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DECLINE IN BLOOD HEMOGLOBIN CONCENTRATION IS ASSOCIATED WITH FAMILIAL LONGEVITY

Yong-Han He[1], Shao-Yan Pu[1], Wen Li[2], Xiao-Qiong Chen[1], Fu-Hui Xiao[1], Dong-Jing Yan[3], Yao-Wen Liu[1], Rong Lin[4], Xiao-Ping Liao[5], Xing-Li Yang[1], Ming-Xia Ge[1], Wang-Wei Cai[3,*] and Qing-Peng Kong[1*]

[1] *State Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology, the Chinese Academy of Sciences*, Kunming 650223, China;

[2] *Department of Endocrinology, Third People's Hospital of Yunnan Province*, Kunming, Yunnan, People's Republic of China;

[3] *Department of Biochemistry and Molecular Biology, Hainan Medical College*, Haikou 571199, China;

[4] *Department of Biology, Hainan Medical College*, Haikou 571199, China;

[5] *Department of Neurology, the Affiliated Hospital of Hainan Medical College*, Haikou 571199, China.

***Corresponding authors**: kongqp@mail.kiz.ac.cn; caiww591020@163.com

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Abstract: Hemoglobin (HGB) in the blood carries oxygen from the lungs to other organs to produce energy. Calorie restriction has been shown to slow aging and extend lifespan. Thus, we hypothesized that HGB may be associated with human longevity as a link to energy metabolism. To test this hypothesis, HGB levels in the blood of 60 centenarian (CEN) families were measured and its association with age (20-80 and 20-100 years) was studied, as well as the associations of CEN HGB with levels in first generation offspring (F1) and their spouses (F1SP). The results showed no association of HGB with age between 20 and 80 years (r=-0.097, p=0.160); however a strikingly inverse relationship with age between 20 and 100 years (r=-0.526, p<0.001) was revealed. After dividing the samples into four age groups (20-39, 40-59, 60-80 and ≥100 years), the HGB in CEN were significantly lower than that of F1SP (p<0.001). Interestingly, the HGB levels of CEN were significantly associated with that of F1 (r=0.379, p=0.015) but not with F1SP (r=0.022, p=0.451), suggesting that HGB could be a heritable phenotype. Furthermore, the genes methylenetetrahydrofolate reductase (*MTHFR*), nuclear receptor subfamily 2, group C, member 1 (*NR2C1*) and *NR2C2* were differentially expressed in CEN when compared to F1SP, which may likely be responsible for the changes in HGB levels. In conclusion, our results suggest that HGB is a heritable phenotype which associates with familial longevity.

Key words: aging; longevity; hemoglobin; centenarian; energy metabolism

INTRODUCTION

Aging is characterized by an increase in the incidence of degenerative disorders, such as cardiovascular disease, cancer, type 2 diabetes and neurodegenerative diseases [1]. There are many theories to explain the aging phenotype. According to one prevailing theory, increasing oxidative stress leads to elevated levels of free radicals, particularly reactive oxygen species (ROS) which are produced during cellular respiration, that damage biological molecules, including proteins, DNA and lipids, thereby accelerating the aging process [2]. Given that ROS are a byproduct of cellular respiration, energy expenditure may have an impact on oxidative stress and the aging process. In fact, some studies have revealed a positive association between ROS production and metabolic rate [3,4] and an inverse association with both of these on maximum lifespan [5]. Moreover, numerous studies also showed that calorie restriction can slow aging and extend lifespan in a variety of species from yeast to mice [6]. Studies in humans suggest that aging markers, such as blood glucose, blood pressure and cholesterol levels, are improved in diets of calorie restriction [7,8]. The

above-mentioned evidence collectively suggests the importance of ROS and energy expenditure in influencing the aging process.

Oxygen is the most important and essential agent in cellular energy metabolism, which is coupled to the generation of ROS. Naturally, it can be inferred that the utilization of oxygen can play vital roles in the aging process. Human hemoglobin (HGB) is an iron-containing oxygen-transport metalloprotein that carries oxygen from the lungs to the other organs to produce energy. We hypothesized that HGB levels are related to human lifespan. To test this hypothesis, we investigated the association of HGB with age and longevity, and also studied its potential of inheritance in 60 centenarian families. Our results showed that HGB levels were significantly lower in centenarians, and could represent a heritable phenotype associated with familial longevity. However, whether low HGB levels are beneficial or detrimental to human longevity needs to be studied further.

MATERIALS AND METHODS

Subjects

Sixty centenarian families comprised of 61 centenarians (CEN), 63 members of the first generation offspring (F1), 47 spouses of F1 (F1SP), 25 second generation of offspring (F2) and 10 spouses of F2 (F2SP) were recruited from Hainan province in China. The average ages are 102.70, 62.23, 59.90, 31.87 and 31.11 years, respectively. Of the 61 CEN, 51 were female and 10 were male. All subjects belong to the Han nationality.

Blood measurement

All subjects were invited to participate in a physical examination. Non-fasting blood samples were collected from each family member in the clinical chemistry department of the hospital. Of the 60 CEN families, 40 were available to test the association of HGB between CEN and F1 as well as F1SP. The study protocol was approved by the Ethics Committee at Kunming Institute of Zoology, Chinese Academy of Sciences. Written informed consent was obtained from each of the participants.

RNA extraction and RNA sequencing analysis

Twenty-seven centenarians, 18 F1 and 18 F1SP were randomly selected for RNA sequencing (RNA-Seq). Total RNA was extracted from 3 mL of fresh blood using the Trizol reagent (Invitrogen). RNA-Seq was performed at the Beijing Genomics Institute at Shenzhen according to the manufacturer's protocol. The reads count was transformed into fragments per kilobase per million mapped (FPKM) reads [9], which reflects the abundance of the gene expression. Gene expression was analyzed using the edgeR package, in which the P value was adjusted by the Benjamini-Hochberg (BH) method.

Data extraction of genes from the RNA-Seq dataset

To understand the changes in HGB, we retrieved genes associated with HGB metabolism by searching the NCBI (http://www.ncbi.nlm.nih.gov/) and the GWAS Catalog (http://www.genome.gov/). Of the 63 samples for RNA-Seq, there were 13 longevity families available for the association analysis of gene expression between the CEN and F1 as well as F1SP.

Statistics

Data were analyzed using Graphpad Prism 5.0 (Graphpad Software, San Diego, CA). HGB levels among the CEN, F1, F1SP, F2 and F2SP groups were analyzed by one-way ANOVA followed by the Bonferroni post hoc test. The associations of blood parameters with age were analyzed using Pearson's correlation coefficient. Blood parameter levels between CEN and the old group (aged 60-80 years) were compared using the Student's t-test. Genetic additive effect (narrow sense), which was measured as the covariance (the slope) of the relationship between centenarians and F1 as well as F1SP, was determined according to the method as described [10]. Data are expressed as means±SD and differences of $p<0.05$ were considered significant.

RESULTS

Blood measurements

As shown in Table 1, there were significant differences in HGB levels among the CEN, F1, F1SP, F2 and F2SP groups ($p<0.05$). Post-hoc comparison revealed that HGB levels in CEN were significant lower than in F1, F1SP, F2 or F2SP. In addition, the levels of white blood cells (WBC), lymphocytes, granulocytes, red blood cells (RBC), hematocrit (HCT), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width coefficient of variation (RDW-CV), red cell distribution standard deviation (RDW-SD) and platelets (PLT) were also significantly lower in CEN compared to F1, F2 and F2SP. Considering F1SP as the control group, HGB levels in F1 and F2 were significantly higher, while comparable in F2SP. Moreover, the levels of HCT, MCH and RDW-SD were higher while those of platelet hematocrit (PCT) were lower in F1 than in F1SP. There were no differences in lymphocyte percentage, neutrophil percentage, granulocyte percentage, mean platelet volume (MPV), platelet distribution width (PDW) and platelet large cell ration (P-LCR) among the groups.

Association of HGB with age, and HGB in CEN with that in F1 and F1SP

We observed a decreasing trend without significance for HGB with age between 20-80 years (r=-0.097, p=0.160, Fig. 1A). However, when centenarians were included in the analysis, the association displayed considerable significance (r=-0.526, $p<0.001$, Fig. 1B). After dividing the samples into four age groups, we observed that the HGB levels in CEN were significantly lower than that in the older group (aged 60-80 years) ($p<0.001$, Fig. 1C), suggesting that the

Table 1. Blood parameters in 60 centenarian families.

	CEN (n=61)	F1 (n=63)	F1SP (n=47)	F2 (n=25)	F2SP (n=10)
WBC (10^9/L)	5.76±1.61	7.22±1.74*	7.04±2.04*	7.37±2.35*	6.84±1.38
Lymph# (10^9/L)	1.99±0.66	2.45±0.79*	2.41±0.64*	2.68±0.80*	2.64±0.45*
Mid# (10^9/L)	0.5±0.22	0.58±0.17	0.56±0.21	0.59±0.20	0.55±0.1
Gran# (10^9/L)	3.27±1.17	4.19±1.48*	4.07±1.63*	4.10±1.83	3.75±1.12
Lymph (%)	34.89±7.86	34.46±8.80	35.27±7.61	37.30±7.74	38.84±7.19
Mid (%)	8.85±2.06	8.24±1.62	7.99±1.85	8.20±2.18	8.25±0.92
Gran (%)	56.18±8.67	57.30±9.7	56.74±8.48	54.50±9.35	52.91±7.50
HGB (g/L)	110.38±14.80	138.51±15.27*#	123.34±13.61*	145.88±14.53*#	126.40±13.01*
RBC (10^{12}/L)	4.14±0.58	5.11±0.66*	4.85±0.51*	5.22±0.31*#	5.11±1.15*
HCT (%)	37.3±4.72	45.62±4.94*#	41.07±4.23*	46.2±4.82*#	41.11±4.64*
MCV (fL)	90.75±8.13	90.09±9.01	85.3±9.7*	89.29±6.79	82.55±11.83*
MCH (pg)	26.78±2.69	27.30±3.02#	25.55±3.16	27.88±2.36#	25.42±4.26
MCHC (g/L)	295.37±11.54	303.06±9.62*	299.7±9.50	312.52±9.48*#	307.50±13.12*
RDW-CV (%)	15.34±1.47	14.36±1.77*	14.01±1.54*	13.46±0.99*	14.47±1.74
RDW-SD (fL)	54.63±4.60	49.84±5.57*#	46.75±4.72*	46.60±4.07*	44.99±6.77*
PLT (10^9/L)	224.60±68.98	251.22±54.71*	281.81±65.66*	258.76±56.36	259.80±23.60
MPV (fL)	9.83±0.87	9.74±0.98	9.95±0.68	9.73±0.59	10.10±0.73
PDW	14.80±0.33	14.76±0.33	14.72±0.26	14.63±0.23	14.71±0.21
PCT (%)	0.22±0.06	0.24±0.05#	0.28±0.06*	0.25±0.05	0.26±0.03
P-LCR (%)	27.05±7.27	26.21±7.97	28.19±6.34	26.19±5.33	29.66±6.31

*$p<0.05$ – compared to the CEN group; #$p<0.05$ – compared to the F1SP group; CEN – centenarians; F1 – centenarians' first generation of offspring; F1SP – F1 spouses; F2 – centenarians' second generation of offspring; F2SP – F2 spouses; WBC – white blood cell; Lymph – lymphocyte; Mid – neutrophil; Gran – granulocyte; HGB – hemoglobin; RBC – red blood cell; HCT – hematocrit; MCV – mean corpuscular volume; MCH – mean corpuscular hemoglobin; MCHC – mean corpuscular hemoglobin concentration; RDW – red cell distribution width; PLT – platelets; MPV – mean platelet volume; PDW – platelet distribution width; PCT – platelet hematocrit; P-LCR – platelet large cell ratio

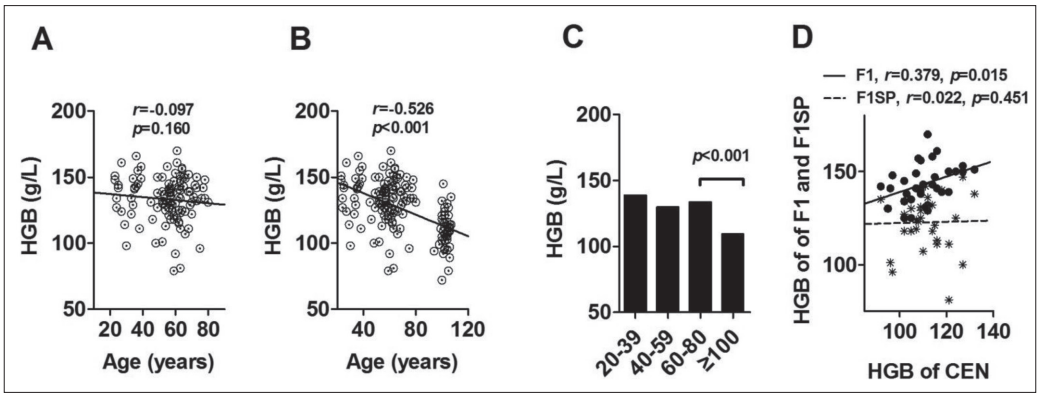

Fig. 1. Association of HGB with age and between generations. (**A**) Association of HGB with age ranging from 20 to 80 years. (**B**) Association of HGB with age ranging from 20 to 100 years. (**C**) Changes in HGB levels in different age groups. (**D**) Association of HGB in CEN with that in F1 and F1SP. HGB – hemoglobin; CEN – centenarians; F1 – centenarians' first generation; F1SP – spouses of F1 offspring.

HGB levels were associated with age, especially in the oldest subjects. Next we analyzed the associations of HGB between generations. Surprisingly, the HGB levels of centenarians were associated with F1 (r=0.379, p=0.015) but not with F1SP (r=0.022, p=0.451), suggesting that HGB could be a heritable index (Fig. 1D).

Expression of genes involved in HGB metabolism

To understand the reason for the difference in HGB among the groups, especially between CEN and F1SP, we collected genes associated with HGB levels. The genes are listed in Table 2. Among them, we identified

Table 2. Expression comparisons of genes involved in HGB metabolism.

	CEN vs. F1SP				F1 vs. F1SP			
Gene name	**baseMean**	**log2FoldChange**	**p value**	**adjusted p value**	**baseMean**	**log2FoldChange**	**p value**	**adjusted p value**
TSHZ2	148.33	-0.52	0.000	0.002	148.33	-0.06	0.649	1.000
ZFPM1	273.31	0.28	0.004	0.034	273.31	0.09	0.377	1.000
NR2C1	504.78	-0.09	0.004	0.266	504.78	0.04	0.537	1.000
MTHFR	957.13	0.19	0.007	0.049	957.13	0.13	0.091	1.000
FGFR1OP	621.65	-0.24	0.010	0.063	621.65	-0.13	0.202	1.000
NR2C2	2510.43	-0.21	0.028	0.119	2510.43	-0.01	0.958	1.000
ATXN2	586.64	-0.12	0.029	0.216	586.64	0.02	0.743	1.000
ITPA	372.06	0.18	0.037	0.143	372.06	0.06	0.504	1.000
GATA1	170.26	0.29	0.048	0.167	170.26	-0.12	0.396	1.000
BRAP	484.36	-0.09	0.086	0.240	484.36	-0.10	0.096	1.000
HK1	2463.29	0.07	0.188	0.383	2463.29	0.01	0.895	1.000
TMPRSS6	27.36	-0.13	0.204	0.405	27.36	-0.08	0.495	1.000
TRAFD1	2296.56	0.13	0.234	0.440	2296.56	-0.14	0.237	1.000
NFE2	3532.67	-0.12	0.343	0.555	3532.67	-0.24	0.070	0.983
HIST1H1T	7.67	0.09	0.525	0.709	7.67	-0.05	0.724	1.000
PRKCE	218.35	0.05	0.533	0.715	218.35	0.08	0.364	1.000
HFE	110.25	0.06	0.577	0.749	110.25	-0.10	0.361	1.000
KLF1	3.32	0.05	0.696	0.832	3.32	-0.17	0.193	1.000
KLF1	3.32	0.05	0.696	0.832	3.32	-0.17	0.193	1.000
MYB	113.18	-0.04	0.782	0.887	113.18	-0.19	0.188	1.000

CEN – centenarians; F1 – centenarians' first generation offspring; F1SP – spouses of F1 offspring.

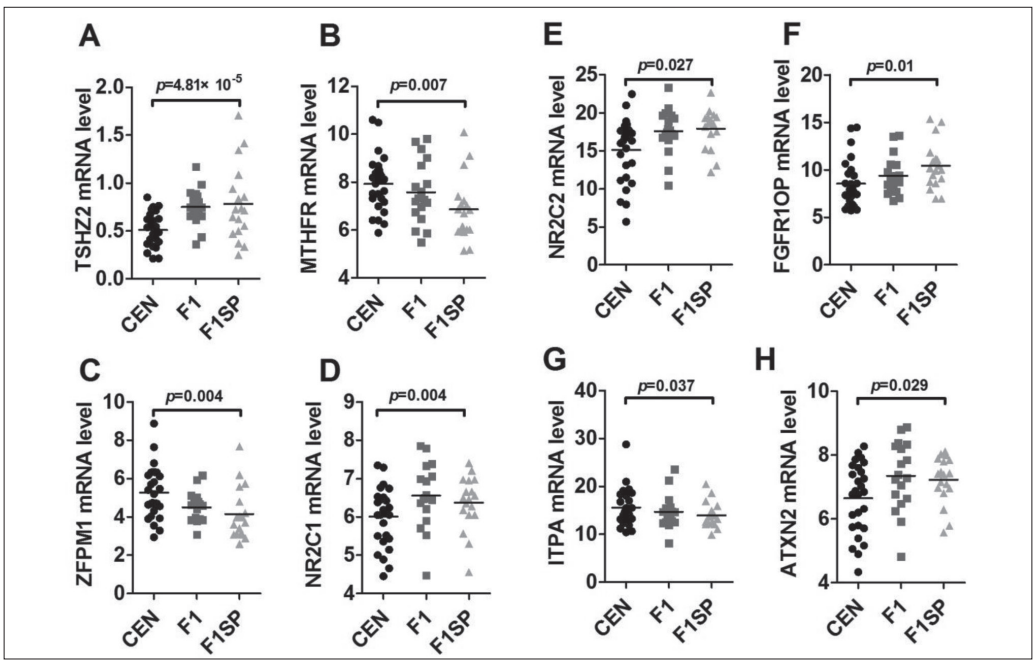

Fig. 2. Expression of genes involved in HGB metabolism. HGB – hemoglobin; CEN – centenarians; F1 – centenarians' first generation; F1SP – spouses of F1 offspring.

the *TSHZ2*, *MTHFR*, *ZFPM1*, *NR2C1*, *NR2C2*, *FGFR1OP*, *ITPA*, *ATXN2* and *GATA1* as potential genes responsible for differential HGB levels (Fig. 2A-2H, Table 2), with significant differences in their expressions between the CEN and F1SP groups ($p<0.05$). After the BH adjustment, *TSHZ2*, *MTHFR* and *ZFPM1* remained significant in both groups. There were no differences in these gene expressions between F1 and F1SP ($p>0.05$, Table 2). The other genes did not differ among the CEN, F1 and F1SP groups (Table 2).

Association of gene expression of HGB metabolism-associated genes in CEN with that in F1 and F1SP

The expression level of the above-mentioned genes in F1 was between the CEN and F1SP, but was not different from that in F1SP. Of them, the expression of *MTHFR*, *NR2C1*, *NR2C2* and *FGFR1OP* of centenarians was significantly associated with F1 but not with F1SP (Fig. 3A-D), indicating they might be involved in the inheritance of HGB of F1 from CEN.

DISCUSSION

In this study, we explored changes in blood HGB levels associated with age and found an inverse relationship between them, with a dramatic decrease in the extreme longevity subjects. We also report for the first time the association of centenarians' HGB levels with their offspring, and identify the genes responsible for the difference in HGB in the longevity subjects and the elderly. As early as 1954, the association of variations in HGB levels with age and sex revealed a similar trend of HGB changes with age (6-98 years) [11] as described in our study. In 2012, a high prevalence of anemia based on HGB levels in octogenarians and centenarians was reported [12]. Reduced hemoglobin has been associated with adverse outcomes in some clinical diseases, such as heart failure [13] and chronic kidney disease [14]. However, a prospective study on 1205 participants revealed that higher HGB concentrations were associated with increased mortality [15]. Moreover, it has been suggested that higher hemoglobin may cause increased blood viscosity, blood pressure and dialysis-access

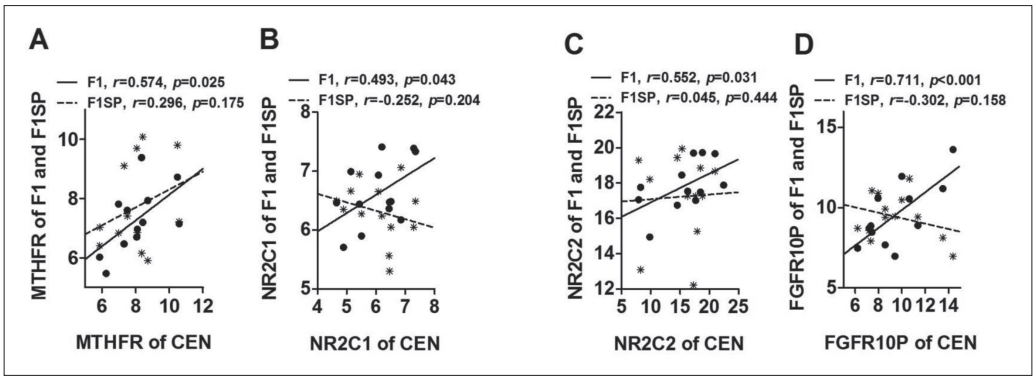

Fig. 3. Association of gene expression in CEN with that in F1 and F1SP. HGB – hemoglobin; CEN – centenarians; F1 – centenarians' first generation; F1SP – spouses of F1 offspring.

thrombosis [16]. In addition, HGB has been shown to be the predominant factor that determines oxidative stress in blood red cells. HGB-generated oxidants could affect cellular membrane and cytoskeleton and cause red-cell aging [17]. Thus it seems that HGB has dual roles depending on its concentrations. A moderate level of HGB might be beneficial to health, but it remains unclear what the optimal HGB level should be [16]. As described above, HGB is in charge of transporting oxygen from the lungs to the other organs to produce energy, which is coupled with the production of ROS. Decreased HGB levels may be responsible for lower level of energy metabolism and an accordingly lower level of ROS production, resembling the state of energy restriction which has been suggested to prolong lifespan [18]. If this is the case, lower HGB levels may be beneficial to human longevity. In addition, the longevity subjects usually have a delayed or reduced prevalence of age-related diseases [19-21]. They should have a higher prevalence of disease if their lower HGB levels were associated with the diseases. Additional evidence came from the study of Haslam et al. [22], who reported that anemia in centenarians was only associated with lower hand-grip and leg strength, but not with physical function in everyday activities. Therefore, it seems that the impact of lower HGB in centenarians is not as simple as was once thought.

To understand why HGB levels were so low in centenarians, we analyzed the expression of HGB-associated genes. Of the candidate genes, the *MTHFR* and *ITPA* gene polymorphisms were associated with plasma hemoglobin levels [23-25]. *NR2C1* and *NR2C2* are two ligand-inducible transcription factors that regulate gene expression by binding to specific DNA sequences within promoters of target genes, which were demonstrated to affect fetal globin transcription in erythroid cells [26]. However, the mechanisms through which these candidate genes regulate blood HGB levels still need to be explored. The high association of *MTHFR*, *NR2C1*, *NR2C2* and *FGFR10P* expression between centenarians and their offspring lends additional support to the heritable potential of HGB.

In this study, several limitations should be acknowledged. One was that the gender ratio in samples were not equivalent. As we all know, females usually live longer than males, resulting in more female centenarians than male. Another was the small sample size of F2 offspring, so that we just focused on the association of HGB between centenarians and F1 offspring. We believe that a larger number of F2 offspring would strengthen the conclusion that HGB is a heritable phenotype. The third limitation was that we investigated the genes at the transcriptional level rather than the translational level: therefore, one should be cautious when interpreting the data.

Overall, our results suggest that centenarians have lower levels of blood HGB in comparison to the general population of elderly subjects. This phenotype is likely to be heritable. However, whether a lower HGB level is beneficial or detrimental to longevity warrants further study.

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Conflict of interest disclosure: The authors declare no conflict of interest.

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