

# Quality and Quantity of Nucleic Acids Extracted from Formalin-Fixed Paraffin-Embedded Lymphoma Biopsies from Nigerian Archived Biopsy

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**ABSTRACT**

**Background:** Integrity of nucleic acids derived from archived formalin-fixed paraffin-embedded (FFPE) cancer specimens affects diagnosis, prognosis, and therapy. Several factors affect the quality and quantity of extracted nucleic acids and one of such factors is storage period. **Aim:** We investigated the impact of storage duration on the quality and quantity of nucleic acids extracted from archived FFPE lymphoma biopsies in Nigeria. **Materials and Methods:** A total of 53 FFPE biopsies diagnosed as lymphoma stored over several years (2008–2019) were analyzed. They were 22 chronic lymphocytic leukemia (CLL) cases, 17 Hodgkin lymphoma (HL) cases, and 14 diffuse large B-cell lymphoma, not otherwise specified (DLBCL, NOS). DNA was extracted from all the lymphoma samples which were analyzed for integrity and amplifiability using the four pairs of control genes polymerase chain reaction (PCR) primers of BIOMED-2 protocol, whereas RNA extraction was from 6 CLL cases used for qPCR analysis of RNU43. **Results:** For CLL, the mean DNA yield was 193.6 ng/μl (range: 3.0-533.0 ng/μl), whereas the mean A260/A280 ratio was 1.7 (1.2-1.9). For DLBCL, NOS, and HL, 255.5 ng/μl (range: 32.9-605.4 ng/μl), 1.8 (1.5-2.0) and 242.7 ng/μl (range: 1.3-886.0 ng/μl), and 1.7 (0.9-1.8), respectively. The extracted DNA gave amplifiable products of at least 200bp, whereas the RNA analysis showed CT values of <38 in all the samples. The mean RNA yield was 462.2 ng/μl (range: 74.7-1082.1), whereas the mean A260/A280 was 1.7 (1.5-1.8). **Conclusion:** Quantity and quality of nucleic acids from FFPE tissues stored for different time periods showed no significant difference in yield and quality.

**KEYWORDS:** Archived biopsies, DNA, FFPE, lymphoma, Nigeria, RNA

## INTRODUCTION

Lymphoid malignancies are common blood cancers and are increasing in incidence globally.<sup>[1]</sup> The heterogeneity of the morphological, genetic, and epigenetic characteristics inherent in the various subtypes makes it necessary for accurate diagnosis and


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prognosis. Formalin-fixed paraffin-embedded (FFPE) tissues are used routinely in most pathology laboratories for morphological and genetic diagnosis of lymphoid neoplasms.<sup>[2]</sup> Archival FFPE tissues serve as a valuable repository for genetic material which is necessary for molecular studies such as identification of biomarkers for accurate diagnosis, for disease monitoring, and response to chemotherapy and for prognosis.<sup>[3]</sup>

Several factors ranging from biopsy procedures, fixation, and tissue processing to storage affect nucleic acids extracted from FFPE samples.<sup>[4]</sup> The fixation of tissues for architectural, molecular, and structural preservation necessitates the use of formalin on biological specimens.<sup>[5]</sup> Formalin, a fixative discovered over a century ago, is commonly used for routine tissue preservation.<sup>[6,7]</sup> The effect of formalin on tissue proteins and nucleic acids is known to significantly impact the integrity and quantity of these molecules, albeit the wide usage of this fixative in routine diagnostics.<sup>[8]</sup> The continuous usage of formalin is attributed to its practicability especially when compared to freezing, another means of tissue fixation that is limited by its ice crystal formation that distorts tissue structure. More so, DNA and RNA together with protein can still be derived from FFPE tissues for downstream applications. Accurate measures to ensure the usage of FFPE tissues for translational research require the adherence to procedures that would aid the preservation of the content and quality of nucleic acids. It is, therefore, important to review and standardize procedures such as surgical biopsy methods, quantity of fixative, duration of fixation, tissue processing protocols, and tissue storage, to ensure the preservation of nucleic acids and proteins needed for the molecular diagnosis of most pathological conditions. The effect of storage duration has been established in some studies which revealed impediments to downstream usage of the tissue blocks, whereas some studies demonstrated no effect on the extracted nucleic acids.<sup>[2,5,9]</sup> In Nigeria, the effect of the duration of storage of archival FFPE tissue blocks on nucleic acids quantity and quality is yet to be established. In this study, we investigated if storage period had any effect on the quantity and quality of extracted nucleic acids from archival lymphoma biopsies. We also evaluated the quantity and quality of genomic DNA and total RNA in the archived tissue biopsies.

## MATERIALS AND METHODS

### Lymphoma samples

We retrieved 53 FFPE tissues from 2008 to 2019 from the Department of Pathology of University College Hospital Ibadan (UCH), Enugu State University

Teaching Hospital, Parklane, Enugu (ESUT), and Meena Histopathology and Cytology Laboratory (MHCL), Jos, Plateau State. All the tissue biopsies were lymphoid malignant tissues. There were 22 chronic lymphocytic leukemia (CLL) cases, 17 Hodgkin lymphoma (HL) cases, and 14 diffuse large B-cell lymphoma, not otherwise specified (DLBCL, NOS). To investigate the effect of storage duration on the yield and quality of extracted nucleic acids, we compared years of storage of the biopsy and the concentration and quality of DNA extracted from the 53 FFPE tissues and RNA extracted from 6 CLL cases.

### Ethical approval

Ethical approvals were obtained from University of Nigeria Teaching Hospital, Enugu (UNTH-NHREC/05/01/2008B), Ministry of Health, Ibadan, Oyo State (AD13/479/1138), and Meena Histopathology and Cytology Laboratory, Jos, Plateau State (MEENALAB/AEC/177).

### DNA and RNA extraction and integrity

Seven sections of 10  $\mu\text{m}$ -thick FFPE were used for DNA and RNA extraction. DNA from 14 FFPE tissues of DLBCL, NOS was extracted using NucleoSpin® DNA FFPE XSin (Macherey-Nagel, Germany), whereas that of 22 CLL and 17 HL were extracted using MagCore Nucleic Acid Automatic Extractor with MagCore Genomic DNA FFPE One-Step Kit 405 kit. The quantity and purity (A260/A280) of extracted DNA were measured using a MagCore Super HF16Plus instrument (RBC Bioscience Corp, Belgium) and a Nanodrop ND-1000 spectrophotometer (Celbio, Milan, Italy). Microscopic dissection of areas with tumor load of more than 60% was done for each specimen. Eleven of 10  $\mu\text{m}$ -thick FFPE from 6 CLL specimens were extracted using miRNeasy FFPE Kit (QIAGEN, Group) for purification of the total RNA and miRNA, from the FFPE tissue sections following the manufacturer's instructions. The quantity and purity (A260/A280) of extracted RNA were evaluated using a Nanodrop ND-1000 spectrophotometer (Celbio, Milan, Italy). This was immediately followed by reverse transcription PCR for each extracted RNA sample.

### Polymerase chain reaction

The DNA samples (100 ng) were analyzed for integrity and amplifiability using the four pairs of control genes PCR primers of BIOMED-2 protocol.<sup>[10]</sup> The genes were human thromboxane synthase gene (*TBXAS1*), human recombination activating gene (*RAG1*), human promyelocytic leukemia zinc-finger gene (*PLZF*), and human *AF4* gene. The primers were multiplexed to amplify products of 100bp, 200bp, 300bp, and 400bp according to the standardized BIOMED-2 protocol.

The primers were used with ABI Buffer II and 2.0 mm MgCl<sub>2</sub> under standardized BIOMED-2 amplification conditions. The products were analyzed on 2% agarose.

**Reverse transcriptase reactions**

The RNA integrity was assessed by reverse transcriptase reactions. The total RNA were reversely transcribed into complementary DNA (cDNA) using TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA). The reaction mixture contained 5 µl of purified total RNA, 10 × RT Buffer (Applied Biosystems) 1.5 µl, Multiscribe™ RT enzyme (50 U/µl, Applied Biosystems) 1.0 µl, stem-loop reverse transcription (RT) primer (10 µmol/l) 3.0 µl, RNase inhibitor (20 U/µl) 0.19 µl, dNTP mix (100 mM total) 0.15 µl, and Nuclease free water 4.16 µl. In this study, 15 µl reactions in a ratio of 5 ul total RNA: 7 µl RT master mix: 3 µl of RT primers were performed with the following thermal cycling parameters: 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then held at 4°C in an Applied Biosystems 2720 thermal cycler.

**Quantitative reverse-transcription PCR for small nucleolar RNA, C/D box 43 gene (RNU43)**

All quantitative reverse-transcription PCR (qRT-PCR) reactions were performed on the Rotor Gene 6000 (Corbet Research). The reaction contained 7 µl of RT product, 10 µl of Master Mix, 1 µl probe, 0.5 µM of each primer, and deionized water to a total volume of 25 µl. The reactions were run with the following thermal cycling parameters: 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 60s.

**Statistical analysis**

We performed a Spearman’s correlation test to assess if storage duration was associated with the quantity and purity of extracted nucleic acids. In addition, a multivariate linear regression model was used to investigate associations between lymphoma subtype, age of FFPE, and measurement of RNA/DNA quantity and quality. The differences in quantity and quality of nucleic acids between categories were evaluated using one-way ANOVA and Kruskal–Wallis test for analysis of distribution for each DNA yield and quality across various years. All statistical analyses were performed using IBM Statistics/Statistical Package for Social Scientists (SPSS) Statistics (Version 25) software packages.

**RESULTS**

**Characteristics and descriptive statistics of archived FFPE lymphoma biopsies**

The characteristics of the 53 FFPE lymphoma biopsies are shown in Table 1. Nucleic acids were

**Table 1: Selected characteristics of malignant lymphoid biopsies**

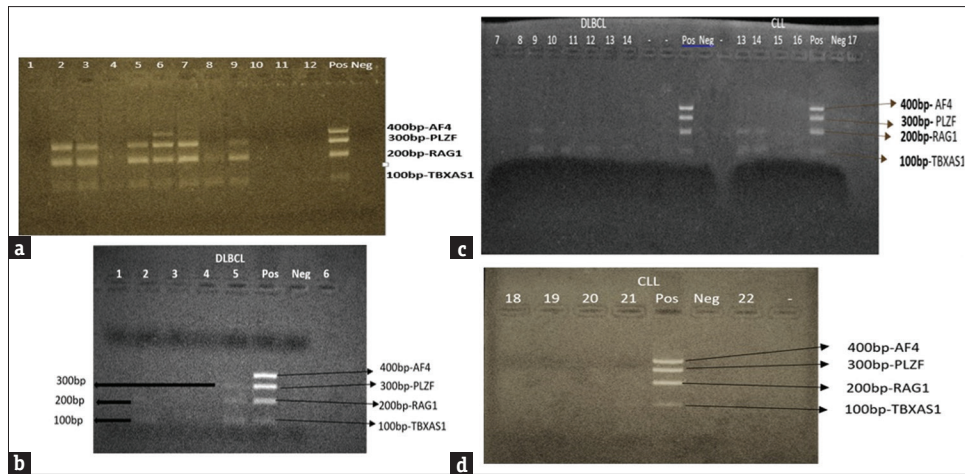
Characteristics	DLBCL, NOS (14)	HL (17)	CLL (22)	ALL FFPE (n=53)
Year of biopsy				
Mean age (yr)	46.1	33.8	56.2	2008-2019
Mean storage duration (yr)	4.4	3.5	4.8	
Biopsy site				
Nodal (%)	85.7	100	81.8	
Extranodal (%)	14.3	0.0	18.2	
Sex				
M (%)	64.3	76.5	50	
F (%)	35.7	23.5	50	

**Table 2: Quantity and quality of DNA and RNA measurements**

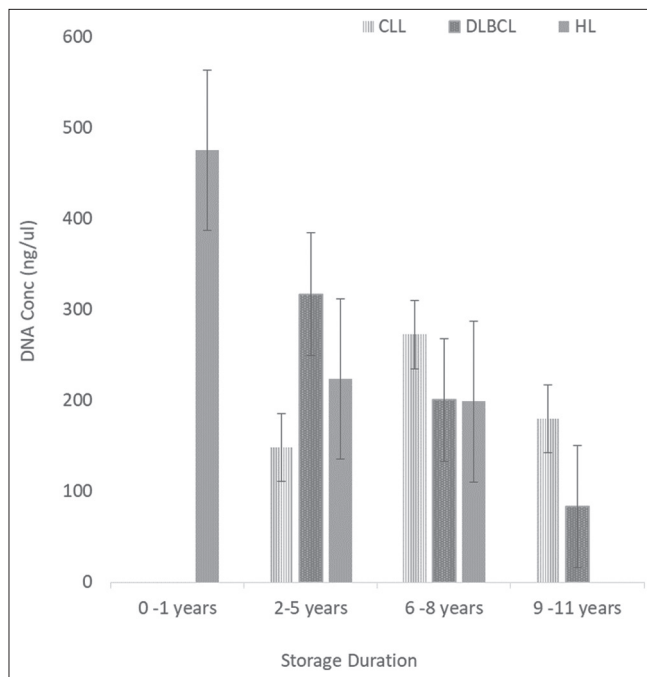
Archived biopsies	DNA	RNA	P
Archived FFPE sections	10 µm	10 µm	
Number extracted	53	6	
Yield (ng/µl) CLL cases			
Minimum	3.0	74.7	0.021
Maximum	533.0	1082.1	
Mean	193.6	462.2	
A260/A280-CLL			
Minimum	1.2	1.5	0.73
Maximum	1.9	1.8	
Mean	1.7	1.7	
C <sub>T</sub> value RNU43 -CLL			
Minimum	N/A	29	N/A
Maximum		31	
Mean		29.9	
SD		0.5	
Yield (ng/µl) DLBCL, NOS			
Minimum	32.9	N/A	0.29
Maximum	605.4		
Mean	255.5		
A260/A280-DLBCL, NOS			
Minimum	1.5	N/A	0.65
Maximum	2.0		
Mean	1.8		
Yield (ng/µl) for HL	1.3		
Minimum	886	N/A	0.80
Maximum	243		
Mean			
A260/A280-HL			
Minimum	0.9	N/A	0.59
Maximum	1.8		
Mean	1.7		

successfully extracted from the 53 archived FFPE lymphoma biopsies. There were differences in the

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**Figure 1:** BIOMED-2 control genes PCR for assessment of amplifiability and integrity of DNA. (a) PCR products from 12 cases of CLL showing strong amplification in 6 samples and weak amplification in 2 samples. (b) PCR products from 6 DLBCL cases showing weak 100bp, 200bp and 300 bp amplification product. (c) More samples of DLBCL and CLL showing weak amplification (d) CLL cases with no amplified products



**Figure 2:** DNA concentration of FFPE lymphoma tissues. Comparison of mean DNA con-centration values obtained over time points for chronic lymphocytic leukemia, diffuse large B-cell lymphoma and Hodgkin lymphoma

yield and quality of DNA in the three lymphoma subtypes. The mean DNA yield for all 22 cases of CLL was 193.6 ng/μl (range: 3.0-533.0 ng/μl) and the mean A260/A280 ratio was 1.7 (1.2-1.9). The mean DNA yield and mean A260/A280 for DLBCL, NOS, and HL were 255.5 ng/μl (range: 32.9-605.4 ng/μl), 1.8 (1.5-2.0) and 242.7 ng/μl (range: 1.3-886.0 ng/μl), and 1.7 (0.9-1.8) respectively. There was a significant difference in the mean DNA yield in CLL cases when compared with the mean DNA yield in DLBCL, NOS and HL,  $P = 0.021$  [Table 2].

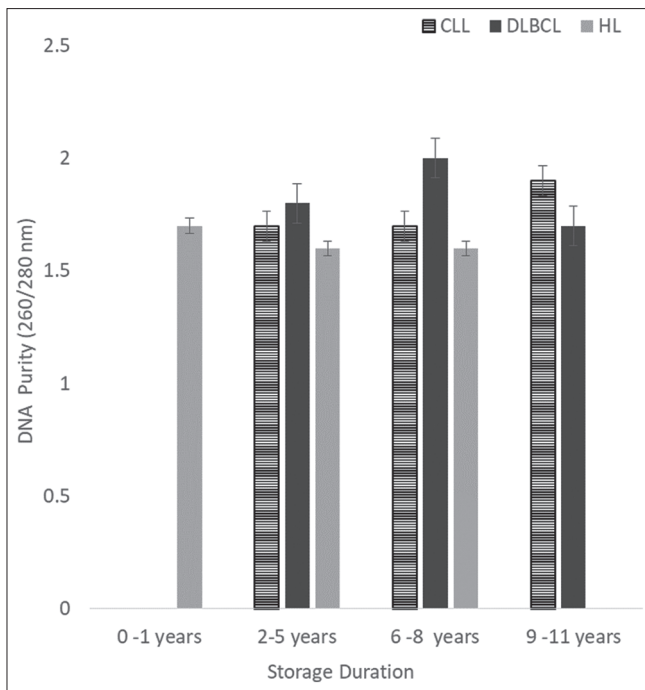
### Assessment of DNA quantity and quality from archived FFPE lymphoma biopsies

The quantity and quality of the extracted DNA from CLL cases were evaluated using the BIOMED-2 protocol control gene PCR. In the PCR reaction of 10 out of the 22 cases of CLL, amplified products were at least 200bp and above and had the distribution as follows: 4 (200bp), 5 (300bp), and 1 (400bp) [Figure 1]. The remaining 12 samples had amplified products that were 100bp [Figure 1]. For the DLBCL, NOS samples, 4 out of the 14 cases had amplified products of at least 200bp (1 case of 300bp and 2 cases of 200bp) [Figure 1].

There was no evidence of correlation between storage duration and DNA concentration ( $r = 0.098$ ,  $P = 0.49$ ). There was also no significant correlation between storage length and the A260/A280 ratios ( $r = 0.099$ ,  $P = 0.479$ ). A multivariate regression analyses established that storage duration had more effect on RNA yield than DNA yield. Each additional year of storage decreased RNA yield by 44.2 ng/μl (95% CI: -377.4 – 289.0), whereas DNA yield is reduced by 5.8 ng/μl (95% CI: -42.9 – 31.3). However, there was no association between Age of FFPE tissues and DNA A260/A280 ratio for purity score ( $\beta = 0.01$ , 95% CI: -0.009-0.028,  $P = 0.30$ ).

### Quality and Quantity of DNA from different lymphoma subtypes stored for different durations

A comparison was made between DNA concentration and DNA purity obtained from FFPE tissues of the three-lymphoma subtypes stored over several time points. Minimal variance was seen in the quantity of DNA extracted from archived FFPE tissues of CLL, DLBCL, NOS, and HL from 9-11 years, 6-8 years, 2-5 years, and 0-1 year [Figure 2]. Only the HL types



**Figure 3:** 260/280 nm values of purity level of extracted DNA from FFPE lymphoma tis-sues. Comparison of mean 260/280 nm values obtained over time points for chronic lympho-cytic leukemia, diffuse large B-cell lymphoma and Hodgkin lymphoma

were found among FFPE tissues stored for a year and below, whereas CLL and DLBCL types were seen amongst those stored for over a nine-year period. In addition, the quality of extracted DNA from FFPE of the three lymphoma subtypes evaluated by the 260/280 nm values did not differ significantly [Figure 3]. A Kruskal–Wallis analysis of distribution for each DNA yield and quality across various years demonstrated an acceptance of the null hypothesis. This implied that there was no statistically significant difference in values obtained across the various years of storage.

### Assessment of RNA quantity and quality from archived FFPE biopsies

There were differences in the yield and quality of RNA from the 6 samples of CLL cases.

The mean RNA yield was 462.2 ng/μl (range: 74.7-1082.1), whereas the mean A260/A280 was 1.7 (1.5-1.8).

The RNA from the six CLL samples was evaluated by qPCR measuring the expression levels of small nucleolar RNA, C/D box 43 gene (RNU43). All the RNA samples had a CT-value of <38 and the mean CT-values were 29.9 [Table 2].

## DISCUSSION

Lymphoid malignancies are essentially diagnosed and characterized by both histological staining and

immunohistochemistry using FFPE tissues.<sup>[11]</sup> It is not known what effect tissue storage may have on the quantity and quality of DNA and RNA extracted from archived FFPE block of lymphoid neoplasms in Nigeria. Although there has been some studies in Nigeria, evaluating the suitability of stored FFPE tissues from lymphoma samples for immunophenotyping, none has studied the effect of storage on nucleic acids for molecular testing.<sup>[12]</sup> We investigated the effect of duration of tissue storage on the quantity and quality of nucleic acids extracted from FFPE of lymphoid neoplasms in Nigeria.

Our results show that DNA extracted from FFPE lymphoma tissue blocks stored for 9-11 years, 6-8 years, and 2-5 years is comparable to that extracted from tissues stored for a year or less. This agrees with the work of Kokkat *et al.*<sup>[5]</sup> that recorded no significant difference in the quantity and quality of DNA extracted from newly processed FFPE tissues and those stored over several years. In addition, Yakovleva *et al.*<sup>[2]</sup> observed no evidence of difference in the fitness of DNA after long-term storage.

Our study shows that the length of storage of FFPE lymphoid malignant tissues predicts RNA quantity more than it did for the DNA quantity. More so, the concentration of extracted RNA from the FFPE tissues decreased with additional years of storage. This may be due to the fact that RNA is more labile than DNA and the effect of formalin fixation on nucleic acids is more deleterious to RNA than DNA.<sup>[8,13]</sup> In contrast, some studies reported comparable RNA quantity and quality in aged and newly processed FFPE biopsies, and that extracted RNA is suitable for microarray analysis.<sup>[14,15]</sup> RNA extracted from FFPE tissues is a valuable source for retrospective and prospective microRNA studies and gene expression studies and can be used for molecular analysis for biomarker identification studies.<sup>[14,16]</sup> The RNA quality and quantity from the six CLL cases were of good yield and quality; therefore, this shows that FFPE lymphoma biopsies from Nigerian archives banks are useful for genomic research in lymphoid neoplasms. Since this type of studies is not available in Nigeria, our current study will encourage the use of FFPE for generation of much needed genomic data in our population. In addition, the qPCR for measuring the expression levels of small nucleolar RNA, C/D box 43 gene (RNU43) from the extracted RNA samples showed good amplification, thus demonstrating presence of adequate quantity of nuclear material.

The mean DNA quantity in CLL FFPE blocks was lower than that of the DLBCL, NOS, and HL. This difference in mean DNA quantity could be because CLL

is a less aggressive tumor with low proliferation index. Also, we evaluated the amplifiability of extracted DNA using BIOMED-2 PCR protocol, and a good percentage of amplified products of at least 200bp were obtained. This shows that DNA extracted from archived FFPE tissues from Nigerian patients with lymphoma can be used for molecular testing and for other downstream applications.

In diagnostic pathology laboratories in Nigeria, archived FFPE blocks are in abundance, especially as they form the major means of making diagnosis of several cancers and other conditions. Lymphoid neoplasms are the commonest blood cancers in Nigeria and molecular investigations are important for arriving at a definite diagnosis, better prognostication, and efficient disease monitoring of patients with lymphoid neoplasms.<sup>[17]</sup> Therefore, use of FFPE tissues for molecular diagnosis of lymphoid neoplasms in Nigeria is achievable.

## STUDY LIMITATIONS

There is a need to study the effect of storage on the quality and quantity of nucleic acids extracted from archival FFPE samples from other types of tumors.

## CONCLUSIONS

This study demonstrated that DNA and RNA could be successfully extracted from archived FFPE tissues of lymphoma patients from Nigeria. Duration of storage did not affect the quantity and quality of the extracted nucleic acids. The use of FFPE to generate genomic and genetic expression data will contribute to evolution of precision and personalized medicine in Nigeria.

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## Ethical approval and consent to participate

The study protocol complied with ethical standards and was approved by the Ethical Board of each study jurisdictions: University of Nigeria Teaching Hospital, Enugu (UNTH-NHREC/05/01/2008B), Ministry of Health, Ibadan, Oyo State (AD13/479/1138), and Meena Histopathology and Cytology Laboratory, Jos, Plateau State (MEENALAB/AEC/177).

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## Conflicts of interest

There are no conflicts of interest.

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