


# Shorter Cilia Length and Aberrant Ciliated Marker DNAI1 in Allergic Rhinitis

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**Purpose:** This study aimed to investigate whether the impaired ciliary length and aberrant ciliary ultrastructure marker, dynein axonemal intermediate chain 1 (DNAI1), are important pathological characteristics in nasal mucosa from patients with allergic rhinitis (AR).

**Patients and Methods:** Biopsies were taken from the inferior turbinate (IT) of controls ( $n = 20$ ) and patients with AR ( $n = 20$ ). The ciliary length and the DNAI1 location patterns were assessed by using immunofluorescent staining. Three patterns of DNAI1 localization were defined using a semi-quantitative scoring system: normal (N), partial (P) and absence (A). Every individual section was assigned a score between 0 and 2 in each high-power field (5 fields per sample). The score of 0 = pattern N >70%; 1 = patterns N + P >70%; and 2 = pattern A  $\geq$ 30%. The receiver operating characteristic (ROC) curve was used to evaluate the predicted value of DNAI1 score for AR.

**Results:** The ciliary length was reduced by 33.3% in patients with AR compared with controls ( $P < 0.0001$ ). The higher DNAI1 score was found in the AR group, with a median (first and third quartile) of 0.9 (0.4 and 1.08), which was 0.1 (0 and 0.76) in the control group ( $P = 0.0071$ ). The ROC of DNAI1 was calculated based on the area under the curve of 0.74 ( $P = 0.0094$ ). The cutoff value of ROC was 0.5833, with a sensitivity and specificity of 70%.

**Conclusion:** These results suggested that the shorter ciliary length and aberrant localization of DNAI1 are potentially important pathological characteristics of the allergic nasal mucosa. The aberrant localization of DNAI1 may provide a novel candidate target for clinical management of AR.

**Keywords:** allergic rhinitis, cilia length, DNAI1, biomarker, ciliated marker

## Introduction

Allergic rhinitis (AR) is a kind of the T-helper type 2-skewed upper airway inflammatory disease, characterized by pruritus, sneezing, rhinorrhea, and nasal congestion.<sup>1</sup> Poorly controlled clinical symptoms not only increase the risk of asthma but also strongly decline the quality of life and productivity at work or school.<sup>2</sup> Besides these symptoms, ciliary impairment would decrease the mucociliary clearance (MCC) function and extend the duration of pathogens and allergens in nasal epithelium, aggravating the severity of AR and its comorbidities.<sup>3-5</sup> A lot of clinical researches showed that the treatment for increasing the MCC, such as nasal irrigation, could relieve the nasal symptoms of AR effectively.<sup>6,7</sup> However, the molecular factors leading to ciliary impairment in mucosa of AR patients still not fully understood.

Currently, the abnormality of cilia length and the ciliated motile structure is considered as important characteristics and pathogenesis in nasal mucosa of airway diseases. Lengthened cilia have been reported in chronic rhinosinusitis with nasal polyps may lead to chronic inflammation or infection in nasal mucosa.<sup>8</sup> Smoking-associated shorter airway epithelial cilia plays a significant role in the pathogenesis of smoking-induced lung disease.<sup>9</sup> Dynein axonemal

intermediate chain 1 (DNAI1) is located on the intermediate chain of outer dynein arm, which is a key marker for ciliary movement and useful for establishing a clinical molecular genetic test for primary ciliary dyskinesia.<sup>10</sup> DNAI1 was reported to be induced by the upregulation of transforming growth factor beta-1 in perennial and seasonal AR.<sup>11</sup> However, thus far there has yet to be a systematic histopathological investigation directly linking abnormal DNAI1 localization with AR, for which abnormal cilia length may also be a key indicator of the disease.

Based on our previous studies, the high-throughput microarray data, Gene Ontology enrichment and Kyoto Encyclopedia of Genes and Genomes pathway enrichment prompted significant functional groups and pathways directly related to the ciliary structure.<sup>12</sup> Furthermore, significant downregulation of DNAI1 was found on microarray and quantitative reverse transcription-polymerase chain reaction in patients with AR compared with control individuals.<sup>13</sup> This study aimed to extend the investigation for the localization of DNAI1 protein as well as the length of cilia in inferior turbinate (IT) from patients with AR and controls, which was used to find the pathological characteristic underlying allergic nasal mucosa.

## Materials and Methods

### Patients Recruited

This study was approved by the Institutional Review Boards of Zhujiang Hospital, Southern Medical University (China), and the National Healthcare Group Domain-Specific Review Board of Singapore (Singapore). All participants provided informed consent, in accordance with the Declaration of Helsinki. Biopsy specimens of IT from control individuals and AR patients with septal deviation were obtained during septal plastic surgery. AR was diagnosed according to the criteria of the Initiative on Allergic Rhinitis and Its Impact on Asthma,<sup>14</sup> based on the skin prick test (Alutard, ALK-Abellórd, Denmark) or serum total immunoglobulin E detected using an allergy screen test (LG Chem, South Korea). All recruited patients with AR were free of rhinosinusitis, lower respiratory tract infection, and self-reported or physician-diagnosed asthma and smoking.

### Immunofluorescence Staining

The tissues were embedded in paraffin and cut into 0.4- $\mu$ m sections. Acetylated- $\alpha$ -tubulin (acet.- $\alpha$ -tubulin) (mouse anti-human acetylated alpha-tubulin monoclonal antibody [clone ab24610], Abcam, MA, USA) was stained for cilia. DNAI1 (rabbit anti-human DNAI1 polyclonal antibody [HPA021649], Sigma-Aldrich, St. Louis, MO, USA) was co-stained with acet.- $\alpha$ -tubulin in the paraffin-embedded specimens from the collected nasal biopsies. The sections were incubated with their respective primary antibodies overnight at 4°C, followed by 1-h incubation with Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies (Sigma) in the dark at 37°C. The coverslips were mounted on the slides using SlowFade Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies, CA, USA). For negative controls, primary antibodies were substituted with the species- and subtype-matched antibodies at the same concentration. The slides were then analyzed with fluorescent microscopy (Olympus IX51, Tokyo, Japan).

### Evaluation of Results by Immunofluorescence Staining

Two researchers independently assessed cases in a blinded manner to obtain a standardized histologic evaluation of the staining. Protein expression by immunofluorescence (IF) staining was quantified using ImageJ with images taken at five randomly selected fields at 400 $\times$  magnification with a fluorescence microscope. The mean value was calculated from positive staining in five fields.

### Measurement of Cilia Length

The cilia length was evaluated in nasal tissues by assessing the positive staining of acet.- $\alpha$ -tubulin, with 20  $\mu$ m (scale bar of 400 $\times$  magnification) used as the standard for measurement. The mean value of the cilia length was calculated from 20 measurements per area for each paraffin-embedded section. The cilia length distribution was analyzed using the proportion from all the fields in AR and control groups.

## Score Evaluation of DNAI1

The score evaluation of DNAI1 was referred to as the semi-quantitative grading system as published.<sup>15</sup> The three patterns of DNAI1 were defined as follows: (1) the localization of DNAI1 was present throughout the entire axoneme (pattern N, normal); (2) the localization of DNAI1 was within the axoneme, except in proximal regions (pattern P, partial); and (3) the localization of DNAI1 was completely missing throughout the entire axoneme, regardless of their presence or absence in the apical cytoplasm (pattern A, absence).

Each individual section was given a score between 0 and 2, where score = 0 (normal DNAI1 presence) was given if the field contained >70% of areas with pattern N; score = 1 (abnormal DNAI1 presence) was given if the field contained a combined area of pattern N + P >70%; and score = 2 (highly abnormal DNAI1 presence) was given if the field contained a combined area of pattern A  $\geq$ 30%.

## Statistical Analysis

All data were analyzed using GraphPad Prism 7. The Mann–Whitney two-tailed nonparametric test was used to compare the cilia length and DNAI1 localization score in paraffin-embedded specimens. The ROC curve analysis [area under the ROC curve (AUC), cutoff value, sensitivity%, and identity%] was employed to assess the predicted value of DNAI1 for AR. The optimal cut point is defined as the point of closest approach to the upper left axes according to the following criterion: minimum  $[(1-\text{sensitivity})^2 + (1-\text{specificity})^2]$ , which was calculated by excel to choose the best cutoff for DNAI1 to predict AR. The *P* value <0.05 indicated a statistically significant difference.

## Results

### Participant Characteristics

The clinical characteristics of control individuals and patients with AR are summarized in Table 1. Control (n = 20) and AR (n = 20) groups with self-reported or physician-diagnosed asthma or chronic rhinosinusitis (CRS) were excluded from this study. Moreover, smokers were also excluded as smoking caused shorter airway cilia. All paraffin specimens with cilia staining were confirmed by IF with acet- $\alpha$ -tubulin and used for cilia length and DNAI1 score analysis. A summary of cilia length and DNAI1 expression pattern statistics in vivo is shown in Table 2.

### Shorter Cilia Length in Nasal Epithelium in Patients with AR

IF was performed to observe the ciliary morphology and assess cilia length in IT biopsies from control and AR groups (Figure 1A and B). The results demonstrated that the median value of cilia length in the control group was 3.96  $\mu\text{m}$ , and the values of first and third quartiles were 3.28  $\mu\text{m}$  and 4.34  $\mu\text{m}$ , respectively. However, the median value of the cilia length in the AR group was 2.64  $\mu\text{m}$ , and the values of the first and third quartiles were 2.42  $\mu\text{m}$  and 2.85  $\mu\text{m}$ ,

**Table 1** Summary of Patient Characteristics and the Methods

	Control Subjects	Patients with AR
Sample size (n)	20	20
Age, years (SD)	39.58 (12.50)	31.76 (9.44)
Male/female	14/6	13/7
Allergen sensitization	0	20
Dust mites	0	16
Allergens other than dust mites	0	4
Paraffin specimens with cilia staining with IF	20	20

**Note:** Values are n.

**Abbreviations:** AR, allergic rhinitis; IT, inferior turbinate; IF, immunofluorescence.

**Table 2** Summary of Cilia Length and DNAI1 Expression Patterns Statistics in vivo

	Control Subjects	Patients with AR
Paraffin specimens (n)	20	20
Cilia length		
Evaluated areas	82	77
Evaluated times	1020	1540
Cilia length median (1st, 3rd quartile)	3.96 (3.28, 4.34)	2.64 (2.42, 2.85)
DNAI1		
Evaluated areas	82	77
DNAI1 score [median (1st, 3rd quartile)]	0.1 (0 and 0.76)	0.9 (0.4 and 1.08)
DNAI1 patterns (n)		
Pattern N	55	27
Pattern P	20	35
Pattern A	7	15

**Note:** Values are n.

respectively (Figure 1C). The cilia length was reduced by 33.3% in patients with AR compared with control individuals ( $P < 0.0001$ ). The distribution of the cilia length weighted equally based on all the fields from control individuals and patients with AR evaluated showed a consistent shift toward shorter cilia (Figure 1D).

### Aberrant DNAI1 Location Was More Frequently Observed in Patients with AR

Tissue paraffin sections from control and AR groups were stained using IF to evaluate the co-location of DNAI1 (red) and acet.- $\alpha$ -tubulin (green) (Figure 2A). The location patterns of DNAI1 were patterns N, patterns P, and pattern A between AR and control groups (Figure 2B–D). According to the semi-quantitative scoring system, the median value (first and third) of the DNAI1 score was 0.1 (0 and 0.76) in control individuals and 0.9 (0.4 and 1.08) in patients with AR. The DNAI1 score was obviously higher in patients with AR than in control individuals ( $P = 0.0071$ ) (Figure 3A). The results showed that aberrant DNAI1 location was more frequently observed in patients with AR.

### DNAI1 Was Considered as a Predictor of AR

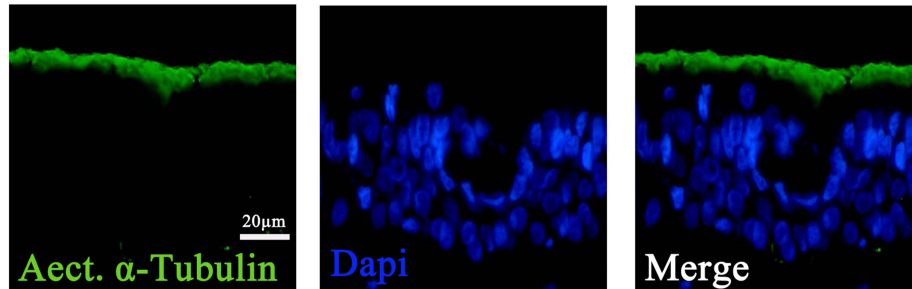
The sensitivity and specificity at different cutoff points of the DNAI1 score for AR are shown in Table 3. The result of AUC was 0.74, and  $P = 0.0094$  demonstrated that DNAI1 could be considered as a predictor of AR. The following formula was used to calculate the cutoff value of ROC:  $[(1 - \text{sensitivity})^2 + (1 - \text{specificity})^2]$ . A score of 0.5833 was chosen as the best cutoff for DNAI1 to predict AR, with a sensitivity and specificity of 70% (Figure 3B).

### Discussion

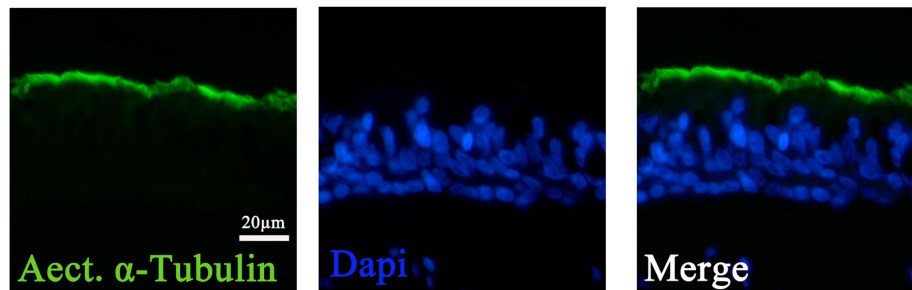
This study was novel in revealing the shorter cilia length as well as increased abnormal DNAI1 location, which might be the important pathological characteristics of allergic nasal mucosa. At the same time, DNAI1 was demonstrated to be a key ciliated marker correlated with AR, which may provide a novel candidate target for clinical management.

Ciliary impairment in lower airway diseases leads to prolonged exposure of aeroallergens or pathogens to the respiratory epithelium and increased susceptibility to infections, which aggravates clinical symptoms and contributes to the disease burden.<sup>16</sup> Shorter cilia length has been reported in various airway-related diseases, which is well known as an important factor for the ciliary impairment. Philip et al found that smoking was associated with shortened airway cilia, which participated in the pathogenesis of smoking-induced lung disease.<sup>9</sup> Furthermore, Lam et al suggested that cilia

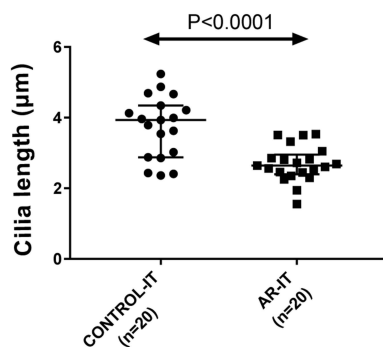
## A Control subjects



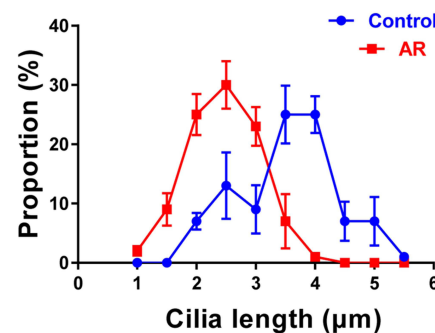
## B AR patients



## C Cilia length measurement



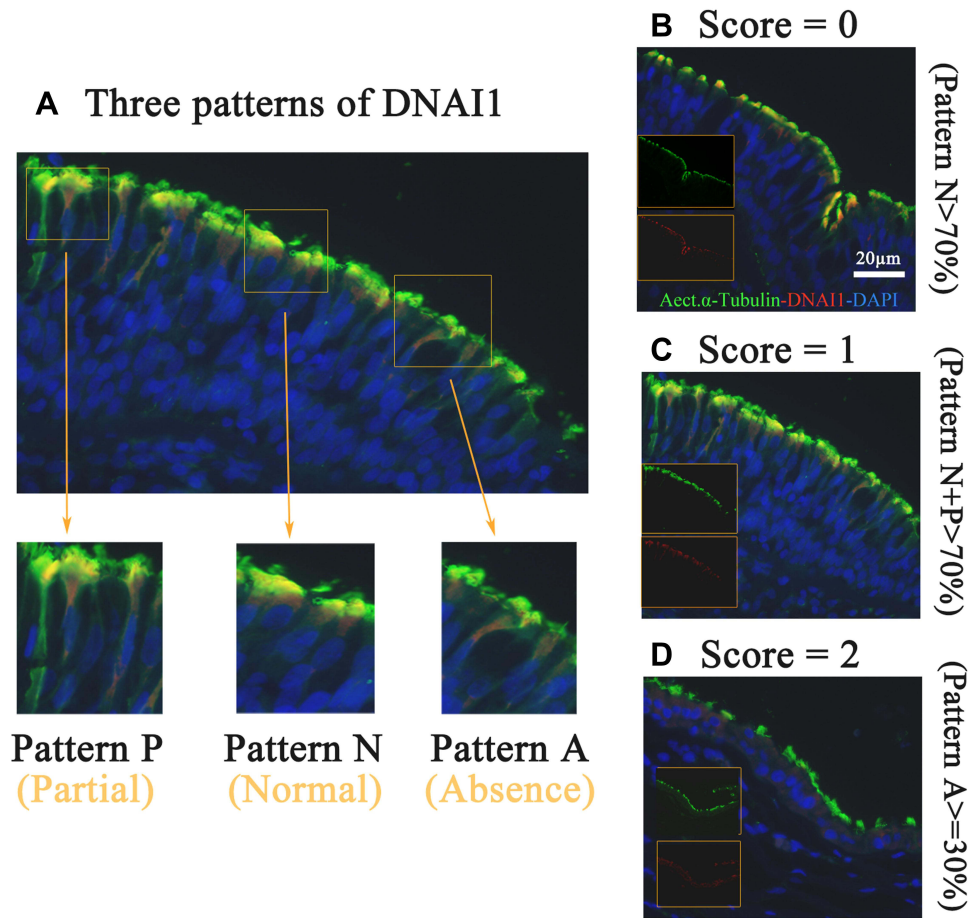
## D Cilia length distribution



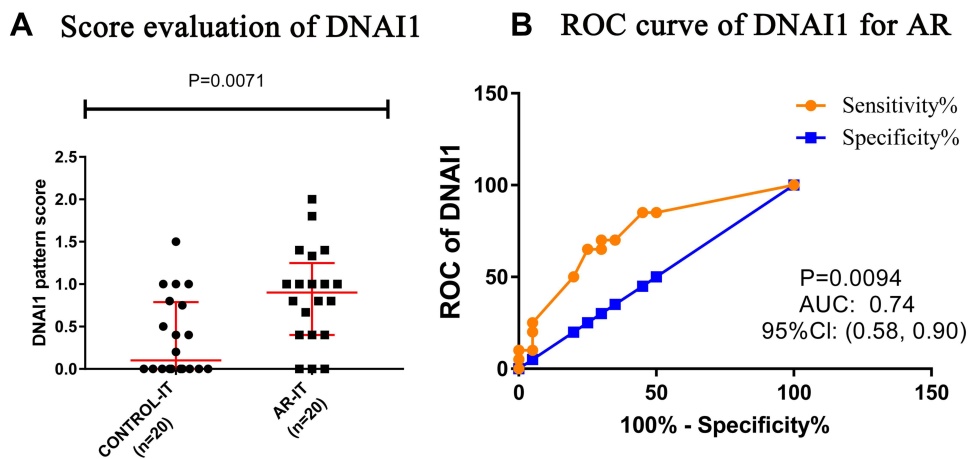
**Figure 1** Shorter cilia length in patients with AR compared with control individuals. **(A and B)** Positive staining of cilia by acet.α-tubulin in patients with AR compared with control individuals under 400× magnification, scale bar 20 μm. Green – acet.α-tubulin, blue – DAPI. **(C)** Shorter cilia length in patients with AR compared with control individuals. Median and first and third quartile values are indicated by the scale bar. **(D)** A higher proportion of shorter cilia length distribution in patients with AR.

shortening in chronic obstructive pulmonary disease (COPD) correlated with autophagy-dependent pathway, which regulated the cilia length and had potential as a therapeutic target for COPD.<sup>17</sup> As upper and lower airways are considered a unified morphological and functional unit, whether shorter cilia length in patients with AR correlates with the progression of AR still needs more evidence.

In motile ciliated cells, DNAI1 has been reported to assess the ciliary structure and provide normal beating motion of the cilia. Djakow et al found at least one mutant allele in over half of patients who had primary ciliary dyskinesia due to dynein axonemal heavy chain 5 (DNAH5) or DNAI1 using selected exon sequencing.<sup>18</sup> Biomarkers play an increasingly important role in clinical application.<sup>19</sup> For the studies of ciliated associated markers in airways, the absence or mislocalization of DNAH5 from motile cilia in chronic rhinosinusitis with nasal polyps may provide novel candidate targets for clinical management.<sup>15</sup> Dynein axonemal heavy chain 9 (DNAH9) was supposed to be a promising candidate gene for early smoke exposure in bronchial hyperresponsiveness.<sup>20</sup> According to the examination at microarray, mRNA, and protein levels, significant downregulation of DNAI1 was found in patients with AR compared with control



**Figure 2** Three location patterns of DNAI1 expression and evaluated scores. (A) Three patterns of DNAI1 localization, normal, N; partial, P; absence, A, were observed in both control and AR groups under 400× magnification, scale bar 20 μm. (B–D) Semi-quantitative scoring system was used in five areas per paraffin section. Every individual section was assigned a score between 0 and 2.



**Figure 3** Score and ROC evaluation of DNAI1 for AR. (A) Higher DNAI1 score in patients with AR compared with control individuals. Median and first and third quartile values are indicated by the scale bar. (B) Predictive ability was calculated based on the AUC. DNAI1 score had an AUC of 0.74 and  $P = 0.0094$ . The cutoff value was 0.5833, and sensitivity and specificity were 70%.

**Table 3** Sensitivity and Specificity at Different Cutoff Points of DNAI1 Score for AR

	Cutoff Value	Sensitivity%	Specificity%	$(1 - \text{Sensitivity})^2 + (1 - \text{Specificity})^2$
DNAI1 score	0.3	85	55	0.225
	0.45	70	65	0.2125
	0.5833	70	70	0.18
	0.7083	65	70	0.2125
	0.775	65	75	0.185

individuals. Thus, DNAI1 might be a symptomatic ciliated marker for AR. With its predicted value of AR, the abnormal location of DNAI1 is potential to be a critical and novel target for future clinical assessment and intervention for patients with AR.

A previous study reported the downregulation and aberrant localization of ciliogenesis-associated marker, forkhead box J1 (FOXJ1), in allergic nasal mucosa so as to further investigate the mechanism underlying shorter cilia length and aberrant DNAI1 location. At the mRNA level, the expression of DNAI1 positively correlated with FOXJ1.<sup>13</sup> Researches of nearly 10 years showed that the function of FOXJ1 was elongating cilia and activating and regulating genes necessary for motile cilia function.<sup>21</sup> Further extension of the aforementioned findings showed that shorter cilia length and abnormal expression of DNAI1 were likely related to the regulation of FOXJ1. Future studies are required to confirm the possible mechanism underlying the regulation of ciliogenesis-associated marker FOXJ1 on ciliated marker DNAI1 and cilia length in AR.

IF imaging revealed the localization of DNAI1 proteins directly and conveniently. With IF imaging now being clinically validated, the possibility of DNAI1 protein helping in the early prediction of AR has increased.<sup>22</sup> However, the limitation of this study was that individual differences from the tissue samples were unavoidable, which might have affected the result interpretation. In future studies, samples from nasal cells and animal models with AR should be used to confirm the findings.

## Conclusion

Shorter cilia length and aberrant localization of ciliated marker DNAI1 played a key role in pathological characteristics of the allergic nasal mucosa. Moreover, the aberrant localization of DNAI1 may provide a novel candidate target for clinical management of AR. Further studies should understand the etiology and molecular mechanisms underlying this pathological condition and explore whether such alteration in nasal mucosa is common among patients with other chronic respiratory diseases (eg, asthma and CRS).

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## Disclosure

The authors report no conflicts of interest in this work.

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