

## Original Article

# Oxidative Stress Markers and Genetic Polymorphisms of Glutathione S-Transferase T1, M1, and P1 in a Subset of Children with Autism Spectrum Disorder in Lagos, Nigeria

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### ABSTRACT

**Background:** The role of oxidative stress has been identified in the development of autism spectrum disorder (ASD), and polymorphisms of glutathione S-transferase have been associated with some diseases linked to oxidative stress. Hence, we evaluated the serum levels of oxidative stress markers and investigated genetic polymorphisms of glutathione S-transferase associated with autism. **Materials and Methods:** Forty-two children clinically diagnosed with ASD using the Diagnostic and Statistical Manual for Mental Disorders (DSM-5) criteria and a clinical interview were included in the study. Twenty-three age-matched controls without any known genetic/developmental disorder were also recruited. Oxidative stress markers along with the genetic polymorphisms of glutathione S-transferase were determined. **Results:** Reduced glutathione in ASD patients was significantly lower than the control ( $P = 0.008$ ), whereas other oxidative stress markers measured were not significantly different in both the control and case populations. The frequencies of *GSTT1* and *GSTMI* null genotypes were lower among the controls compared with the cases, however, no association risk was observed. The observed risk of carrying Val/Val genotype among the cases was approximately six times that of the controls. **Conclusion:** Individuals with ASD showed a significant diminished level of reduced glutathione, however, the distribution of *GSTT1*, *GSTMI*, and *GSTPI* polymorphisms was not found to be associated with autism in this study population.

**KEYWORDS:** Autism, genetic polymorphisms, glutathione S-transferase, Nigeria, oxidative stress

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## INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that presents with developmental abnormalities that are usually evident early in childhood with certain deficits at variance with typical development. Though often observed before the age of 3 years, it may not be diagnosed by 3 years, especially in the African setting where late presentation is common in clinics. It is usually characterized by social communicative and language impairments along with restricted and repetitive behavior and interests.<sup>[1]</sup> It is approximately four times more common in males than females, and affects 1 in 68 children; the average

recurrence risk of autism in siblings of affected children is approximately 10%.<sup>[2,3]</sup>

Although the pathophysiology of autism is largely unknown, the role of oxidative stress has been identified.<sup>[4]</sup> Oxidative stress occurs when there is an imbalance between levels of reactive oxygen species (ROS) and antioxidants as a consequence of depletion

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of the antioxidants or excessive generation of ROS. ROS are usually produced during normal cellular metabolism and are highly reactive. Several antioxidant defense mechanisms, which can either be enzymatic (catalase, glutathione peroxidase, superoxide dismutase) or nonenzymatic (vitamin C and E, zinc, reduced glutathione, selenium, riboflavin, carotenoids, etc.), help detoxify ROS.<sup>[5]</sup> ROS may impair biological molecules such as carbohydrates, lipids, proteins, and DNA, which may result in cell dysfunction. Accumulation of ROS may also lead to production of pro-inflammatory and anti-inflammatory cytokines.<sup>[6]</sup> Glutathione-S-transferases (GST) are multifunctional enzymes that are involved in the detoxification of toxic substances caused by oxidative stress products, electrophilic compounds, and carcinogens. They are conjugated to glutathione during phase II of xenobiotic metabolism.<sup>[7]</sup> In humans, GSTs are classified into eight classes, including  $\alpha$  (alpha),  $\mu$  (mu),  $\kappa$  (kappa),  $\omega$  (omega),  $\pi$  (pi),  $\sigma$  (sigma),  $\theta$  (theta), and  $\zeta$  (zeta), that are encoded by the *GSTA*, *GSTM*, *GSTK*, *GSTO*, *GSTP*, *GSTS*, *GSTT*, and *GSTZ* genes, respectively. Glutathione S-transferase-mu (*GSTM1*), glutathione S-transferase-theta (*GSTT1*), and glutathione S-transferase-pi (*GSTP1*) are located on chromosome 1p13.3, 22q11.2, and 11q13, respectively,<sup>[8]</sup> and are the most widely studied GSTs. They play significant roles in the cellular protection against toxic products of oxidative stress.<sup>[9]</sup> *GSTT1* and *GSTM1* polymorphisms are caused by gene deletion which results in the absence of enzyme activity in individuals with the *GSTT1* and *GSTM1* null genotypes.<sup>[10]</sup> Polymorphisms of GSTs have been associated with some diseases linked to oxidative stress such as asthma, lung cancer, and type 2 diabetes mellitus.<sup>[11-13]</sup>

Although autism has a strong genetic background, prevailing evidence supports the involvement of genetic, epigenetic, and environmental factors. Little is known regarding the degree to which these genetic variations underlie symptom variability in typical ASD cases; single-nucleotide polymorphisms (SNPs) are observed genetic markers which have assisted researchers to search for genes associated with complex diseases. SNPs have been used to help predict whether an individual has a certain disease or a subtype of a certain disease.<sup>[14]</sup> SNP-diagnostic models have been constructed for several diseases, which have been useful in classifying diseases such as hypertension and asthma.<sup>[15]</sup> Some researchers have suggested that SNPs have the potential to offer accurate classification of ASD symptom severity.

In a systematic literature review examining oxidative stress markers and polymorphisms in autism population, ASD patients showed decreased blood levels of

reduced glutathione (27%), glutathione peroxidase (18%), methionine (13%), and cysteine (14%), as well as increased concentrations of oxidized glutathione (45%) relative to controls, whereas superoxide dismutase, homocysteine, and cystathionine showed no association with ASDs. These researchers suggested that in explaining Autism aetiology, there is a role for glutathione metabolism, the transmethylation cycle, and the transsulfuration pathway, although these findings should be interpreted with caution, and calls for larger, more standardized studies have been made.<sup>[16]</sup>

There are little or no studies available to the knowledge of the authors that explore biological or genetic markers in individuals with neurodevelopmental disorders such as ASD in Africa generally and Nigeria in particular. While we are faced with multiple challenges of diagnosis and interventions locally, there is a need to also begin to explore what could be the biomedical associations identified in our local populations with ASD. This will be useful in helping researchers better understand the role such metabolic and genetic associations play in the ASD condition within our own local population of interest, and hopefully assist in the development of useful solutions.

Hence, we aimed to evaluate serum levels of reduced glutathione, catalase, malondialdehyde (MDA), and GST as biomarkers of oxidative stress in relation to ASD patients, as well as investigated genetic polymorphisms of glutathione S-transferase M1, T1, and P1 associated with autism in Lagos, Nigeria.

## MATERIALS AND METHODS

### Participants

Eligible cases were enrolled from the Child and adolescent Unit of the Department of Psychiatry, Lagos University Teaching Hospital (LUTH) and Federal Neuro-Psychiatry Hospital in Lagos. Age-matched controls were also selected from the paediatric and general outpatient clinic of LUTH. Eligibility criteria included age 4-14 years and having a diagnosis of ASD. Individuals with observable comorbid congenital anomalies, chronic medical disorders, or other psychiatric disorders were excluded. Forty-two study participants were diagnosed as having ASD along with 23 age-matched controls without any known genetic/developmental disorder.

### Participants' assessment

All participants had a basic sociodemographic proforma completed for them by their accompanying caregiver. A diagnosis of autism was confirmed in ASD participants enrolled following a clinical interview and assessment

by a child psychiatrist. Assessments involved an in-depth clinical interview and evaluation based on the ASD criteria of the Diagnostic and Statistical Manual for Mental Disorders Fifth Edition (DSM 5).<sup>[1]</sup>

### Sample collection

Five milliliters of fasting blood sample was collected from each participant following standard laboratory procedure; 3 ml blood sample into heparin bottle to assay for oxidative stress markers and 2 ml into EDTA bottle for DNA analysis. All samples were processed and stored in a -80°C freezer till it was time for sample analysis.

### Evaluation of oxidative stress markers

#### Determination of superoxide dismutase activity

Superoxide dismutase (EC 1.15.1.1) catalyzes the dismutation of superoxide anions converting them to hydrogen peroxides. Superoxide dismutase activity was determined by its ability to inhibit the auto-oxidation of epinephrine determined by the increase in absorbance at 480 nm, as described by Sun and Zigma.<sup>[17]</sup>

#### Reduced glutathione determination

The reduced glutathione (GSH) content was estimated according to the method described by Sedlak and Lindsay.<sup>[18]</sup>

#### Determination of Lipidperoxidation

MDA, an index of lipid peroxidation was determined using the method of Buege and Aust.<sup>[19]</sup>

#### Glutathione S-transferase activity assay

GST activity was assayed spectrophotometrically by monitoring the conjugation of 1-chloro-2, 4-dinitro benzene (CDNB) with GSH at 340 nm.<sup>[20]</sup>

### Genomic DNA extraction

DNA extraction was done using column extraction kits (Jena Bioscience, Germany) following manufacturer's instruction.

### Genotyping of Glutathione S-transferases polymorphism

Polymorphisms of *GSTM1* and *GSTT1* were detected by single tube multiplex amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) according to the method described by Abdel-Rahman *et al.*<sup>[21]</sup> and Arand *et al.*<sup>[22]</sup> using the following primers:  $\beta$ -globin gene as an internal control with the forward primer: 5'-GAA GAG CCA AGG ACA GGT AC-3' and reverse primer: 5'-CAA CTT CAT CCA CGT TCA CC-3'; 5'-GAA CTC CCT GAA AAG CTA AAG C-3' and 5'-GTT GGG CTC AAA TAT ACG GTG G-3' for *GSTM1*; 5'-TTC CTT ACT GGT CCT CAC ATC T-3' and 5'-TCA CCG GAT CAT GGC CAG CA-3' for *GSTT1*. Genomic DNA (50–100 ng) was amplified in a

total reaction volume of 25  $\mu$ l with 10 pmol of each of the primers, 5X Taq master containing Taq polymerase, dNTPs, KCl and MgCl<sub>2</sub> (Jena Bioscience, Germany), and PCR grade water. Cycling conditions were 2 min at 94°C for initial denaturation, followed by 35 cycles of 94°C for 1 min, 62°C for 1 min for annealing, 72°C for 1 min (extension), and a final extension at 72°C for 2 min to complete the process. PCR products were separated on a 2% agarose gel, and *GSTM1* was identified by a 215 bp band, *GSTT1* by a 473 bp band, and  $\beta$ -globin gene by a 268 bp band.

### GSTP1 genotyping

*GSTP1* polymorphism was genotyped according to the method described by Nock *et al.*<sup>[23]</sup> Briefly, the *GSTP1* Ile105Val (rs947894) polymorphism was detected using these primers: 5'-ACC CCA GGG CTC TAT GGG AA-3' and 5'-TGA GGG CAC AAG AAG CCC CT-3'. 25  $\mu$ l PCR reaction containing 50–100 ng DNA, 10 picomoles of each primer, 5X Taq Master containing Taq polymerase, dNTPs, KCl and MgCl<sub>2</sub> (Jena Bioscience, Germany), and PCR grade water. Cycling conditions: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, extension at 72°C for 1 min. A final extension step of 72°C at 2 min was carried out to terminate the process. Ten microliters of PCR product was digested with 5 U BsmA1 (New England Biolabs, USA) at 55°C for 1 hour. The digested samples were run on 2.5% agarose gel stained with ethidium bromide. A 176 bp (Ile/Ile), 91 and 85 bp (Val/Val) and 85-, 92-, and 176-bp fragments (Ile/Val) were identified.

### Ethical consideration

Ethical approval was obtained from the ethical Committees of the Lagos University Teaching Hospital and Federal Neuro-psychiatric hospital prior to commencement of the study. A written informed consent was obtained from the participant's care giver who was in all cases the accompanying parent. Parents were assured of confidentiality, and purpose of the study was explained to them in simple clear language. Standard sterile and health procedures were observed during blood sample collection.

### Statistical analysis

Results are shown as median (interquartile range) and are compared with the Mann-Whitney U test. Genotypes and allele frequency between case and control populations were compared using Fisher's exact test. Odds ratio and 95% confidence interval were calculated to estimate risk caused by the polymorphisms. Hardy-Weinberg equilibrium analysis was performed to compare the observed and expected frequencies. A *P*-value of <0.05 was considered to be statistically significant.

GraphPadPrism 5.0 software (GraphPad software, California, USA)<sup>[24]</sup> was used for the analysis.

## RESULTS

Age of the participants ranged from 3 years to 14 years with a mean age of 8.4 years ± 3.6 standard deviations. Of the cases, 30 (71.4%) were aged below 12 years and 18 (78.3%) of the controls were of the same age range. The others were aged between 12 and 14 years. The ages were not statistically significantly different ( $P = 0.549$ ). Male participants were 30 (71.4%) in the cases and 14 (60.9%) in the control group. Therefore, gender differences among the cases and controls were not statistically significant with  $P$  values of 0.549 and 0.617, respectively.

The levels of oxidative stress markers examined in this study included GSH, SOD, GST, and MDA [Table 1]. The GSH value of the autistic group (0.45) was lower than the control (0.83) with  $P = 0.008$ . Median SOD value of the autistic group (1.50) was higher than the control group (1.20) but was not significant ( $P = 0.194$ ). GST values of autistic and control populations were 0.03 and 0.02, respectively. The difference, however, did not result in any statistical significance ( $P = 0.955$ ). Autistic

group had higher level of MDA (0.05) compared with the control (0.04); this, however, did not show any statistical significance ( $P = 0.523$ ).

Table 2 shows the distribution of *GSTT1* and *GSTM1* genotypes among the cases and the controls. The frequency of *GSTT1* null and *GSTM1* null genotypes were 4.3% and 13%, respectively, in the control population whereas the autistic group had 11.9% for the *GSTT1* null genotype and 33.3% for the *GSTM1* null genotype. No association was observed between *GSTT1* polymorphism and the risk of developing autism ( $P = 0.411$ ). In the case of *GSTM1* polymorphism, despite the higher frequency of *GSTM1* null in the case group than the controls, no association was found, although the  $P$  value (0.087) approached a statistical significance. The combined effect of *GSTT1* and *GSTM1* was investigated. A total of 7.1% of the cases had double null genotype, and 61.9% had double present genotypes. In the control population, none had double null genotype whereas the frequency of double present genotype was 82.6%. When *GSTT1* and *GSTM1* present was used as the reference, the combined *GSTT1/GSTM1* genotypes showed no statistical significance [Table 2].

**Table 1: Oxidative stress markers of study participants**

Oxidative stress markers		N	Median [IQR]	P-value
GSH level (U/mg protein)	Cases	42	0.46 [0.30–0.63]	0.008
	Control	23	0.83 [0.47–1.23]	
SOD activity (U/mg protein)	Cases	42	1.50 [1.10–2.10]	0.194
	Control	18	1.20 [0.78–2.25]	
GST activity (U/mg protein)	Cases	42	0.03 [0.02–0.03]	0.955
	Control	23	0.02 [0.02–0.04]	
MDA level (U/mg protein)	Cases	42	0.05 [0.03–0.07]	0.523
	Control	19	0.04 [0.03–0.07]	

GSH, Reduced glutathione; SOD, Superoxide dismutase; GST, Glutathione S-transferase; MDA, Malondialdehyde; IQR, Interquartile range

**Table 2: Frequency distribution of *GSTT1* and *GSTM1* genotypes among the patients and control populations**

Genotype	Cases, n (%)	Control, n (%)	P-value	OR (95%CI)
<i>GSTT1</i>				
[+]	37 (88.1)	22 (95.7)	0.411	2.97 (0.33–27.14)
[-]	5 (11.9)	1 (4.3)		
<i>GSTM1</i>				
[+]	28 (66.7)	20 (87.0)	0.087	3.33 (0.84–13.16)
[-]	14 (33.3)	3 (13.0)		
<i>GSTT1/M1</i>				
[+]/[+]	26 (61.9)	19 (82.6)	-	1 (Reference)
[+]/[-]	11 (26.2)	3 (13)	0.213	2.68(0.66–10.95)
[-]/[+]	2 (4.8)	1 (4.4)	1.00	1.46 (0.12–17.33)
[-]/[-]	3 (7.1)	-	0.267	5.15 (0.25–105.7)

*GSTT1*, Gltathione S-transferase T1; *GSTM1*, Gltathione S-transferase M1; Wild [+] and null [-] genotypes; OR, Odds ratio; CI, Confidence interval

**Table 3: Distribution of *GSTP1* genotypes among patients and control participants**

	Cases, <i>n</i> (%)	Control, <i>n</i> (%)	<i>P</i> -value	OR (95% CI)
<b>Genotypes</b>				
Ile/Ile	16 (38.1)	11 (47.8)	Reference	1.0
Ile/Val	18 (42.9)	11 (47.8)	1.00	1.13 (0.38–3.29)
Val/Val	8 (19.0)	1 (4.4)	0.22	5.5 (0.6–50.47)
Ile/Val + Val/Val	26 (61.9)	12 (52.2)	0.59	1.49 (0.53–4.17)
<b>Allele</b>				
Ile	0.595	0.717		
Val	0.405	0.283	0.166	1.73 (0.795–3.75)
<b>HWE</b>				
<i>P</i> -value	0.474	0.389		

*GSTP1*, Glutathione S-transferase P1; OR, Odds ratio; CI, Confidence interval.  $\chi^2$  test for the genotypic distribution between the cases and the control = 2.74 and the *P*-value = 0.254. OR for *GSTP1* genotypes were calculated using Ile/Ile as reference

The distribution of all the *GSTP1* genotypes among the cases and controls were in agreement with the Hardy–Weinberg equilibrium (*P* = 0.474 and 0.389 for cases and controls, respectively). The frequency of Ile/Ile genotype among the cases was 38.1% whereas the control was 47.8%. Ile/Val genotype was observed in 42.9% of the cases whereas the controls had 47.8%. The overall distribution of *GSTP1* polymorphism in both the case and control populations did not show any statistical significance. The frequency of having Val/Val genotype among the cases (19%) was more than the control (4.4%), showing that the risk of carrying Val/Val genotype among the cases was approximately six times that of the controls using Ile/Ile as the reference. However, the difference between the cases and controls were not statistically significant. The allelic frequency of Ile was 0.595 and 0.717 among the cases and controls, respectively, whereas the Val allele was 0.405 among the cases and 0.283 for the control population [Table 3].

## DISCUSSION

This study explored the presence of oxidative biomarkers and specific genetic polymorphisms. Our modest finding in this environment does implicate certain oxidative stress markers, in this case glutathione in particular, with none of the explored polymorphisms identified.

The disequilibrium that exists between the oxidants and antioxidants in favor of the former leads to oxidative stress. Increased production of oxidants and/or decreased availability of antioxidants, therefore, triggers a cascade of oxidative reactions with pathological consequences, which include damage to lipids, proteins, and DNA ultimately leading to cell death.<sup>[25]</sup>

Glutathione is mainly concerned with the removal of oxygen free radicals and maintenance of membrane protein

thiols. It prevents cells against apoptosis and regulates activation of transcription factor such as NF- $\kappa$ B.<sup>[6]</sup> Decreased glutathione antioxidant capacity may result in increased superoxide production and a devastating inflammatory state.<sup>[26]</sup> Reduced level of GSH in autistic children found in this study is similar to reports of James *et al.*,<sup>[27]</sup> showing that elevated levels of free radicals that causes imbalance of GSH/GSSG redox antioxidant system may have deleterious implications in autistic children.

Nonsignificant but slight increase in the level of GST in children with autism observed in this study though at variance with that of Alabdali *et al.*,<sup>[28]</sup> could be a consequence of GST initiation to offset the effect of increased oxidative stress. MDA, an end product of peroxidation, was used as a marker of lipid peroxidation in this study. Free radicals are produced under normal biological conditions, however, when in excess, they bring about oxidative degradation of polyunsaturated fatty acids in the brain causing formation of lipids peroxides and hydrocarbon polymers, and ultimately membrane damage.<sup>[29]</sup> Similar but statistically significant results have been reported in autistic children in previous studies.<sup>[30]</sup> Decreased levels observed in this present study is consistent with the resulting lipid peroxidation. Serum MDA level found to be higher in children with autism reveals the extent of oxidative degradation of polyunsaturated fatty acids in the brain which causes formation of lipids peroxides and hydrocarbon polymers, leading to membrane impairment.

Prevalence of *GSTT1* and *GSTTM1* null genotypes is dependent on ethnic diversity and geographical locations of the population. For instance, frequency of *GSTT1* null genotype among the Caucasians, Asians, and Africans was 13-26%, 35-52%, and 29-50%, respectively, whereas the frequencies of *GSTMI* null genotype was

10-63%, 42-54%, and 42-60% among the Africans, Asians, and Caucasians, respectively.<sup>[31,32]</sup> The frequency of *GSTT1* null genotype among the controls in this present study was lower than the previous studies. The higher frequency of *GSTT1* null genotype in the case group was not associated with the risk of developing autism, even though the risk of having *GSTT1* null genotype among the cases was three times the controls. The frequency of *GSTMI* among the cases did not show any statistically different finding with the controls indicating that *GSTMI* polymorphism was not found to be associated with autism here. This is similar to other studies in chronic disorders that found no association between *GSTT1/GSTMI* polymorphisms and type 2 diabetes mellitus and prostate cancer.<sup>[7,33]</sup>

The frequency of Val/Val genotype was higher among the Egyptian control subjects than the asthmatic patients (12% vs. 6%)<sup>[34]</sup> while one study from Iran showed higher frequency in subjects with prostate cancer (14.3%) than the controls (2.4%) and reported that carriage of Val/Val genotype was associated with prostate cancer.<sup>[35]</sup> A southern Indian study found out that *GSTP1* Ile/Val genotype was significantly associated with a decreased risk for prostate cancer.<sup>[36]</sup> This study, however, showed that the risk of having *GSTP1* Val/Val genotype among the cases was five-fold higher than the controls though with no statistical association with autism. Limited studies of these polymorphisms in autism were available for a detailed comparison.

### Clinical implications

An understanding of the psychological deficits has helped to guide the development of interventions for these children with ASD. Such interventions are often behavioral, family-based, or educational. The exploration here into metabolic or genetic associations in our local population can lead to a better understanding of possible biologic vulnerabilities in children and adolescents with ASD and further guide research with the aim of discovering effective evidence-based interventions. Locally, there are potential benefits in establishing a role, if any, of oxidative and metabolic biomarkers in autism. These may include, for example, the emphasis on maternal folate supplementation, as have been suggested by other researchers. The inadequate resource burden required in diagnosis, treatment, and other interventions for ASD locally make the prospects of possible preventative strategies of huge relevance. The knowledge that ASD is a condition of public health significance, which has not been explored in-depth in the African setting and specifically in Nigeria, makes us believe that our findings here contribute to the body of knowledge locally in this field and will drive further research in the field.

### Study limitations

The study had a few limitations which the researchers are aware of. Our small study sample, due to cost of sample analysis, is a limitation in generalizing these findings to the general population; second, the sample size mandates a cautious interpretation of our findings. However, the description of our findings present a useful first step and obtaining some reference of what pattern of genetic and metabolic markers are observed in a local Nigerian population of ASD children. Second, the DSM 5 clinical diagnosis is reliable and sufficed to be used here; it is noteworthy that followed a structured interview schedule is useful for patient description and research uniformity. The non-use of a formal diagnostic schedule such as the Autism Diagnostic Observation Schedule (ADOS) or the Autism diagnostic interview (ADI) in making the ASD diagnosis is a limitation in supporting the ASD diagnosis; however, we do know that ASD diagnosis is primarily a clinical diagnosis. The lack of such formal diagnostic instruments in the study environment made it impossible to use these instruments, and as such may raise concern for some as to the accuracy of our diagnosis.

Finally, comorbid behavioral features among the individuals with autism could not be conclusively explored or disorders ruled out; this may have implications on our study findings as most of the individuals may have comorbid neurodevelopmental disorders, which though were clinically ruled out, metabolic disorders or unknown genetic disorders with ASD like phenotypes unknown to the researchers may still be present.

### CONCLUSIONS

Individuals with ASD in this study location showed a diminished level of reduced glutathione but the distribution of *GSTT1*, *GSTMI*, and *GSTP1* polymorphisms was not found to be associated with autism in this study population. There is a need for a larger study sample size in this Nigerian ASD population to further explore our findings.

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### Conflicts of interest

The authors declare no conflicts of interest.

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