9432-40-7, and 9433-40-7), approved on 7 November 2024.

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** The raw data supporting the conclusions of this article are included within the article and its Supplementary Materials, and further inquiries can be directed to the corresponding author.

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**Conflicts of Interest:** The authors declare no conflicts of interest.

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