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# Expression and promoter methylation status of two DNA repair genes in leukocytes from patients undergoing propofol or isoflurane anaesthesia

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## Abstract

Despite the widespread use of the anaesthetics propofol (PROP) and isoflurane (ISO), data about their toxicogenomic potential and interference in epigenetic events are unknown. This study evaluated the expression and methylation profile of two important DNA-repair genes (*XRCC1* and *hOGG1*) in 40 patients undergoing elective and minimally invasive surgery (tympanoplasty and septoplasty) under ISO or PROP anaesthesia. The endpoints were examined at three sampling times: before anaesthesia (T0), 2 h after the beginning of anaesthesia (T2) and 24 h after the beginning of surgery (T24). Both gene expressions were assessed by quantitative real-time polymerase chain reaction (qRT-PCR), whereas methylation specific-PCR (MS-PCR) evaluated the DNA methylation patterns. Increased expression of *XRCC1* was observed at T2 only in the PROP group. On the other hand, *hOGG1* and *XRCC1* expressions were decreased at T24 in both groups. There were no statistical significant differences between the two anaesthetics at the respective sampling times. The methylation status of *XRCC1* (methylated at T0) and *hOGG1* (unmethylated at T0) remained unchanged in the three sampling times. In conclusion, this study showed modulations of *hOGG1* and *XRCC1* expression especially 1 day after elective surgery in patients undergoing PROP and ISO anaesthesia. However, the data indicated that methylation was not the mechanism by which the genes were regulated. More studies are warranted to further investigate the possible epigenetic mechanisms involved after exposure to anaesthetics.


## Introduction

Due to its low metabolism rate and blood-gas partition coefficient, isoflurane (ISO) is one of the most widely used inhaled halogenated anaesthetics. Its introduction in clinical practice represented a significant advance for inhalation anaesthesia (1). Propofol (PROP), another widely used compound, is a short-acting intravenous anaesthetic with the advantages of its limited severe side effects, favourable operating conditions and recovery profile (2). Some studies have demonstrated that ISO is a potentially genotoxic agent, capable of inducing DNA damage in patients undergoing general anaesthesia (3,4) while PROP can induce toxicogenomic effects, such as the inhibition of DNA repair genes expression in

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RAW264.7 cells (5). However, the literature also presents contrary data. No genotoxicity was detected in patients undergoing minor surgeries with ISO anaesthesia (6,7) whereas PROP, instead of displaying toxicity, presented cytoprotective properties in cardiomyocytes exposed to $H_2O_2$ (8).

Indeed, the genome is constantly exposed to endogenous and exogenous agents that can interact with DNA, generating lesions and modifying the cell fate (9). However, the DNA repair system is a highly orchestrated cellular mechanism that responds to that genetic damage in order to ensure genome stability by recognising and repairing DNA insults (10). Depending on the type of damage, a variety of DNA repair strategies can be activated to restore the lesion and the lost information. The base excision repair (BER) pathway acts on damage promoted by some endogenous and exogenous agents, including reactive oxygen species (ROS), alkylating compounds and ionising radiation (9,11) and is dependent on some gene expression. The *hOGG1* gene encodes an enzyme with DNA glycosylase/AP-lyase activity, which is able to catalyze the excision of 8-oxo-7,8-dihydroguanine (8-oxoG), a highly mutagenic base lesion induced by the oxidation of guanine (12). On the other hand, *XRCC1* encodes a scaffolding protein that directly associates with DNA polymerase β, PARP (ADP-ribose polymerase) and DNA ligase III in a complex of proteins to facilitate the repair pathway (13). It is well known that gene expression is mainly regulated by epigenetic mechanisms (DNA methylation, histone modification, chromatin remodelling and non-coding RNAs), without altering the underlying DNA sequence (14,15). In mammalian cells, DNA methylation occurs almost exclusively at the fifth carbon atom of the cytosine residues within cytosine–phosphate–guanine (CpG) dinucleotides, and takes place by transferring a methyl moiety from S-adenosylmethionine to the 5′ position of the cytidine ring. The CpG dinucleotides tend to form CG-rich clusters called CpG islands that are distributed mainly in the core promoter sequence and transcription start site of structural genes. DNA methylation may induce changes in chromatin structure, DNA conformation, DNA stability and interactions between DNA and protein, resulting in transcription inhibition (16,17). Despite the worldwide use of ISO and PROP, their toxicogenomic mechanism and interference on the pattern of DNA methylation are still not well understood. Therefore, the aim of this study was to evaluate whether methylation is a mechanism of *hOGG1* and *XRCC1* modulation in patients undergoing PROP or ISO anaesthesia.

## Material and methods

### Study population

The Ethics Committee for Human Research from the Botucatu Medical School - UNESP approved the protocol used in the present study (3909-2011). After signing the informed consent form, all of the patients completed a detailed questionnaire about their lifestyle, health status and previous exposure to environmental pollutants.

A total of 40 (male and female) subjects aged 19–50 years, scheduled for elective and minimally invasive surgeries and classified as physical status I (healthy patient with no disease other than a surgical abnormality) according to the American Society of Anaesthesiologists (ASA), were enrolled in the study.

### Anaesthesia procedure

After standard clinical monitoring including electrocardiogram, peripheral oxygen saturation, non-invasive arterial pressure (systolic and diastolic) and end-tidal $CO_2$ ($P_{ET}CO_2$), patients were randomly allocated into one of the two groups: under ISO or PROP anaesthesia

**Table 1.** Demographic features of patients underwent propofol or isoflurane anaesthesia and anaesthesia duration

| Variables/anaesthesia | Propofol ($n$ = 20) | Isoflurane ($n$ = 20) | $p$ value |
|---|---|---|---|
| Age (years) | 26 ± 10 | 24 ± 5 | 0.5 |
| Body mass index (kg/m$^2$) | 23 ± 3 | 23 ± 4 | 0.9 |
| Gender (male/female) | 10/10 | 8/12 | 0.3 |
| Anaesthesia duration (min) | 159 ± 42 | 146 ± 33 | 0.4 |

Data are expressed as means ± SD or absolute values.

(Table 1). In the operating room, all patients were intravenously (iv) premedicated with midazolam and for both groups, anaesthesia was induced using the opioid fentanyl, the hypnotic propofol and the neuromuscular blocker rocuronium. Anaesthesia was maintained with ISO inhalation at approximately 1.0 minimum alveolar concentration equivalent to 1.2% (ISO group) while for PROP group (iv anaesthesia), propofol (Diprivan®) was administered by a computer-controlled infusion pump. The estimated plasma concentration of PROP was maintained at 3–5 µg/ml until the end of surgery. The lungs were mechanically ventilated using the volume-controlled mode with a tidal volume of 8 ml/kg of 40% oxygen (0.8 l/min) in air (1.2 l/min) and a respiratory rate of 10–12 breaths/min to maintain the $P_{ET}CO_2$ concentration at 30–35 mmHg. The effectiveness of anaesthesia was monitored by assessing the haemodynamic responses. Additional doses of fentanyl and rocuronium were administered if the patient was considered to be inadequately anaesthetized. Among the patients, none needed blood transfusion or had surgical complications, and all were discharged from the hospital according to the guidelines established for their surgical procedure protocol.

### Blood sampling and DNA and RNA isolation

Venous blood was collected in EDTA anticoagulant tubes and in PAXgene Blood RNA Tubes (PreAnalytiX - Qiagen) at three sampling times: before anaesthesia (T0); 2 h after the beginning of anaesthesia (T2); and 24 h after the beginning of surgery (T24). All samples were coded and blindly analyzed for the investigated endpoints. DNA and RNA were isolated using the DNA Illustra Blood kit (GE Healthcare) and the PAXgene Blood RNA kit (Qiagen/PreAnalytiX), respectively, according to the manufacturer's protocols. The concentrations of nucleic acids were determined in a spectrophotometer, and each sample was assessed for purity at A260/A280 nm absorbance. DNA integrity was verified in 1.5% agarose gel, and RNA in 1.5% agarose gel with Tris/borate/EDTA buffer.

### Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was reverse-transcribed to complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems), according to the manufacturer's instructions. Briefly, the reactions were firstly incubated at 25°C for 10 min, 37°C for 120 min and then at 4°C. The cDNA samples were stored at −80°C until the qRT-PCR using Taqman® (*hOGG1* and *XRCC1*). The β-actin gene (*ACTB*) was employed as an endogenous control. Thermal cycling and real-time were carried out in an ABI Prism 7500 FAST Applied Biosystems equipment, using the following amplification parameters: denaturation at 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Duplicate negative controls were added into each plate to ensure no contamination. Fold change was calculated using the formula $2^{-\Delta\Delta Ct}$ (18).

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### Methylation-specific polymerase chain reaction (MSP-PCR)

The MethylSEQr™ bisulfite conversion kit (Applied Biosystems) was used to perform chemical conversion of unmethylated cytosine to uracil, thus preserving the chemical structure of methylated cytosine. Gene specific MS-PCR assays were based on the genomic regions spanning 2 Kb upstream to transcription start site of each gene retrieved from the UCSC Genome Browser on Human (GRCh38/hg38) assembly [references ncbiRefSeqCurated NM006297.2 (nucleotide position chromosome 19: 43,575,579-43,577.578) and NM002542.5 (nucleotide position chromosome 3:9,747,944-9,749,943) for *XRCC1* and *hOGG1*, respectively]. Promoter-associated CpG islands were refined according the parameters: minimum length of 300 bp, C+G content equal to 50%, and CpG observed/CpG expected ratio of 0.6. Then, primer sequences were designed using the Methyl Primer Express® software v1.0 (Applied Biosystem) for detection of methylated and unmethylated alleles. The target regions were narrowed to 145bp for *XRCC1* (nucleotide position chromosome 19: 43,576,030–43,576,174) and 250bp for *hOGG1* (nucleotide position chromosome 3: 9,749,754–9,749,993) genes. Bisulfite-modified DNA was eluted out in 50 µL of TE (10 mM Tris/HCl pH 8, 0.1 mM EDTA) and stored at −20°C. Methylation status of CpG islands in *hOGG1* and *XRCC1* promoter regions was verified by the MSP-PCR technique. For *XRCC1* amplification, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1.5 U Platinum enzyme (Applied Biosystems) and 0.2 µM primers (Integrated DNA Technologies - IDT) were used. For *hOGG1* were employed 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1.5 U Platinum enzyme (Applied Biosystems) and 0.1 µM primers (IDT). The reactions were carried out at the following thermocycling conditions: denaturation at 95°C for 5 min and 35 cycles of 30 s at 95°C. The annealing temperatures were 55°C for unmethylated sequences and 60°C for methylated ones, followed by 1 min at 72°C for extension. Table 2 shows the primers used for each gene, the product size and the annealing temperature for each methylated and unmethylated sequence. The products were resolved on 2% agarose gel and stained with Gel Red. Commercial samples containing totally methylated DNA were used as the positive control (Cells-to-CpG methylated DNA kit, Applied Biosystem). Reactions were performed using the PTC200 Peltier Thermal Cycler (MJ Research) and in all PCR reactions a negative (no template) control was used as a routine quality control for the assay.

### Statistical analysis

The statistical power of the study was 82% considering 20 patients per group. The Student's *t* test was used to evaluate differences in age and body mass index while the Fisher's test was used to evaluate differences in gender between the two groups (propofol and isoflurane). For gene expression, repeated-measure analysis using the Gamma distribution followed by Wald multiple analyses were done considering the interaction between sampling time and anaesthetic exposure, with adjustment for gender and age. Pearson correlation was performed to detect possible effects of anaesthesia duration on gene expression. Any $P$ value < 0.05 was considered significant.

## Results

Figure 1 shows a significant decrease of expression of *XRCC1* and *hOGG1* in those individuals under PROP or ISO anaesthesia 1 day after surgery (T24), when compared to the other two sampling times (T0 and T2). An increased expression of *XRCC1* was only detected 2 hours after the beginning of anaesthesia (T2) in PROP group in comparison to the basal levels (T0). However, there were no significant differences ($P$ > 0.05) between the two anaesthetics at the respective sampling times. The Pearson's analysis showed a mild positive correlation between ISO anaesthesia duration and *XRCC1* and *hOGG1*expressions at T24 (Figure 2).

*XRCC1* (methylated at T0) and *hOGG1* (unmethylated at T0) methylation status remained unchanged at the three sampling times for both anaesthesia protocols.

## Discussion

In general, a unifying mechanism of toxicity for various classes of anaesthetics is based on metabolic formation of ROS-oxidative stress (19). Furthermore, literature correlates the toxicogenetics events caused by some anaesthetics mainly to free radicals, which are capable of directly attacking DNA (20–23). Thus, this study was designed to investigate whether ISO or PROP could modulate DNA repair genes (*hOGG1* and *XRCC1*) expression acting through methylation mechanism.

It is known that the protein encoded by *XRCC1* is involved in the repair of DNA single-strand breaks formed by exposure to ionising radiation and alkylating agents, while that encodes by *hOGG1*

**Table 2.** Primers, expected product size and annealing temperature used in the methylation-specific polymerase chain reaction (MS-PCR) assays for *XRCC1* and *hOGG1* genes

| Gene | Primer sequences | Product size (bp) | Annealing temperature (°C) |
|---|---|---|---|
| *XRCC1* | Methylated allele (M) | 145 | 60 |
| | F-5′AGAGGTTGAGGTAGGTGGATT3′ | | |
| | R- 5′AAATAACTAAAAATTACACACACAC3′ | | |
| | Unmethylated allele (U) | | |
| | F-5′CGTCGAGGTAGGTGGATC3′ | 145 | 55 |
| | R-5′TAACTAAAATTACACGCACGC3′ | | |
| *hOGG1* | Methylated allele (M) | 250 | 60 |
| | F-5′TAGATGGAATTTGTTAGTGTTTT3′ | | |
| | R-5′AAACACACACAACTATATTCTTCT3′ | | |
| | Unmethylated (U) | | |
| | F-5′ATGGAATTCGTTAGCGTTTC3′ | 250 | 55 |
| | R-5′AACGCGCACAACTATATTCTT3′ | | |

F, forward primer; *hOGG1*, human 8-oxoguanine DNA glycosylase gene; M, methylated sequence; R, reverse primer; U, unmethylated sequence; *XRCC1*, X-ray cross complementing group 1 protein gene.

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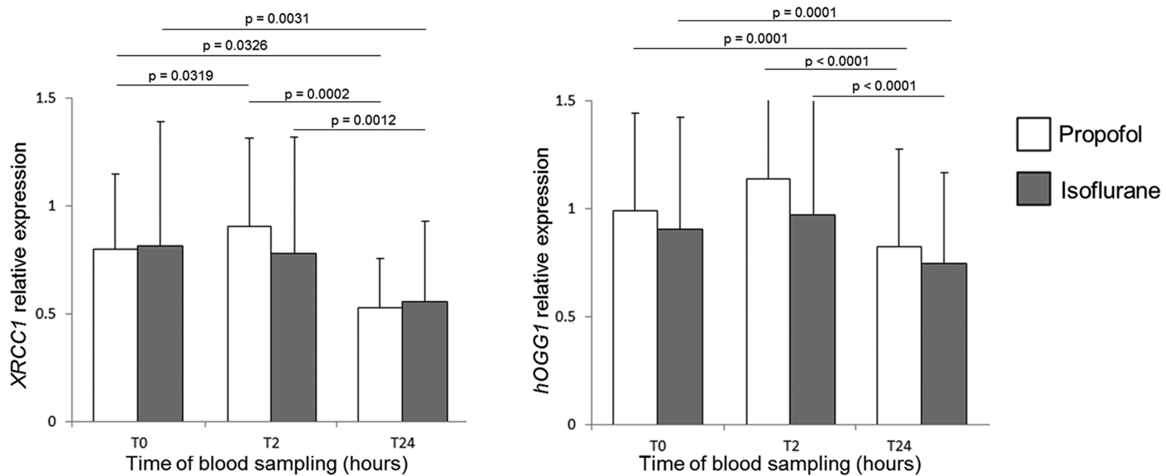


**Figure 1.** *XRCC1* and *hOGG1* relative expressions in peripheral blood cells from patients undergoing anaesthesia with propofol or isoflurane. Data are expressed as mean and standard deviation; T0 – before anaesthesia; T2 – 2 h after the beginning of anaesthesia; T24 – 24 h after the beginning of surgery. Absence of *P* values mean > 0.05.

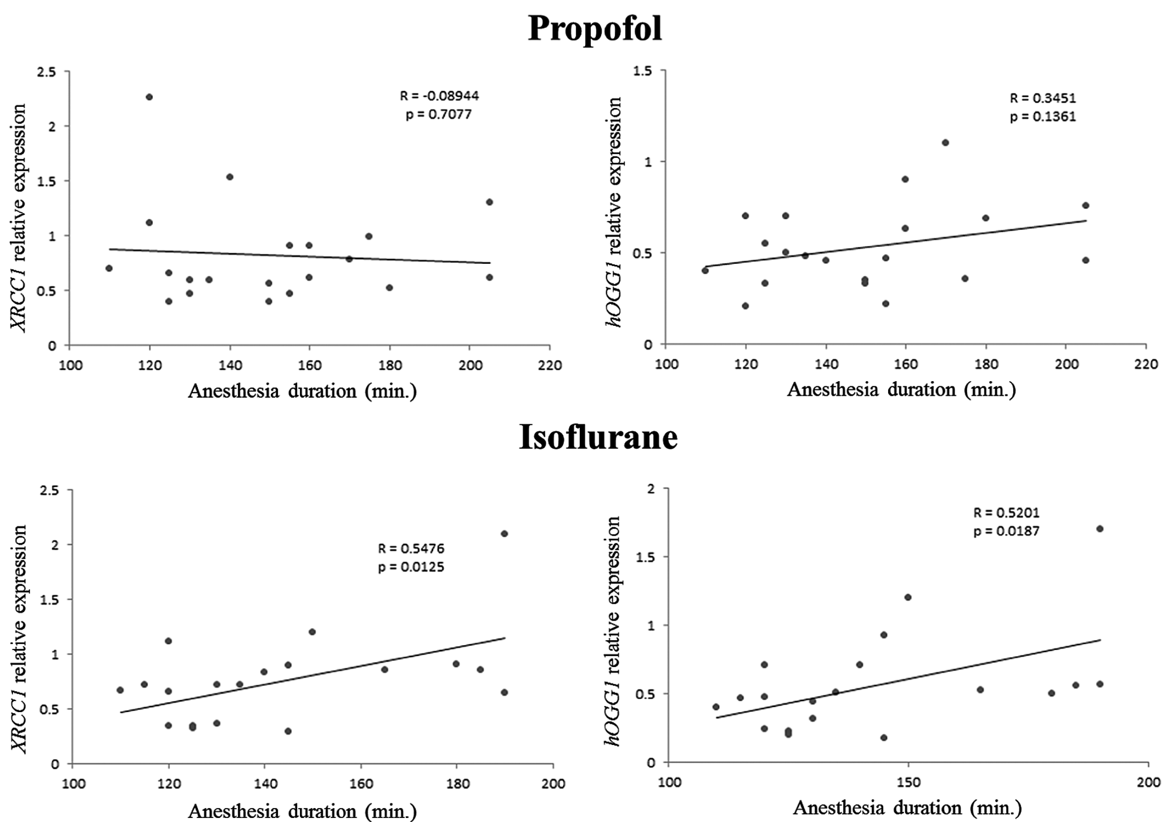


**Figure 2.** Pearson's correlation between anaesthesia duration and *XRCC1* and *hOGG1* relative expressions in peripheral blood cells 24 h after the beginning of surgery under propofol or isoflurane anaesthesia.

is responsible for the excision of 8-oxoguanine, a mutagenic base byproduct which occurs as a result of exposure to ROS. Therefore, since these methylation-sensitive genes (24,25) play important role for preventing mutations associated with oxidative damage, it would not be surprising *XRCC1* and *hOGG1* were modulated during/after ISO and PROP exposure.

Actually, not only the anaesthetics, but also the surgical stress can generate ROS and be harmful do DNA. A surgical procedure can lead to a variety of profound physiological alterations characterized by changes in haemodynamics, endocrine and immune functions, and in the production and release of ROS (26–28). Several factors, including severity of injury and genetic predisposition to adverse outcome are known to contribute to the magnitude of the surgical stress response (29). Furthermore, distressing situations of the preoperative period can also dysregulate immune and inflammatory functions, whereas anxiety and fear are associated with increased blood fluid concentrations of epinephrine and norepinephrine (30). Literature shows that stress hormones can increase DNA damage by

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generating ROS and regulating the cell cycle (29,31). In fact, a study showed that genes directly related to DNA damage and repair pathways were modulated by epinephrine and norepinephrine in murine 3T3 cells (32). Therefore, independently of the anaesthetic activity, pre-surgical and surgical stress *per se* could have induced systemic response responsible for genomic alterations and DNA repair gene modulation.

Perhaps the slight *XRCC1* overexpression observed during surgery can be related to our previous findings (33) that showed a decrease of oxidative DNA breaks during PROP anaesthesia. Indeed, it was previously demonstrated an inversely correlation between the expression levels of *XRCC1* (mRNA) and the levels of FPG-sensitive sites (34). However, when PROP and ISO anaesthetics were compared, no statistical significant differences were detected between them. In fact, one day after surgery, when patients were not under the effect of the anaesthetics since both of them are quickly eliminated, both genes were down-regulated. These findings partially confirm our previous studies (6,33).

The decreased expression of both *XRCC1* and *hOGG1* on the first post-operative day in comparison to the other two sampling times might be explained by the lack of events to potentially induce DNA damage (no surgical stress or chemical exposure). Furthermore, it was demonstrated that anaesthesia maintained with PROP or ISO are able to increase both hydrophilic and total antioxidant capacity in plasma, possibly preventing ROS attack to DNA (26). Additionally, the ability of PROP to reduce oxidative stress and improve post-operation recovery through induction of heme oxygenase (HO-1) expression was also reported (35). The HO-1 upregulation in response to prior injurious stimuli may confer protection to cells and organs against subsequent injury (36).

Interestingly, our data evidenced a mild positive correlation between anaesthesia duration and relative expression of both *XRCC1* and *hOGG1* genes 24 hours after the beginning of surgical procedure in those patients exposed to ISO, but not to PROP. In the case of PROP, the lack of correlation in both situations could be due to its stronger antioxidant capacity (8,26,33,36), avoiding the interaction between ROS and DNA.

In relation to the mechanism of regulation, reports about the methylated/unmethylated pattern of *hOGG1* and *XRCC1* are scarce. Trying to understand the molecular mechanism of the observed transcriptional modulation of *hOGG1* and *XRCC1*, the methylation patterns were determined. Nevertheless, we showed, for the first time, that no changes were detected, suggesting other mechanism(s) than methylation as responsible by these genes regulation after surgical procedure under general anaesthesia with intravenous or inhalation anaesthetics.

It lacks in the literature studies concerning anaesthetics and microRNA (miRNA) expression in humans. However, it has been reported that PROP and ISO are able to modulate miRNA expression in animal models (37,38). But, as far as we know, there is no study demonstrating that *XRCC1* can be regulated by some miRNA in anesthetized patients. Differently, a recent study showed that *hOGG1* is a direct target of *miR-4673*, which may accomplish its function through down-regulating *hOGG1* by specifically binds to 'CUGUUGA' in 3′UTR site of the gene (39). Therefore, instead of DNA methylation, the lower expression of *hOGG1* after surgery might be regulated by an ISO/PROP-mediated miRNA. However, other mechanism(s) might be involved (40), especially in the case of *XRCC1*.

In conclusion, this study showed changes in the *hOGG1* and *XRCC1* expression in leukocytes of patients under PROP and ISO anaesthesia for minimally invasive surgery. The gene modulation happened especially in the post-anaesthesia period (recovering time), and DNA methylation was not the mechanism involved in the genes regulation. Gene expression regulation by ISO/PROP-mediated other epigenetic mechanisms are the matter of future investigations.

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Conflict of interest statement: None declared.

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