RESEARCH ARTICLE

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Diagnostic Efficacy of *PSMA* and *PSCA* mRNAs Combined to PSA in Prostate Cancer Patients

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Abstract

Background: Serum Prostate-specific antigen (PSA) has been used for screening and diagnosis of prostate cancer (PCa) but it is burdened by its low accuracy, creating a need for reliable diagnostic markers. Despite prostate-specific membrane antigen (PSMA) and prostate stem cell antigen (PSCA) being widely expressed in the tissue of PCa, no definite conclusion regarding their use as clinical biomarkers due to their lacking organ specificity. Therefore, this study aimed to evaluate the peripheral blood levels of *PSMA* and *PSCA* mRNAs and examine their diagnostic significance as non-invasive integrated markers. **Materials and Methods:** 125 subjects were enrolled in this study. They were divided into 25 healthy controls, 25 BPH patients, and 75 PCa patients. The expression levels of *PSMA* and *PSCA* were determined using quantitative RT- PCR, in addition to measuring serum PSA. **Results:** Levels of *PSMA* and *PSCA* were over-expressed in PCa patients compared to controls and BPH patients and were found to be associated with increased susceptibility to PCa. Moreover, the diagnostic values of *PSMA* and *PSCA* to distinguish PCa patients from BPH patients and controls were inferior to that of PSA. However, the combination of *PSMA* and *PSCA* with PSA enhanced the efficacy of the latter. **Conclusion:** This study suggests that these genes were associated with malignant susceptibility. Concerning the duality of *PSMA*-PSA or *PSCA*-PSA, this implies the significance of their investigation together in peripheral blood of prostate patients.

Keywords: Prostate cancer- *PSMA*- *PSCA*- PSA

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Introduction

Prostate cancer (PCa) is the second most common cancer and considered the fifth common cause of death among men worldwide. PCa fatality is caused by the progression of benign prostatic hyperplasia to metastatic cancer (Rawla, 2019). In 2018, globally more than 1.3 million men were diagnosed with prostate cancer. Moreover, approximately 1.4 million new cases and 375,000 deaths worldwide were diagnosed in 2020; age-standardized incidence and mortality (world) rates of prostate cancer were 13.9/100,000 and 7.9/100,000 (Sung et al.,2021).

Prostate-specific antigen (PSA) is a 33kDa glycoprotein, belonging to the kallikrein family, consisting of chymotrypsin-like proteins (Yousef and Diamandis, 2001). It is secreted from epithelial cells in the prostate. PSA is commonly used as a diagnostic marker for PCa. Nonetheless, serum PSA exhibits low accuracy because of its organ-specific rather than tumor-specific; where it is found in normal prostate tissue and benign prostatic hyperplasia (Ross et al., 2016; Ezenwa et al., 2012). To improve this, other markers are urgently required for early diagnosis and prognosis as well as prospective therapeutic targets.

Prostate-specific membrane antigen (PSMA) is a promising biomarker that is more specific than PSA (Ross et al., 2016). PSMA is a 100kDa non-soluble type II transmembrane glycoprotein that is expressed on the apical surface of endothelial cells. It has enzymatic functions as carboxypeptidase in prostate tissue and as folate hydrolase activity, which plays a role in folic acid utilization and metabolism (Pinto et al., 1996).

Despite its name, PSMA is not fully prostate-specific, it is widely expressed in various non-prostatic solid tumors, including urothelial, renal, gastrointestinal, and breast carcinomas. However, the expression levels in these tissues are lower than in the prostate tissue (Chang et al., 1999; Haffner et al., 2009; Samplaski et al., 2011).

Notably, tissue PSMA expression rises significantly in primary PCa as compared to benign and is significantly elevated in lymph node and distant metastases when

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compared to the primary tumor; which suggests it may have a role in PCa progression (Queisser et al., 2015). Although the use of *PSMA* is widely spread, its role and/or benefit in PCa diagnosis and evolution is still needed to be confirmed.

Prostate stem cell antigen (PSCA) is a cell-surface antigen, located on chromosome 8q24.2 and belongs to the Ly-6/Ty-1 family of glycosyl-phosphatidyl inositol-anchored proteins. *PSCA* was expressed mainly in the basal cell epithelium of the normal prostate (Reiter et al., 1998).

Authors displayed that the expression levels of PSCA protein and *PSCA* gene were elevated in metastatic patients, using immunohistochemistry and in situ hybridization analysis, and found an association between the expression levels and Gleason grade, stage, metastases, and higher risk for biochemical recurrence (Lam et al., 2005; Gu et al., 2000). Additionally, few studies showed that *PSCA* levels were upregulated in PCa when compared to benign tumors (Hara et al., 2002; Zhigang and Wenlu, 2008; Fawzy et al., 2015). Otherwise, PSCA is also over-expressed in other cancers like urothelial, kidney, and lung (Amara et al., 2001; ELsamman et al., 2006; Kawaguchi et al., 2010). Thus, these data indicate that PSCA expression is not prostate-specific.

Although the research revelations about PSCA and its potential in prostate cancer, no definitive conclusions have been reached regarding it being a clinical biomarker due to the little number of published researches and the lack of better measuring techniques. Accordingly, there is a need for integrated biomarkers to better diagnosis and prediction of disease progression.

Therefore, this study was conducted to evaluate gene expression levels of *PSMA* and *PSCA* in the peripheral blood of newly diagnosed patients and to study their potential utility as an integrated non-invasive diagnostic markers.

Materials and Methods

Subjects

This study included 125 subjects; 75 newly diagnosed patients with pathologically and radiological proven PCa and 25 benign prostatic hyperplasia (BPH) who had no history of prostate carcinoma nor previous prostate surgery, with mean age (67.4 ± 7.65 and 65.7 ± 6.12, respectively), were recruited from outpatient medical oncology clinic at the National Cancer Institute (NCI), Cairo University. In addition to, 25 healthy volunteer as a control group. This study was performed according to the declaration of Helsinki. Institutional Review Board of NCI approved the study. A written informed consent was obtained from all participants.

Three mL whole blood samples were withdrawn from all participates. Blood samples were divided into two fractions; the first one was collected into evacuated tubes contained ethylenediaminetetraacetic acid (EDTA) for molecular analyses. The other fraction (serum samples) were used for analysis of total and free PSA using the fully automated chemiluminescence analyzer Cobas e400 with the corresponding kit according to manufacturer's protocol (Roche Diagnostic, IN, USA).

Methods

Mononuclear cells were separated from whole blood by Ficoll- Plaque™ density gradient media (GE Healthcare UK, Buckinghamshire, UK). Total RNA was extracted using QIAamp RNA Blood Mini Kit according to manufacturer's instructions (Cat no. 52304, Qiagen, Germany), then Nano-Drop (Thermo Scientific, Wilmington, USA) was used to detect RNA purity and concentration.

cDNA synthesis was performed using High-Capacity cDNA reverse transcription kit QuantiTect® Reverse Transcription Kit (Cat no. 205311,Qiagen). Firstly (genomic wipeout process); 14 µl reaction mixture containing 2 µl of genomic DNA wipeout buffer 7x, 1µg of template RNA and then the volume completed with RNAase free water. Mixture was incubated for 10 minutes at 42°C. Second: reverse transcription was performed in 20 µl reaction mixture containing 1µl Reverse Transcriptase, 4µl RT buffer 5x, 1 µl RT primer mix, and 14 µl of the sample. QuantiTect® Primer Assays for *PSMA*, and *PSCA* (Cat no. QT00052647 and QT02394329, respectively; Qiagen) were used for real-time reactions.

Real-time quantitative PCR was carried out in a total volume of 25 µl; each reaction contained 12.5 µl 2x Quanti-Tect SYBR® green PCR master mix (Qiagen), 2.5 µl primer, 8 µl of RNAase free water , and 2 µl cDNA. RQ- PCR reactions using Step One™ Real Time PCR System (Applied Biosystems) were performed as follow: initial denaturation step at 95˚C for 15 min, followed by 45 cycles of 15 s for denaturation at 95˚C, annealing at 55˚C for 30s and finally extension for 15s at 72˚C. The expression level of mRNAs was normalized to the expression of housekeeping gene *β-actin* (Cat no. QT00095431, Qiagen). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical analysis

Data were analyzed using SPSS (ver. 20; IBM, Chicago, IL, USA). Shapiro-Wilk test was performed to identify the type of data distribution. Quantitative data was displayed as mean±SD for normally distributed data while median and inter-quartile rang (25th and 75th percentile) for non-normally distributed. Qualitative data was demonstrated as frequency and percentage. For continuous variables, multiple comparisons between groups was performed using one way ANOVA test followed by Tukey's post hoc or Kruskel Wallis test followed by Mann Whitney as appropriate. Chi square test was used to assess the association between categorical variables. To evaluate the diagnostic value of *PSMA*, and *PSCA* relative expression levels, receiver operating characteristic (ROC) curve analysis was performed. Logistic regression analysis was used to assess the strength of the association between, *PSMA*, and *PSCA* relative expression levels and the susceptibility to prostate cancer. P-value< 0.05 was considered statistically significant.

Results

Basic characteristics of study population

Basic characteristics of the study population are listed in (Table 1), PCa patients and BPH were older when compared to normal control group (P<0.001). Compared to the controls, serum total PSA levels were significantly elevated in the BPH and PCa patients (P=0.001 and P<0.001, respectively), in addition to increased levels of free PSA (P<0.001). PCa patients exhibited significantly increased levels of total PSA and free PSA (P<0.001) in comparison to BPH patients. Regarding gene expression levels, *PSMA* and *PSCA* genes were significantly over-expressed in peripheral blood of PCa patients compared to BPH patients and normal controls (P<0.001). In contrast, there was no significant differences between BPH patients and the control group for both *PSMA* and *PSCA* (P=1.0 and P=0.051, respectively). Further, more than of 70% of PCa patients had a high Gleason score (≥7).

Association of PSMA and PSCA expression levels with the studied parameters

PCa patients were assigned into two groups based on the median of gene expression levels. Only a significant correlation was found between higher *PSCA* expression levels and metastasis (P<0.045). In addition, high *PSMA* and *PSCA* expressers sub-group had higher Gleason score than those in the lower expression sub-group. While, there were non-significant correlations between *PSMA*, *PSCA* expression levels and PSA levels (Table 2).

PSMA and PSCA as risk factors for PCa

Logistic regression analysis showed that older age, elevated levels of total PSA, high expression levels of both *PSMA* and *PSCA* were associated with increasing the risk of PCa development, whereas both *PSMA* and *PSCA* expression levels were remained significant after adjustment for age and total PSA (P=0.049 and P=0.015, respectively). *PSMA* and *PSCA* levels increase the risk of cancer development by 1.4 and 4.49 fold, respectively

Table 1. Demographic Data of the Studied Groups

	Control (n=25)	BPH (n=25)	Pca (n=75)	p-value
Age (years)	35 ± 5.97	65.7 ± 6.12	67.4 ± 7.65	<0.001
Gleason score				
< 7			20 (26.7%)	
≥7			55 (73.3%)	
Tumor localization				
Localized			42 (56%)	
Distant metastasis			33 (44%)	
Total PSA (ng/ml)	1.04 (0.75 - 1.56)	2.47 (1.24 - 6.80)[a]	50.54 (11 - 115)[ab]	<0.001
Free PSA (ng/ml)	0.27 (0.21 - 0.32)	0.69 (0.41 - 1.36)[a]	8.14 (1.83 - 17.37)[ab]	<0.001
PSMA	0.6 (0.42 - 1.00)	0.61 (0.36 - 1.81)	1.85 (1.39 - 10.04)[ab]	<0.001
PSCA	0.42 (0.27 - 0.51)	1.02 (0.34 - 1.47)	1.53 (1.33 - 1.91)[ab]	<0.001

Data are expressed as mean ± SD for Gaussian data, median (inter-quartile range) for non-Gaussian data, and frequency (percentage) for categorical data. BPH, benign prostate hyperplasia; PCa, prostate cancer; PSA, prostate specific antigen; *PSMA,* prostate specific membrane antigen; *PSCA,* prostate stem cell antigen. In multiple comparisons, ap<0.05 vs. control, and bp<0.05 vs. BPH

Table 2. Association of *PSMA* and *PSCA* Expression Levels with Metastasis, Gleason Score, and PSA Score in PCa Group

	PSMA		*PSCA*	
	Low expression (n=37)	High expression (n=38)	Low expression (n=37)	High expression (n=38)
Metastasis n (%)				
Absent	22 (59.5)	20 (52.6)	25 (67.6)	17 (44.7)
Present	15 (40.5)	18 (47.4)	12 (32.4)	21 (55.3)
P-value	0.551		0.045	
Gleason score n (%)				
Gleason <7	16 (43.2)	4 (10.5)	15 (40.5)	5 (13.2)
Gleason ≥7	21 (56.8)	34 (89.5)	22 (59.5)	33 (86.8)
P-value	0.001		0.007	
PSA score n (%)				
<4	3 (8.1)	2 (5.3)	3 (8.1)	2 (5.3)
4-10	4 (10.8)	8 (21.1)	5 (13.5)	7 (18.4)
>10	30 (81.1)	28 (73.7)	29 (78.4)	29 (76.3)
P-value	0.452		0.771	

PSA, prostate specific antigen; *PSMA*, prostate specific membrane antigen; *PSCA*, prostate stem cell antigen.

Table 3. Binary Logistic Regression Analysis of Total PSA, *PSMA,* and *PSCA* as Potential Risk Factors for Prostate Cancer

Variables	OR (95% CI)	p-value	†Adjusted OR (95% CI)	p-value
Age	1.12 (1.07-1.16)	<0.001		
Total PSA	1.4 (1.2-1.64)	<0.001		
PSMA	1.45 (1.1-1.91)	0.008	1.3 (1-1.7)	0.049
PSCA	9.28 (4.1-21.03)	<0.001	4.49 (1.3-15.1)	0.015

PSA, prostate specific antigen; *PSMA*, prostate specific membrane antigen; *PSCA*, prostate stem cell antigen; OR, odd ratio; CI, Confidence interval; †, adjusted for age and total PSA

(Table 3).

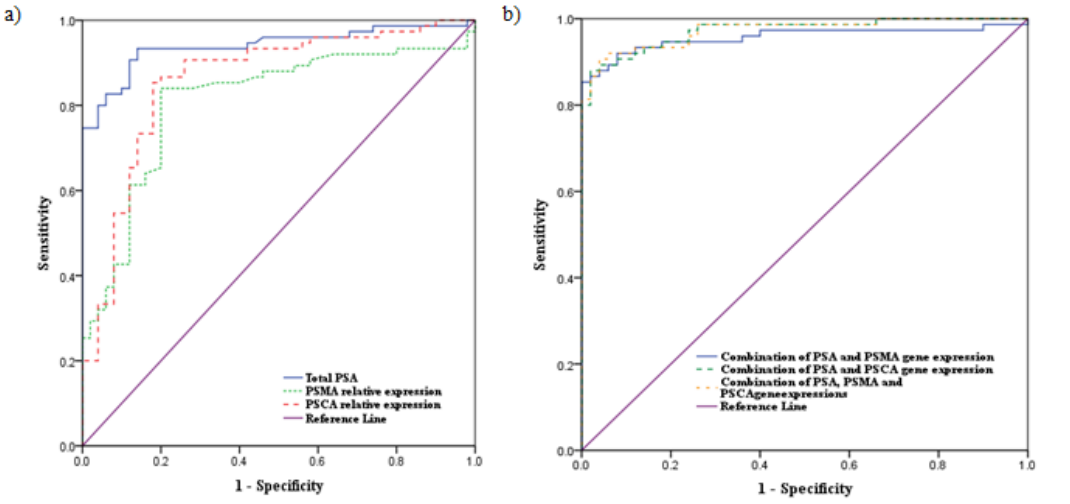
Efficacy of PSMA and PSCA as potential diagnostic biomarkers for PCa

Figure 1 demonstrates the ROC curves of *PSMA* and *PSCA* expression levels in addition to total PSA, and their combinations to discriminate PCa patients from the control and BPH groups. The figure showed that total PSA had the highest diagnostic value for PCa with an area under curve (AUC) of 0.94 (95% confidence interval [CI]: 0.898–0.982, p < 0.001) at an optimal cutoff point of 5.42 ng/ml which could yield 93% sensitivity and 86% specificity. However, the AUC of *PSCA* was 0.859 (95% CI: 0.789–0.929, p < 0.001) at a cutoff point of 1.1, which is associated with a sensitivity of 85% and specificity of 82%. Further, *PSMA* showed slightly lower sensitivity than the other two markers with an AUC of 0.809 (95% CI: 0.729–0.888, p < 0.001) that can give 83% sensitivity and 80% specificity at a cutoff point of 1.23.

Regarding the combination of the different 3 parameters together, an increase in the AUC values was noticed. Further, the combinational ROC analysis illustrated that the combination of the PSA with *PSMA* and/or *PSCA* increased sensitivity and specificity values. The highest elevation resulted from the combination of the three variables, which yielded 91% sensitivity and 96% specificity while the combination of PSA and *PSCA* resulted in sensitivity and specificity of 89% and 96%, respectively followed by the combination of PSA and *PSMA* with 85% sensitivity and 100% specificity.

Discussion

Although there are gradual advances in knowledge about PCa, biology of PCa has not been completely apparent, and understanding accurate molecular basis of



	AUC	95%CI		*p*-value	Cutoff	Sensitivity (%)	Specificity (%)
		Lower bound	Upper bound				
Total PSA (ng/ml)	.940	.897	.982	<.001	≥5.42	93%	86%
PSMA relative expression	.809	.729	.888	<.001	≥1.23	83%	80%
PSCA relative expression	.859	.789	.929	<.001	≥1.10	85%	82%
Combination of PSA and *PSMA*	.957	.919	.995	<.001	-	85%	100%
Combination of PSA and *PSCA*	.972	.948	.996	<.001	-	89%	96%
Combination of PSA, *PSMA* and *PSCA*	.973	.949	.997	<.001	-	91%	96%

Figure 1. ROC Curves of a) *PSMA, PSCA* Relative Expression and Total PSA Concentration and b) their combinations for discriminating prostate cancer patients. PSA, prostate specific antigen; *PSMA,* prostate specific membrane antigen; *PSCA*, prostate stem cell antigen; AUC, area under curve; CI, Confidence interval

the tumor is needed to improve the diagnosis, monitoring, and treatment of the patients. Nowadays, Digital Rectal Examination (DRE) and serum PSA assay have been used in screening and diagnosis, although they are not completely satisfactory. Where PSA expression levels were increased in benign prostatic hyperplasia and prostatitis, indicating that PSA has a high sensitivity but low specificity. Therefore, search for companion biomarkers to make early diagnosis feasible is needed. Accordingly, this study was designed to assess the potential utility of *PSMA* and *PSCA* as non-invasive integrated diagnostic markers.

This work showed an increase in levels of PSA in PCa patients when compared to BPH and controls. This result is in agreement with that of Igbokwe et al., 2021 who found an elevation in its level in Nigerian patients.

In the present study, *PSMA* was overexpressed in peripheral blood of PCa patients in comparison with BPH and control subjects, which is consistent with the results of previous studies that reported up-regulated levels of *PSMA* mRNA in prostate cancer patients when compared to benign and healthy controls (Hara et al., 2002; Zhang et al., 2008; Joung et al., 2010). In addition, authors found that tissue PSMA expression is significantly increased in primary PCa when compared to the benign group and significantly higher in distant metastases as compared to primary tumors using immunohistochemical techniques (Queisser et al., 2015; Hupe et al., 2018).

Given its enzymatic ability to yield glutamate and folate from polyglutamated substrates, this aberrant overexpression of PSMA may be attributed to the deleterious aberrations in DNA damage repair genes of tumor cells. These aberrations are associated with increased demand for metabolic precursors such as folate and glutamate, which are crucial to DNA synthesis and repair (Paschalis et al., 2019).

High expression levels of *PSMA* is associated with high Gleason score in our work, which is supported with other studies that showed a significant relation between *PSMA* mRNA and protein expression with Gleason score reflecting tumor aggressiveness (Joung et al., 2010; Kasperzyk et al., 2013; Bravaccini et al., 2018).

Regarding *PSCA*, a significant increase in expression levels was observed in PCa patients compared to BPH group which concurs with the results of Hara et al. and Fawzy et al. who found that *PSCA* was up-regulated in PCa patients in comparison with nonmalignant group using nested PCR technique (Hara et al., 2002; Fawzy et al., 2015). This upregulation may be due to *PSCA* is located close to Myc oncogene on chromosome 8q24.2, which is one of the most frequently amplified regions in cancers. Therefore, gene amplification may be the major cause of *PSCA* overexpression (Reiter et al., 2000).

In this study, high level of *PSCA* was associated with presence of distant metastases that seems in line with the results of Fawzy et al., (2015) who revealed that *PSCA* was markedly increased in peripheral blood of metastatic PCa patients than localized patients using nested PCR. Additionally, Lam et al and Dannull et al. reported that the level of tissue *PSCA* mRNA and protein expression were elevated in bone metastases than lymph node and primary tumor specimens (Lam et al., 2005; Dannull et al., 2000). Moreover, this work reported that the elevated expression level of *PSCA* was associated with high Gleason score which in agreement with several studies (Gu et al., 2000; Hara et al., 2002; Fawzy et al., 2015). On the other hand, Heinrich et al., (2018) found that tissue PSCA expression was associated with low Gleason score and low PSA levels in PCa patients.

Although the biological role of PSMA and PSCA in prostate cancer remained elusive, our findings revealed that increased *PSMA* and *PSCA* expression levels were associated with increased risk of PCa. This can be explained through that PSMA activates signaling pathway via G protein-coupled receptor specifically the metabotropic glutamate receptor (mGluR) at the plasma membrane of prostate cells through the release of free glutamate from vitamin B9 as a glutamate substrate via its zinc metalloproteinase enzymatic activity, where PSMA colocalized tightly with two members of the mGluR I family. Up-regulation of mGluR's subsequently stimulates phosphorylation of p110β isoform of phosphoinositide 3-kinase (PI3K), leading to Akt signaling pathway activation that supports prostate cancer pathogenesis and progression (Kaittanis et al., 2018). Despite PSCA has a different role in tumor promotion or suppression depending on the cellular context (Saeki et al., 2010). Li et al., (2017) reported that PSCA promoted PCa cells proliferation and robust tumor growth through stimulation of c-Myc expression resulting in the activation of downstream genes cyclin D1 and cyclin E2. As PSCA mediated c-Myc expression depends on PI3K/Akt pathway, and PSCA lacks a transmembrane domain and cytosolic domain; therefore, it can be speculated that PSCA may form a complex with PSMA as a protein with transmembrane and intracellular domains to be involved in intracellular signaling. The exact mechanism underlying this process needs to be investigated.

Herein, the diagnostic efficacy of *PSMA* and *PSCA* was examined to discriminate PCa patients from non-cancerous patients. The results showed that both parameters have diagnostic utility which seem to be in line with the previous study used tissue samples (Li et al., 2017). However, PSA in this study was found to have a superior diagnostic impact to detect PCa over *PSMA* and *PSCA* although the combinational analysis enhanced the efficacy of the former.

Study limitation, due to the small number of subjects in our study, further studies are needed using a larger sample size to elucidate the molecular pathway linking *PSMA* and *PSCA* with PCa progression. Despite younger control group is another limitation of the current study, but the susceptibility to prostate diseases increases with age where the risk to develop prostatitis and BPH is increased at the age of 45.

In conclusion, this work revealed that the high expression levels of *PSMA* and *PSCA* are associated with higher risk to PCa development and may be valuable therapeutic targets. Their blood levels imply a reliable diagnostic value when integrated with PSA.

Author Contribution Statement

Fatma Abdel Hamid and Iman Abdelgawad contributed to the study conception and design. Material preparation, samples, and data collection were performed by Abeer Ismail and Ibrahim Malash. Mustafa Mahmoud was responsible for practical analysis. Data analysis and the frist draft of the manuscript were made by Doaa Ibrahim. All authors revised, read and approved the final manuscript.

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Ethics approval and consent to participate

The study was conducted according to the Research Ethics Committee of Institutional Review Board of NCI. Written informed consent was obtained from every participant.

Availability of data and material

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

Conflict of interests

The authors declare that they have no conflict of interest.

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