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Original Research

MOLECULAR DETECTION OF HUMAN PAPILLOMAVIRUS GENOTYPE 16 AND 18 IN WOMEN WITH CERVICAL ABNORMALITIES IN OSOGBO SOUTH WEST NIGERIA

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ABSTRACT

Aim: Cervical cancer, a potentially preventable disease poses a disproportionate high burden of incidence and mortality in low-middle income (LMIC) country due to suboptimal linkages for confirmatory diagnosis and subsequent treatment in many LMIC's. This study investigates the prevalence of high-risk HPV genotypes 16 and 18 in women with cervical abnormalities in Osogbo, South West Nigeria. **Method: A** cross-sectional study conducted on 221 women aged 18-65 who attended UniOsun Teaching Hospital for cervical screening in 2023. Cytology was performed using the Papanicolaou staining method and classified with the 2014 Bethesda system, while HPV DNA was detected using real-time PCR. **Results**: Out of 221 women, 135 (61.1%) had normal cytology, while 86 (38.9%) had abnormalities, including 39 (17.65%) with Low-grade Squamous Intraepithelial Lesions (LSIL), 28 (12.67%) with High-grade Squamous Intraepithelial Lesions (HSIL), and 19 (8.60%) with Atypical Squamous Cells of Undetermined Significance (ASC-US). Age-specific analysis indicated LSIL peaked in women aged 30-39, HSIL in those aged 40-49, and ASC-US in those aged 20-29. HPV 16 was found in 3 (1.4%) samples and HPV 18 in 2 (0.9%) samples, all from women with normal cytology. **Conclusion:** The study found no significant association between the presence of HPV 16/18 and cytological abnormalities ($p>0.05$), despite a high prevalence of HPV DNA across all cytology categories (75.0-79.5% for Relative Fluorescence Units) but without significant differences ($p=0.977$). This is supported by a meta-analysis, but contradicts a longitudinal study that showed higher HPV DNA levels were associated with increased risk of more severe cytological abnormalities. The study concluded that other high-risk HPV types or non-HPV factors may be involved in cervical pathogenesis in this region, and further research on a broader range of HPV genotypes and risk factors is needed to guide targeted screening methods.

Keywords: HPV 16, HPV 18, cervical abnormalities, Pap smear, molecular detection

INTRODUCTION

Cervical cancer is predominantly caused Persistent infection with high-risk human papillomavirus (HPV) genotypes, predominantly HPV 16 and HPV 18, is the main causative agent in the growth of cervical cancer and its sign lesions [1]. These two HPV genotypes are liable for approximately 70% of all cervical cancer cases worldwide [2]. The detection and accurate genotyping of HPV 16 and HPV 18 are essential for cervical cancer screening, triage, and management strategies. Cytology-based screening methods, such as the Papanicolaou (Pap) test, have been the traditional approach for detecting cervical abnormalities. However, these methods have limited sensitivity and specificity, particularly for detecting precancerous lesions [3]. Molecular techniques for HPV detection and genotyping have appeared as valuable adjuncts or alternatives to cytology-based

screening, providing enhanced sensitivity and specificity in identifying women at risk for cervical cancer [4]. Various molecular methods have been developed for the detection and genotyping of HPV, including polymerase chain reaction (PCR)-based techniques, hybrid capture assays, and signal amplification techniques [4]. These methods differ in their sensitivity, specificity, and ability to detect and distinguish specific HPV genotypes, particularly the high-risk types HPV 16 and HPV 18 [5].

Understanding the prevalence and distribution of HPV genotypes, especially HPV 16 and HPV 18, among women with cervical abnormalities is important for altering screening and management strategies. This information can guide the appropriate use of HPV testing, triage algorithms, and management decisions, ultimately contributing to the prevention and early detection of cervical cancer [5]. Currently, there has been an increasing focus on the implementation of main HPV screening, where HPV testing is used as the initial screening technique, rather than as a follow-up test for abnormal cytology results [5]. Primarily, HPV screening has shown superior performance in detecting precancerous lesions and reducing the incidence of invasive cervical cancer compared to cytology-based screening [5].

This study investigated the molecular detection and genotyping of HPV 16 and HPV 18 in women with cervical abnormalities in Osogbo South West Nigeria by employing sensitive and specific molecular techniques. The prevalence and distribution of these two high-risk HPV genotypes in relation to various cervical cytological abnormalities were determined. The findings from this study will contribute to the existing knowledge and aid in the development of effective cervical cancer prevention and control strategies tailored to the potential implementation of primary HPV screening.

MATERIALS AND METHODS

Ethical Consideration

Ethical clearance for the study was granted by the Ethics and Research Committee of Uniosun Teaching Hospital, Osogbo, Osun State, on March 2, 2023, with reference number UTH/REC/23/01/20/747

Study Design and Population

This cross-sectional study was conducted at Uniosun teaching hospital, involving 221 women aged 18-65 who presented for routine cervical screening from January 2023 to December 2023

Sample Size

The sample size (N) was determined using the formula $N= Z^2 P (1-p) / d2$ (6) with prevalence rate of (14.7%) by [6]. Confidence interval of (1.96) and desired level of significance taken as 0.05. This calculation resulted at 198 women sample which happened to be the minimum samples [7].

Samples and Data Collection

Demographic and clinical data, including age, sexual history, and other status, were collected. Cervical samples were obtained for cytology (Pap smear) and HPV DNA testing. Cervical smear sample were collected using a previously described method of Kavatkar *et al*. [8, 9]. The cytobrush was *inserted* into the endocervical canal with sterile speculum, rotated clock wisely, withdrawn and inserted into fixative liquid base cytology prepared [6]. The fixed smear allowed standing for 30 min, labeled accordingly and spun at 1500rpm for 30 sec, decanted and diluted with cellular solution [10]. 50ul part of the diluted mixture was used to prepare smear on dried

clean sides with the use of cytobrush as applicator while the remaining was stored in refrigerator for molecular analysis [11]

Data were extracted from the analyzed research records, including demographic information [12] clinical contraceptive use with history, age at first sexual intercourse, number of sexual partners, and as required structured questionnaire [13].

Definitions and Outcome

Cervical abnormalities, the abnormalities in the cells of the cervix that range from precancerous lesions to invasive cervical cancer [8] and can be typically detected through cervical cancer screening methods, such as the Papanicolaou (Pap) test [14] or HPV testing. Precancerous cervical lesions, also known as cervical intraepithelial neoplasia (CIN) under Cytological Examination, PAP smears are classified into three grades according to the Bethesda System [15], as:

- Normal

- Atypical squamous cells of undetermined significance (ASC-US)

- Low-grade squamous intraepithelial lesion (LSIL)

- High-grade squamous intraepithelial lesion (HSIL)

1. CIN 1 (low-grade lesion): Mild dysplasia, representing early precancerous changes in the cervical cells; 2. CIN 2 (moderate-grade lesion): Moderate dysplasia, indicating more advanced precancerous changes; and3. CIN 3 (high-grade lesion or carcinoma in situ): Severe dysplasia or carcinoma in situ, which is considered a precursor to invasive cervical cancer if left untreated [8].

The outcomes of cervical abnormalities can vary depending on the severity of the lesion and the management approach taken, particularly low-grade lesions (CIN 1), may regress spontaneously without treatment, especially in younger women [10]. The Precancerous lesions that do not regress may persist and require close monitoring or treatment to prevent progression to invasive cancer [11]. If precancerous lesions are left undetected or untreated, they may progress to invasive cervical cancer, which can metastasize to other parts of the body and lead to significant morbidity and mortality [12]. The Untreated high-grade precancerous lesions (CIN 2 and CIN 3) have a higher risk of progressing to invasive cervical cancer over time (16). The appropriate management approach for cervical abnormalities depends on the severity of the lesion, age, reproductive status, and other factors. [17].The prevalence and distribution of these cytological categories, along with associated risk factors, are reported in the study population.

HPV DNA Testing

HPV DNA testing was performed using multiplex PCR to detect the presence of HPV genotypes 16 and 18. DNA was extracted from cervical samples using QIAamp Fast DNA Mini Kit (qiagen

Using a QIAamp Fast DNA Mini Kit (qiagen Germany), the extracted DNA was stored at -20 oC prior to amplification (14, 18) and PCR- Fluorescence probing primers by Guagdong Huayin Medicine Science Co., Ltd for HPV 16 and 18 were used in the PCR assay by Guangdong, 2023 with real time qPCR, [19].Preparation of the reaction mix and polymerase chain reactions (PCR) to detect high-risk human papillomavirus (HPV) genotypes 16 and 18.

The HPV PCR Master Mix 1 and 2, along with a re-dissolved diluent, were obtained from Guangdong Huayin Medicine Science Co. Ltd, China (www.huayinbio.com, reference number

HY06238). The master mix was aliquoted into PCR reaction tubes, and the tested sample, negative control, and positive control were added to the respective tubes, bringing the total reaction volume to 25µl. After centrifugation, the reaction tubes were placed in an automatic fluorescent PCR instrument, and the sample parameters were set according to the instrument's operating instructions. The sample was considered positive for HPV genotypes 16 and 18 when the cycle threshold (Ct) value of HPV-DNA via the FAM and VIC channels, respectively, was less than or equal to 38 [20, 21].

Statistical Analysis

Descriptive statistics summarized demographic and clinical characteristics. The association between HPV genotypes and cytological abnormalities was analyzed using chi-square tests and logistic regression [3, 14]. Odds ratios (OR) and 95% confidence intervals (CI) were calculated.

Results

Cytological Findings

Among the 221 Women: - Normal: 135 Cases (61.1%), - ASC-US: 19 Cases (8.60%),- LSIL: 39 Cases (17.65%),- HSIL: 28 Cases (12.67%)

Table 1: Showing Papanicolaou (Pap) smear Cytological changes of Epithelia cell abnormalities distributions among the participants

	CERVICAL CYTOLOGICAL	**Frequency**	**Percentage**
	Subjects Recruited	*n=221*	
	Category		
A	Negative or Normal for intraepithelial lesion	135	61.1
B	Atypical Squamous cells of Undetermined significance (ASC-US)	19	8.60
C	Low- grade Squamous intraepithelial lesion(LSIL)	39	17.65
B	Low- grade Squamous intraepithelial lesion(LSIL)	28	12.67
	Total	*221*	100

Table 1: shows the distribution of cervical cytology results. Normal squamous epithelial cells were found in 135 women (61.1%), the highest percentage. of the 86 (38.9%) with abnormalities, 39 (17.65%) had LSIL, 28 (12.67%) had HSIL, and 19 (8.60%) had ASC-US.

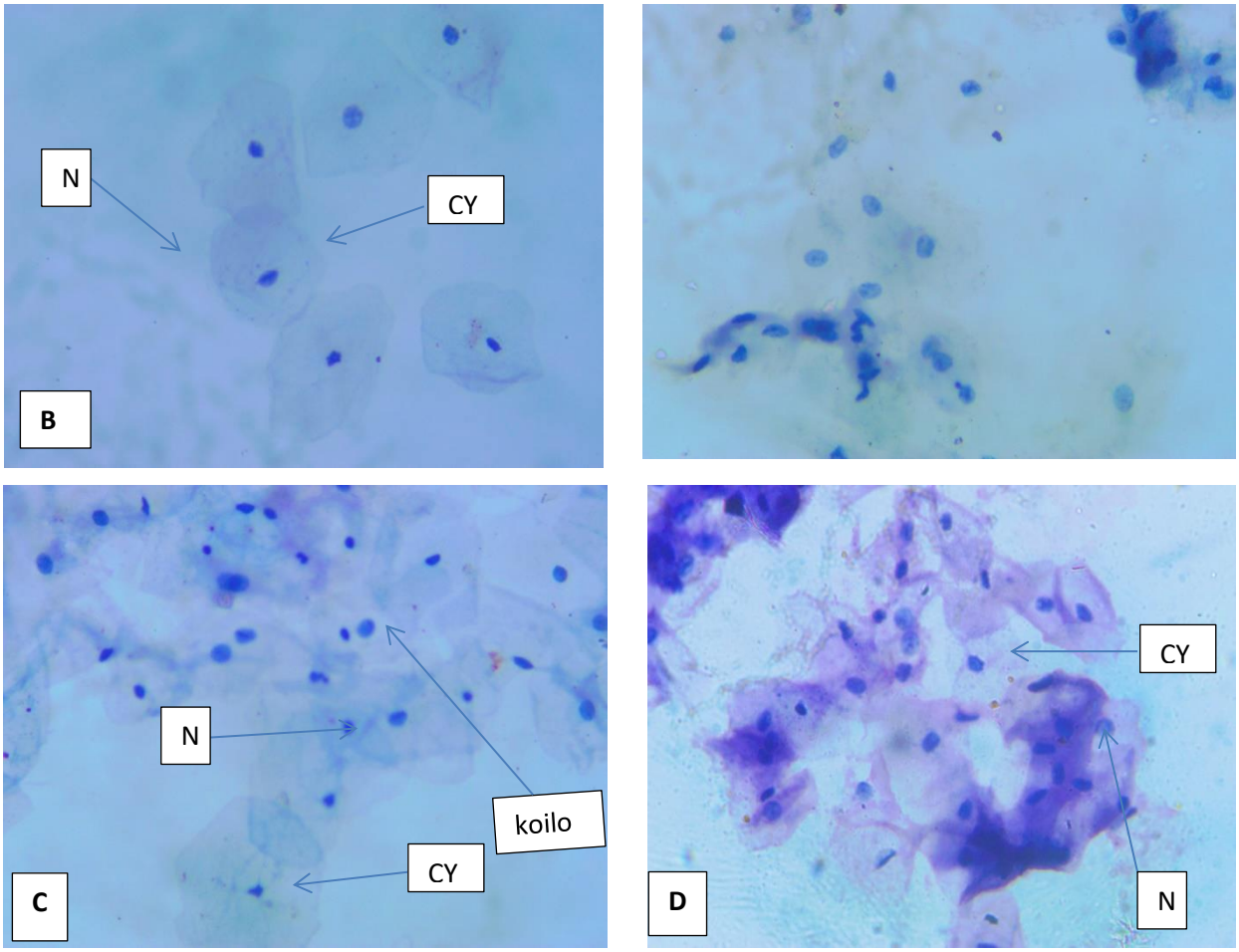

Plate A; (Mg X400). The photomicrograph features suggestive of Normal (no abnormity) cervical cell. It showed clean, none inflammatory, none necrotic, cell types, arrangement, and pattern and membranes with any special features.

Plate B; (Mg X400). The photomicrograph features suggestive of ASC-US." which shows several squamous cells with slightly enlarged nuclei size of normal intermediate cell nuclei and mildly irregular nuclear membranes There is minimal hyperchromasia, and the cytoplasm looks relatively normal to nuclei ratio. These features are suggestive of ASC-US."

Plate C; (Mg X400). "This photomicrograph demonstrates several squamous cells typical of LSIL. The nuclei are enlarged of about 3-4 times the size of normal, with irregular nuclear membranes. The chromatin is coarsely granular and hyperchromatic. Notably, some cells show koilocytes. Some cells are bi-nucleated. Despite nuclear changes, the cytoplasm remains relatively abundant.

Plate D; (Mg X400). The photomicrograph reveals some partially cluster of cells characteristic of HSIL. The some nuclei are enlarged than the size of normal when compared Nucleus /Cytoplasm ratios. The majority of nuclear membranes are highly irregular with hyperchromatic and dark appearance to the nuclei.

Table 2 Showing HPV Genotype Prevalence HPV DNA testing revealed:

	Variables	Frequency	Percentage
	Subjects Recruited	*n=221*	
A	HPV 16		
	NEG	218	98.6
	POS	3	1.4
B	HPV 18		
	NEG	219	99.1
	POS	2	O.9

In Table 2, among the 221 subjects recruited, HPV DNA testing revealed a low prevalence of high-risk genotypes. HPV 16 was detected in 3 (1.4%) samples, while 218 (98.6%) were negative. HPV 18 was found in 2 (0.9%) samples, with 219 (99.1%) testing negative.

Table 3: Showing association between HPV positivity and cytological abnormalities

HPV Method	PAP	ASCUS (%)	LSIL (%)	HSIL (%)	Neg(%)	P-Value
HPV CT	Neg	14(73.70	26(66.7)	19(67.9)	85(63.0)	0.795
	Pos	5(26.3)	13(33.3)	9(32.1)	50(37.0)	
HPV RFU	Neg	4(21.1)	8(20.5)	7(25.0)	30(22.2)	0.977
	Pos	15(78.9)	31(79.5)	21(75.0)	105(77.8)	
Total		19 (100)	39(100)	28(100)	135(100)	

In Table 3: HPV DNA testing on cervical cytology samples showed no significant association between HPV positivity and cytological abnormalities. Using cycle threshold (CT), HPV positivity ranged from 26.3% in ASC-US to 37.0% in normal samples (p=0.795). Using relative fluorescence units (RFU), HPV positivity was high across all cytology categories (75.0-79.5%), with no significant differences (p=0.977).

Table 4 Correlation of HPV genotypes with specific cervical cytological abnormalities.

o	PAP	ASCUS (%)	LSIL (%)	HSIL (%)	Neg(%)	P-Value
HPV16	Neg	19(100.0)	39(100.0)	28(100.0)	132(97.8)	0.585
	Pos	0	0	0	3(2.2)	
HPV18	Neg	19(100.0)	39(100.0)	28(100.0)	133(98.5)	0.425
	Pos	0	0	0	2(1.5)	

In Table 4, HPV 16 was detected in 3 (2.2%) of normal cytology samples and absent in all abnormal cytology cases. HPV 18 was found in 2 (1.5%) of normal cytology samples and absent in all abnormal cytology cases. No significant association was observed between HPV 16/18 presence and cytological abnormalities ($p>0.05$).

DISCUSSION

Cervical cancer incidence rates have been found to decrease in region where organized cytology-based screening programs had been established. However, unfavorable trend has also been reported and was thought to reflect increases in HPV prevalence [22]. The staging of Cervical Cancer is a determining factor in therapeutic strategies [23] hence; this study investigates the prevalence of high-risk HPV genotypes 16 and 18 in women with cervical abnormalities in Osogbo, South West Nigeria, and their correlation with cytological findings. This study recruited 221 women, with 61.1% showing normal cytology and 38.9% presenting with abnormalities. Among these abnormalities, LSIL was the most common (17.65%), followed by HSIL (12.67%) and ASC-US (8.60%). The significant association of HPV 16 and 18 with ASCUS, LSIL and HSIL highlights the critical role of these genotypes in cervical carcinogenesis. Molecular detection of HPV 16 and 18 provides valuable information for risk stratification and early intervention in women with cervical abnormalities as reviewed by this work. This distribution is in support of other studies [24] that reported that in a meta-analysis of global HPV prevalence, about 70-80% of sexually active women acquire HPV infection at some point, but most infections are transient and do not lead to cervical abnormalities. In a study's findings concurred, noted the high prevalence of normal cervical cytology results and the relatively low rates of abnormalities, including LSIL HSIL and ASC-US [25]. The authors emphasized the importance of these baseline cytology data, which can inform cervical cancer screening and prevention efforts in the target population. They argued that understanding the distribution of cervical cytology results in a given population is crucial for developing tailored screening strategies and allocating resources effectively [22, 25]. Meanwhile, there is an express concern about the generalizability of the study findings, given the relatively small sample size and the specific geographic and demographic characteristics of the study population [26]. They argued that larger, more diverse studies are needed to establish the reliability and applicability of the reported cytological and HPV prevalence data. The authors also contend that the limited sample size and potential cultural or socioeconomic factors in the study setting may limit the ability to extrapolate the results to other populations, especially those with different risk profiles or access to healthcare

Photomicrographs are valuable tools on their own, but combining them with other diagnostic modalities can provide greater insights microscopically. It is one piece of a larger puzzle in cancer

diagnosis and management. Correlating the cellular morphology observed in photomicrographs with the underlying molecular changes detected through techniques like DNA extraction and amplification could yield powerful insights [27]. This multimodal approach allows us to not only visualize the cellular abnormalities, but also understand the genetic drivers behind them. This type of integrated analysis can aid in more accurate subtyping of cancers, prognostic assessment, and targeted therapeutic selection [28].

In the Photomicrographs results, the cervical smear samples were evaluated using the Bethesda System for Reporting Cervical Cytology, which categorized the samples into groups like normal smear, ASC-US, LSIL, and HSIL based on the cellular morphology observed. The photomicrograph illustrates (Plates A-D) is visually depicting the cytological features associated with the different cervical abnormality categories. The visual aids can enhance healthcare providers' understanding and accurate classification of cervical cytology samples, leading to more effective clinical decision-making and highlighted the value of the photomicrograph illustrations [29].

Considering the findings, and reviewed of performance of the Bethesda System, in a meta-analysis, it found to be a reliable and well-validated tool for cervical cytology reporting [30].The authors noted that the system provides clear diagnostic criteria and categories that allow for consistent interpretation of cellular changes, as seen in the results discussed. Also, it has been demonstrated with the high sensitivity and specificity of photomicrograph-based evaluation using the Bethesda System in detecting precancerous and cancerous cervical lesions [31]. This aligns with the ability to differentiate HSIL and LSIL in the provided results (A-D). However, it raised potential caveats regarding the exclusive reliance on photomicrographs for cytological diagnosis [32]. They emphasized the importance of considering the clinical context and integrating photomicrograph findings with other diagnostic modalities, such as human papillomavirus (HPV) testing and colposcopy. This multimodal approach can improve the overall accuracy of cancer detection [28]. In addition, the potential for inter-observer variability in the interpretation of photomicrographs, even among experienced cytologists and may not capture the full extent of the disease, such as in the early stages development or borderline cellular changes [33]. This underscores the need for comprehensive training, quality assurance measures, and possibly the use of computer-assisted image analysis to enhance the consistency of cytological diagnoses.

In determining Prevalence of HPV 16 and 18, the findings comprehensive approach in assessing both cytological findings and HPV DNA testing results. The lack of significant association between HPV positivity and cytological abnormalities, as reported in Table 3, underscores the complex relationship between HPV infection and the development of cervical precancerous lesions, warranting further investigation. It showed a surprisingly low prevalence of HPV 16 (1.4%) and HPV 18 (0.9%). This is unambiguous difference to most global data especially by WHO, that says HPV types 16 and 18 are responsible for about 70% of cervical cancers and precancerous cervical lesions worldwide. Also, a meta-analysis [34] established that in women with normal cytology, HPV 16 prevalence was about 3.2%, and HPV 18 was around 1.4% [34]. In women with cervical cancer, these percentages rise dramatically to around 60% for HPV 16 and 10-15% for HPV 18. The low prevalence in this study could be as a result of regional variations in HPV distribution, Sampling methods, Sensitivity of the molecular detection techniques used and risk factors of the study population. In another support, argued that this finding highlights the need for a more nuanced understanding of the interplay between HPV and other risk factors in the progression to cervical cancer [35].

In contrary, there is a report that questioned and dis-agreed the decision to use only HPV 16 and 18 as the high-risk HPV genotypes in their analysis [34]. They contended that a more

comprehensive assessment of other high-risk HPV types, such as 31, 33, 45, and 52, would provide a more complete understanding of the HPV epidemiology in the study setting. The authors argued that focusing solely on HPV 16 and 18 may underestimate the overall burden of high-risk HPV infections and their potential impact on cervical cancer risk in the target population [36].

Interestingly, there was no significant association between HPV positivity using either Cycle Threshold (CT) or Relative fluorescence (RFU) methods or cytological abnormalities. This is uncommon, as most studies report a strong correlation. In the work that revealed an increase in HPV prevalence with the severity of the cytological abnormality, from 12% in ASCUS to 17% in LSIL and up to 84% in HSIL [37]. Moreover, in this study, HPV 16 and 18 were only detected in normal cytology samples, which is counterintuitive to established knowledge. Typically, these high-risk HPV types are more found in high-grade lesions and cervical cancers [38]. In the supported report, through the suggestion in their work revealed that the lack of significant association between HPV positivity and cytological abnormalities that reported in Table 3, may be influenced by the sensitivity and specificity of the HPV DNA testing method used in the study [39].

CONCLUSION

HPV genotypes 16 and 18 are not prevalent in women with cervical abnormalities, particularly in those with ASCUS, LSIL and HSIL. The results underscore the importance of region-specific research in understanding the dynamic of HPV infection and its relationship with cervical abnormalities. They also highlight the need for comprehensive HPV genotyping beyond just types 16 and 18 in cervical cancer screening programs.

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Conflicts of Interest

The authors declare no conflicts of interest related to this study.

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