

## REVIEW ARTICLE

# DNA Methylation Markers in Lung Cancer

Yoonki Hong<sup>1</sup> and Woo Jin Kim<sup>1,\*</sup><sup>1</sup>Department of Internal Medicine, School of Medicine, Kangwon National University, Chuncheon, South Korea

**Abstract:** Lung cancer is the most common cancer and the leading cause of cancer-related morbidity and mortality worldwide. As early symptoms of lung cancer are minimal and non-specific, many patients are diagnosed at an advanced stage. Despite a concerted effort to diagnose lung cancer early, no biomarkers that can be used for lung cancer screening and prognosis prediction have been established so far. As global DNA demethylation and gene-specific promoter DNA methylation are present in lung cancer, DNA methylation biomarkers have become a major area of research as potential alternative diagnostic methods to detect lung cancer at an early stage. This review summarizes the emerging DNA methylation changes in lung cancer tumorigenesis, focusing on biomarkers for early detection and their potential clinical applications in lung cancer.

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## 1. INTRODUCTION

Lung cancer is the most common cancer and the leading cause of cancer-related morbidity and mortality worldwide, with about 2 million new cases and deaths per year [1]. More than 80% of lung cancer cases are attributed to smoking, and about 70% of lung cancer deaths are known to be smoking-related [2, 3]. Although the smoking rate has decreased mainly because of the application of extensive smoking cessation policies, the incidence of lung cancer appears to be increasing in some regions [4, 5]. Lung cancer rates in never-smokers have recently increased [6], which is partly explained by the life span increase.

Lung cancer is clinically expressed in a variety of ways, and it often presents with a few or no symptoms at an early stage. Since early symptoms of lung cancer are minimal and non-specific, many patients are diagnosed at an advanced stage [1]. This can partly explain why lung cancer mortality remains high despite the development and recent clinical application of new therapeutic agents, such as molecular targeting drugs and immune checkpoint inhibitors [7]. Based on the fact that early diagnosis is important in increasing the survival of patients with lung cancer, low-dose chest computed tomography (LDCT) screening has been introduced as a potentially useful tool for early detection of tumors [8, 9]. LDCT screening rates are increasing, especially among patients at high risk of developing lung cancer [10]. However, the role of LDCT in increasing the survival of patients with lung cancer seems to be limited according to a recent systematic literature review [11]. Whether annual routine examination with LDCT should be performed as a screening test

for lung cancer is a topic of much debate mainly because there are several unresolved issues, like false positive detection, lead-time bias, and potential radiation hazard [12].

Despite a concerted effort to diagnose lung cancer early, no biomarkers for lung cancer screening and prognosis prediction have been established yet. Effective noninvasive screening methods for early diagnosis of lung cancer using a molecular signature are therefore highly desirable. Epigenetic biomarkers, including DNA methylation, have become a major area of research as potential alternative diagnostic methods to detect early-stage lung cancer [13, 14].

As global DNA demethylation and gene-specific promoter DNA methylation have been found in lung cancer, [14-16] insights on DNA methylation changes have led to a broader understanding of lung cancer tumorigenesis and have shown promising candidates for early lung cancer detection or for selection of high-risk subjects with further specific screening methods, such as LDCT. This review summarizes the emerging DNA methylation changes in lung cancer tumorigenesis, focusing on biomarkers for early detection and their potential clinical applications in lung cancer.

### 1.1. DNA Methylation as Biomarkers for Lung Cancer

All human cancers present epigenetic abnormalities. Cancer onset and progression are considered to result from the interaction of genetic changes, epigenetic modifications, and environmental factors [14]. Among the epigenetic changes, DNA methylation prevents recombination events between repetitive sequences, resulting in repressed gene activity and sustained genomic stability [17]. Cancer cells have been shown to present an aberrant methylation signature, occurring either as a global hypomethylation or specific hypermethylation on the promoter of tumor suppressor genes [17-19]. Many aberrant DNA methylation signatures have also been identified in lung cancer, especially for gene-specific promoter DNA methylation [20].

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\*Address correspondence to this author at the Department of Internal Medicine, School of Medicine, Kangwon National University, Chuncheon, South Korea; Tel: 82-32-258-9364; Fax: 82-32-258-2404; E-mail: [pulmo2@kangwon.ac.kr](mailto:pulmo2@kangwon.ac.kr)

Inactivation of transcription factors, having roles in cell differentiation and fate [21], has been found to be a key mechanism in cancer development [22, 23]. Although it has been identified that somatic mutations, copy number variation loss, and promoter hypermethylation were associated with the inactivation of transcription factors in cancer, little is known about the main mechanism. By an integrative multi-omic analysis, a recent study shows that inactivation of transcription factors, bivalently or polycomb repressive complex 2 marked, specifying cell fate plays a key role in carcinogenesis and that the predominant mechanism is promoter hypermethylation rather than an inactivating mutation or copy number variation loss [24]. Although several mutations and copy number variation loss could change the expression of driver genes and reduce the impact of tumor suppressor genes, it seems to be difficult to identify cancer driver genes or target for biomarkers of early cancer detection because these events are infrequently found [25]. In contrast, promoter hypermethylation on tumor suppressor genes is found more frequently and could be more relevant for early cancer detection or risk prediction [26]. Therefore, to date, DNA methylation changes on promoter seem to be a promising target for biomarkers in the detection of lung cancer.

### ***1.1.1. DNA Methylation Status for Early Lung Cancer Detection***

Based on the hypermethylated promoter region of the cell cycle regulator CDKN2A (also known as p16INK4a) found in precursor lesions of lung cancer tumors, it was suggested for the first time that aberrant DNA methylation might constitute a new biomarker for early lung cancer detection [27]. Subsequently, aberrant methylation of the promoter of the CDKN2A was observed in 24% of bronchoalveolar lavage (BAL) samples from patients with non-small-cell lung cancer (NSCLC) [28]. A number of studies identified abnormal methylation of many genes in various samples of lung tumor, BAL, pleural fluid, and blood of patients with lung cancer. As will be well described later, DNA methylation in lung tumor tissue has been mainly evaluated in prognostic studies. Studies using tumor tissues for early lung cancer detection focused mainly on four genes (CDKN2A EX2, CDX2, HOXA1, and OPCML) that were methylated differently in lung adenocarcinoma compared with normal lung tissue, with a sensitivity of 67%-86% and specificity of 74%-82% [29]. NEUROG2, NID2 were hypermethylated in tumors and unmethylated in lung tissues of controls, and a five-gene panel (NEUROG2, NID2, RASSF1A, APC, and HOXC9) analyzed by methylation-specific polymerase chain reaction (PCR) detected stage I NSCLC, with a sensitivity of 91.26% and a specificity of 84.62% [30]. Three methylated genes (CDH1, APC, and RASSF1A) were identified in 80% of samples of 31 patients with primary lung cancer by quantitative methylation-specific PCR (QMSP) [31]. The study also showed that 68% of patients with aberrant tumor DNA methylation exhibited aberrant methylation in BAL DNA.

In bronchial aspirates or BAL, aberrant promoter methylation of APC, p16(INK4a), and RASSF1A were detected by QMSP in patients with peripherally located primary lung cancers [32]. RASSF1A hypermethylation by QMSP was also found in 88% of bronchial aspirates from patients with small-cell lung cancer [33] and was detected in 24% of the

bronchial washing samples from patients with peripheral tumors [34]. Hypermethylation of SHOX2 in bronchial aspirates allowed the distinction between patients with lung cancer and benign lung disease (e.g., pneumonia, asthma, and sarcoidosis), with a sensitivity of 68% and a specificity of 95% [35].

In sputum samples, DNA hypermethylation of RASSF1A and 3OST2 promoter could be useful to distinguish lung cancer patients from controls, with a sensitivity of 85% and a specificity of 74% [36]. The promoter methylation changes on seven-gene panels (PAX5 $\beta$ , PAX5 $\alpha$ , Dal-1, GATA5, SULF2, and CXCL14) predicted 71%-77% of lesions in sputum of asymptomatic patients with stage I lung cancer compared to controls in two independent cohorts [37]. Additionally, promoter hypermethylation of MLH1 was observed in 38% of sputum samples from early-stage NSCLC patient compared with 55% of the tumor samples [38]. In that study, the overall concordance in methylation changes between sputum samples and matched tumor samples was reported to be 72%. Meanwhile, hypermethylation of CDKN2A was mostly evaluated to establish a correlation between tumor and sputum samples, and a higher degree of methylation was shown in tumor samples [39-41]. Additionally, a methylation profile (ASSF1A, p16INK4a, RARb, MGMT, DAPK) obtained from pleural fluid DNA was useful to differentiate between malignant pleural mesothelioma and lung cancer [42].

### ***1.1.2. DNA Methylation Status for the Prognosis of Lung Cancer***

Predicting prognosis in lung cancer patients is important in establishing future treatment and monitoring plans. Although TNM staging has conventionally been used to predict prognosis, molecular markers such as EGFR, ALK, and PD-L1 have been recently adopted for predicting prognosis. In addition to these prognostic factors, aberrant DNA methylation in lung cancer has also been studied as a potential biomarker for prognostic prediction. Most of the studies evaluating the prognostic application of methylation in lung cancer were performed in patients who underwent surgery for early-stage lung cancer and targeted methylation changes of known gene promoters using methylation-specific PCR. Promoter methylation of a four-gene panel consisting of APC, RASSF1A, p16, CDH13 in patients with stage I NSCLC treated with surgery was associated with early tumor recurrence [43]. This study was meaningful in that it was the first study to provide evidence that lung cancer prognosis could be predicted by DNA methylation markers, independently of traditional prognostic factors (*i.e.* staging, age, smoking history and histologic types). Methylation in p16 and CDH13 was also reported as an effective prognostic biomarker in lung adenocarcinoma, supporting data presented in the previously mentioned study [44].

Promoter hypermethylation of tumor suppressor genes has been mostly associated with worse outcomes. Simultaneous methylation of more than four tumor suppressor genes was associated with poor 2-year progression-free survival in paired NSCLC tumor tissue samples and adjacent normal tissue samples in the Chinese population [45]. Promoter methylation of BRMS1 was correlated with poor disease-free survival in 325 NSCLC patients treated with surgery

[46], while high methylation of SHOX2 and PITX2 significantly predicted progression-free survival in patients with NSCLC who underwent surgery [47]. Additionally, from DNA methylation profiling using microarrays covering 27,578 cytosine-phosphate-guanine (CpG)s, a set of genes (10 CpGs in 10 genes) was associated with improved survival in 48 patients with stage I NSCLC [48].

### **1.1.3. DNA Methylation Status in Blood for Early Lung Cancer Detection**

Blood samples are an easily accessible and highly feasible source of biomarker data for early lung cancer detection. Based on the hypothesis that DNA from apoptotic cancer cells could be detected in serum and plasma, DNA methylation changes on several target genes have been evaluated in blood samples of patients with lung cancer. Additionally, some evidence suggests that certain aberrant DNA methylation in blood might reflect pathological changes in target tissues that could not be obtained easily or safely [49]. In a previous study of blood DNA methylation, the methylation status of six genes (RASSF1A, CDKN2A, RARb, CDH13, FHIT, and BLU) showed 75%–87% concordance between tumor tissues and plasma samples [50]. Similar levels of promoter methylation on four genes (ECAD, p16, MGMT, and DAPK) were concurrently found in bronchial epithelial cells and blood samples from smokers with lung cancer [51]. Additionally, these results showed that the odds ratio for lung cancer was especially high for p16 methylation and RASSF1A methylation in plasma, indicating a possible application of such sampling for early lung cancer detection. Thereafter, DNA methylation of SHOX2 by QMSP in 411 plasma samples from patients with lung cancer and controls allowed the distinction between lung cancer from controls, yielding a sensitivity of 60% and a specificity of 90% [52]. A recent meta-analysis showed that the pooled diagnostic performances of P16INK4a gene promoter methylation in serum had a sensitivity of 37%, a specificity of 95%, a positive likelihood ratio of 5.46, a negative likelihood ratio of 0.64, and a diagnostic odds ratio of 9.41 [53]. However, other biomarker studies using blood samples have reported inconsistent results. Serum was reported to be inferior to sputum in classifying lung cancer and tumor biopsy samples were classed as normal according to the methylation status of CDKN2A, DAPK, PAX5b, and GATA5 [35]. The methylation status of CDKN2A in blood samples of patients with lung cancer ranged from 22.2%–75.7% in different studies [54–56]. Promoter methylation for DAPK was not affected in blood samples from nonsmokers with NSCLC although DAPK was found in 41% of blood samples of smokers with NSCLC, showing differential methylation changes according to smoking status in NSCLC [51]. Furthermore, some reports indicated that promoter methylation for some selected genes in blood samples was not correlated with tumor stage [20].

Blood biomarkers could have clinical significance in the early lung cancer detection or prediction of prognosis. As DNA methylation changes in cell-free DNA (cfDNA) extracted from plasma were identified to be similar to changes in DNA in cancer tissue [57, 58], it might be suggested that methylation changes on cfDNA could be useful for early lung cancer detection [59]. Recent studies focusing on DNA methylation changes from cfDNA of serum or plasma are as

follows. Using 96 gene markers screened by bisulfite conversion-based genome-wide methylation test (Illumina 450k arrays) in cfDNA from the sera of 204 patients, lung cancer was distinguished from controls and benign lung disease (interstitial lung disease and chronic obstructive pulmonary disease) by QMSP, with a sensitivity of 87.8% and a specificity of 90.2% [60]. Promoter methylation of a six-gene panel (CDO1, HOXA9, AJAP1, PTGDR, UNCX, and MARCH11) selected from The Cancer Genome Atlas (TCGA; [61]) dataset, led to the differentiation of 43 patients with stage IA NSCLC from 42 population-matched controls by QMSP, with a sensitivity of 72.1% and a specificity of 71.4% [62]. QMSP for another six-gene set from the TCGA dataset (SOX17, TAC1, HOXA7, CDO1, HOXA9, and ZFP42) showed that the sensitivity and specificity of the best individual genes ranged from 65%–76% and from 74%–84%, respectively, for lung cancer diagnosis in plasma obtained preoperatively from patients with NSCLC (stage I and IIA) and controls [63]. The study also showed that a three-gene combination (SOX17, TAC1, HOXA7) in plasma had a sensitivity of 93% and a specificity of 62%. Meanwhile, a recent research applied a new cost-effective DNA methylation profiling approach, methylated DNA immunoprecipitation was coupled with high-throughput sequencing to comprehensively characterize the methylation pattern of cfDNA in lung cancer [64]. The analysis yielded a set of ten significantly different methylated genes (B3GAT2, BCAR1, HLF, HOPX, HOXD11, MIR1203, MYL9, SLC9A3R2, SYT5, and VTRNA1-3) in lung cancer from 330 differentially methylated regions at gene promoters identified in patients with lung cancer. Of these, 33 hypermethylated genes were validated against the public DNA methylation data. These studies may help find new biomarkers for early lung cancer detection using cfDNA from blood samples. However, concerns remain in that methylation changes detected in serum or plasma, probably from apoptotic tumor DNA, are indicative of an already advanced cancer stage [65].

Meanwhile, research for DNA methylation changes in buccal cells may provide more understanding for the development of lung cancer, in that buccal cells are directly exposed to most potent carcinogens like smoking. An EWAS study in buccal and blood cells showed that differentially methylated CpGs in buccal cells were much increased compared with matched blood samples [66]. This study has meaningful results and the alterations of DNA methylation in buccal tissue may be more important for lung cancer development or the transformation of precancerous lesions, suggesting a potential to target biomarkers for early detection of lung cancer. Further research seems to be needed in high risk subjects or patients of lung cancer.

### **1.1.4. Development of Methods for the Detection of Genome-wide DNA Methylation Changes**

Identification for methylation changes in specific known gene sites is limited in terms of the identification and development of biomarkers in lung cancer. Simultaneous measurements of methylation across the whole genome may broaden our understanding further on DNA methylation changes induced by lung cancer. Although whole-genome bisulfite sequencing is considered the gold standard for detecting methylation changes in the entire genome, this meth-

od has limited application in clinical research as it requires a high level of technical expertise, and is costly [67]. Alternatively, a popular and informative method, namely microarray, has been developed for accurate estimation of DNA methylation changes in multiple genomic regions using a minimal amount of DNA, as that obtained from blood samples [14]. The Illumina Infinium BeadChips, the HumanMethylation27k, and 450k BeadChip, have been applied in clinical research as easy and effective tools for genome-wide DNA methylation profiling [68]. The 27k microarray allowed researchers to understand the contribution of DNA methylation changes in tumorigenesis and find biomarkers associated with cancer [69]. Using the 27k microarray, large-scale epigenome-wide association studies (EWAS) could be performed for the first time; these found relevant associations with DNA methylation changes and disease risk, with smoking or aging [70-74]. Thereafter, with improved coverage, the 450k microarray has become a popular tool for EWAS studies and has been used for the generation of international epigenetic consortiums, as the International Cancer Genome Consortium, the International Human Epigenome Consortium and TCGA [75]. Especially, the 450k microarray has been used to identify noteworthy gene sites associated with the risk of further lung cancer development in recent important studies, as described below. A more advanced tool, the Infinium MethylationEPIC (EPIC) BeadChip, containing over 850,000 probes, was recently introduced. The EPIC array showed a significant improvement over the 450k microarray with high reproducibility and reliability, making it possible to apply high-throughput human EWAS analyses to diverse clinical samples [76]. In a pilot study in blood samples obtained for three consecutive years from lung cancer patients, the serial changes of genome-wide DNA methylation using EPIC array were evaluated and identified with two significant CpG sites; cg21126229 (RNF212) and cg27098574 (BCAR1) [77]. However, there are few studies showing clinical applications for lung cancer using EPIC.

### ***1.1.5. DNA Methylation Changes in Blood for the Risk of Lung Cancer Development***

A preliminary study reported that aberrant methylation of p16 could be detected from the sputum of tobacco smokers with lung cancer 3 years before a clinical diagnosis could be predicted from tumor tissue with early recurrence of NSCLC [43, 78]. To date, these results have not been applied in clinical practice, partly because the method was not suitable for easily accessible samples, such as blood samples. Meanwhile, there have been many pieces of evidence from recent EWAS studies confirming that DNA methylation changes in blood were related to tobacco smoking [79-81]. Further, it was reported that although most DNA methylation changes reverted in non-smokers, changes in other genomic regions persisted long after smoking cessation in tobacco smokers [82]. Based on these smoking-association EWAS studies using blood samples, important clinical implications were recently reported in several population-based cohorts, including the European Prospective Investigation into Cancer (EPIC) [83], the Melbourne Collaborative Cohort Study (MCCS) [84], the Norwegian Women and Cancer Study (NOWAC) [85], the Northern Sweden Health and Disease Study (NSHDS) [86], and the ESTHER study in the German federal state of Saarland [87]. These studies showed that

DNA methylation changes in blood samples taken years ago from patients diagnosed with cancer were associated with cancer risk or mortality. These results are meaningful because samples were obtained from prospectively enrolled cohorts and mostly used data from the recent EWAS platform using 450k microarray.

F2RL3, repeatedly found to be related to smoking, was evaluated for the association with lung cancer using data from the ESTHER study cohort by specific mass spectrometry [88]. Hypomethylation at F2RL3 was strongly associated with both lung cancer incidence and mortality. Moreover, after adjusting for smoking exposure and other confounders, the associations persisted. This study was meaningful as it was based on blood samples obtained years before lung cancer diagnosis was established in a population-based prospective cohort study, unlike the previous studies of cross-sectional designs. Further research by the same group, using data from the ESTHER cohort, simultaneously investigated the individual and joint predictive values for the lung cancer death of smoking- and lung cancer-related methylation markers, with a case-cohort design and a median follow-up of 13.8 years [89]. Unlike previous studies using mass spectrometry to investigate DNA methylation changes in specific gene regions of F2RL3, this study used the 450k microarray for EWAS to predict lung cancer mortality. The results showed that hypomethylation at 77 CpGs associated with smoking and 121 CpGs associated with lung cancer were thus associated with mortality. These findings suggested that CpGs are promising candidates for identifying high-risk subjects with lung cancer. Another recent EWAS, using the 450k microarray, showed the importance of DNA methylation changes in peripheral blood DNA in lung cancer development. Cg05575921 in aryl hydrocarbon receptor repressor (AHRR), AHRR and cg03636183 in F2RL3 were identified as the most significant associations with lung cancer risk in the NOWAC cohort and were validated in additional case-control pairs from the MCCS, NSHDS, and EPIC HD cohorts [85]. The study showed that methylation changes in the AHRR and F2RL3 genes were significant even after adjusting for smoking, although it had been demonstrated previously that DNA methylation patterns of the AHRR and F2RL3 genes were altered by tobacco smoking [90]. Thus, these findings suggest that DNA methylation changes in the AHRR and F2RL3 genes might mediate the role of tobacco smoking in lung cancer development.

Notwithstanding, the above-mentioned studies are mostly on blood samples of patients with NSCLC, and relatively few studies have been conducted in small-cell lung cancer. The nine validated CpG sites discriminated patients with small cell lung cancer from controls in 85.8%, using pyrosequencing technology in peripheral blood DNA [91].

On the other hand, there is a research that the above findings that DNA methylation changes in blood are associated with lung cancer development may result from confounding [92]. To identify the possible causal role of these methylation changes, an appraising study was performed using a meta-analysis of four EWAS studies and a two-sample Mendelian randomization analysis [93]. The study concluded that there was little evidence for playing a causal role of blood DNA methylation changes at locus including AHRR, identified

from previous EWAS, in lung cancer development. More research is required with solid evidence for a causal role of DNA methylation changes in blood

### 1.1.6. The Causal Relevance of DNA Methylation for Risk of Lung Cancer

Although DNA methylation changes in smoking-associated differentially methylated CpGs, including the AHR and F2RL3 locus, were identified and validated as the associations with lung cancer [85] and were suggested to the mediating role of smoking in lung cancer development [90], the biological mechanisms still remain elusive. A novel systems-epigenomics analysis inferred the action mechanism of lung-specific transcription factors in lung cancer development [94]. The study identified that a transcription factor (AHR) and its repressor (AHR) were implicated in lung carcinogenesis. AHR were not overexpressed in lung carcinoma and AHR were gradually inactivated from dysplasia to carcinoma in site and lung carcinoma, suggesting AHR overexpression to the reflection of response to smoke and AHR inactivation to a condition for the onset of lung cancer. Given that, the AHR-pathway is implicated in controlling a healthy immune response [95, 96], and the gradual inactivation of AHR may lead to changes in the immune response, which facilitates carcinogenesis. Another transcription factor of FOXJ1 which generates airway ciliated cells and clear inhaled pathogens and FOXA2, which have roles in lung morphogenesis were also found to be inactivated in lung cancer and carcinoma *in situ*, suggesting that inactivation of FOXJ1/FOXA2 may contribute causally to lung cancer progression. In addition to the AHR and the FOXJ1 pathway, HIF3A, which is involved in hypoxia was exhibited by gradual inactivation between dysplasia, carcinoma *in situ* and lung carcinoma, suggesting potential causal pathways in lung cancer development.

Chronological age, another important risk factor as well as smoking, can be considered to be a target for the causal relevance of DNA methylation for risk of lung cancer. An aging biomarker was developed by measuring DNA methylation levels on human tissues including blood [97]. Using the method on 2,029 females, the study showed that age acceleration based on DNA methylation levels of blood were significantly associated with subsequent lung cancer incidence, suggesting that the aging marker with DNA methylation could be a promising biomarker for lung cancer susceptibility.

More research is required on mechanism and causality in pathways linking blood DNA methylation changes with risk factors, as aging and smoking exposure, and lung cancer development.

## CONCLUSION

Although lung cancer is the leading cause of cancer-related morbidity and mortality worldwide, there are no biomarkers for lung cancer screening and prognosis prediction. As aberrant DNA methylation patterns have been identified in lung cancer, DNA methylation biomarkers have been heavily researched as potential diagnostic markers to detect early-stage lung cancer. Preliminary studies have identified promising candidates as biomarkers for early lung cancer

detection and prognosis prediction from the aberrant promoter methylation changes in various samples. However, to date, no DNA methylation biomarker is being applied in clinical practice. Recent research using blood samples, an easily accessible and feasible sample source, especially prospectively collected, pre-diagnostic peripheral blood samples from lung cancer patients, and new popular and informative methods, such as EWAS (*e.g.*, the EPIC array), is expected to provide useful prognostic and predictive marker data. DNA methylation changes are promising candidates for the development of biomarkers in lung cancer. Further investigation of DNA methylated regions and affected genes may lead to the development of a biomarker with clinical applications for lung cancer.

## CONSENT FOR PUBLICATION

Not applicable.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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