

# Genotype-based changes in serum uric acid affect blood pressure

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Elevated serum levels of uric acid consistently correlate with hypertension, but the directionality of the association remains debated. To help define this relationship, we used a controlled setting within a homogeneous Amish community and the Mendelian randomization of a nonsynonymous coding single-nucleotide polymorphism, rs16890979 (Val253Ile), in the *SLC2A9* gene. This gene expresses the GLUT9 transporter that also transports uric acid and is associated with lower serum uric acid levels. We studied the unconfounded association between genotype and blood pressure in 516 Amish adults, each placed for 6 days on standardized diets, first with high sodium, followed by low sodium, with an intervening washout period. Blood pressure, measured using 24-h ambulatory monitoring, during both diet periods was used as the primary outcome. All participants were free of diuretic or other antihypertensive medications and the relationships between GLUT9 genotype and both serum uric acid and blood pressure were assessed. Each copy of the GLUT9 minor Ile allele was found to confer a significant 0.44 mg/dl reduction in serum uric acid and was associated with a significant mean decrease in the systolic blood pressure of 2.2 and 1.5 mm Hg on the high- and low-sodium diet, respectively. Thus, a Mendelian randomization analysis using variants in the *GLUT9* gene indicates that a decrease in serum uric acid has a causal effect of lowering blood pressure.

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An association between serum uric acid (UA) and blood pressure (BP) has long been established; over 40 years have passed since the observation that >25% of untreated hypertensive patients were hyperuricemic.<sup>1</sup> Numerous large-scale observational studies have shown persistent independent associations between serum UA and BP, as well as with various other cardiovascular (CV) outcomes.<sup>2–4</sup> However, the complex interplay among metabolic syndrome components, as well as renal function, has produced strong debate about the causality and directionality of the observed associations. Indeed, although UA initially gained much attention as an important mediator of vascular disease, it subsequently was felt to be a secondary marker of disease and was dropped from standard metabolic panels. More recently, there has been resurgence in the interest of the likely causal association between serum UA and a variety of CV-related outcomes, most notably hypertension. For example, both animal and human studies have shown that the presence of hyperuricemia precedes the development of hypertension and that, after adjusting for multiple independent risk factors, hyperuricemia carries an increased relative risk of developing hypertension.<sup>3,5–7</sup> However, these studies, although often providing compelling arguments, are not sufficient to fully resolve concerns regarding the temporality of UA change along with more subtle potential confounders and BP. There have also been some small trials showing an improvement in BP, C-reactive protein, and renal function in response to treatment with allopurinol.<sup>8,9</sup> However, it remains unclear whether these benefits were related to the ability of allopurinol to inhibit xanthine oxidase and/or lower serum UA.

Although diet is well known to modulate serum UA concentration, genetic variability is also significantly associated with serum UA levels. The gene most strongly associated with UA levels resides on chromosome 4 and codes for *GLUT9* (also known as *SLC2A9*), a recently discovered key urate transporter. Genetic variants of *GLUT9* have been strongly associated with reduced levels of serum UA in several Caucasian cohorts, including ours,<sup>10–16</sup> and in non-Caucasian cohorts.<sup>17–19</sup> Specifically, in our cohort, out of a total of 102 genotyped single-nucleotide polymorphisms (SNPs), a missense variant that codes for a Val to

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Ile amino-acid substitution at position 253 on the *GLUT9* gene (GLUT9 Val253Ile) was most significantly associated with a reduction in UA concentration of  $\sim 0.5$  mg/dl in women and  $\sim 0.25$  mg/dl in men per copy,<sup>12</sup> and is associated with increased renal clearance of UA.<sup>13,20</sup>

The discovery of the aforementioned GLUT9 Val253Ile variant allows for an opportunity to study whether UA levels are, in fact, causal of changes in BP, by using a method known as Mendelian randomization. The Mendelian randomization principle relies on the tenet that alleles, and hence genotypes, are randomly assigned during gamete formation. The main advantage of this method is that gamete formation occurs before birth and is therefore unaffected by traditional confounders that occur after conception, such as diet, socioeconomic status, access to health care, and all other environmental factors. Because relationships between genotypes and outcomes have only limited susceptibility to confounding and are not subject to reverse causality, genetic variation may be used to establish directionality and infer causality between a certain gene product and a specific outcome. Therefore, Mendelian randomization is akin to a randomized trial design with genetic variation used as a proxy for an exposure that leads to a given outcome. In a theoretical randomized trial design, inheritance of the GLUT9 Ile allele would be analogous to randomly being assigned probenecid, a uricosuric agent, from birth, whereas inheritance of the wild-type genotype would be analogous to receiving placebo. In this paper, we set out to determine whether exposure to a genotype-associated lowering in serum UA concentration, while on standardized prepared high- and low-salt diets, and free of any diuretic or other antihypertensive medication use, underlies decreases in mean 24-h BP measurements.

## RESULTS

### Sample characteristics

The 868 participants of the HAPI (Hereditary and Phenotype Intervention) Heart Study ranged in age from 20 to 80 years at the time of the study, and 53% were men. Table 1 gives the baseline clinical characteristics of the sample.

### Association of the GLUT9 Val253Ile genotype with serum UA level

We previously performed a genome-wide association study of serum UA and found SNPs within the *GLUT9* gene to be most strongly associated with serum UA.<sup>12</sup> Included in the genome-wide association study were 98 GLUT9 variants. However, as these SNPs were not viewed as functional, we then identified four nonsynonymous SNPs within GLUT9 and genotyped those to see whether a putative SNP could be identified. Of these, a missense SNP rs16890979 (Val245Ile) in exon 8 proved to be the best associated marker and the only independently associated one, when included with the other SNPs in the same regression model.<sup>12</sup> On the basis of these findings, we selected this variant for our Mendelian randomization analysis. No deviation from Hardy–Weinberg

**Table 1 | Clinical characteristics (mean (s.d.)) of the 868 Old Order Amish enrolled in the HAPI Heart Study, Lancaster County, Pennsylvania**

Characteristic	Men (n=460)	Women (n=408)
Age (years)	42.2 (13.6)	45.4 (14.2)
BMI (kg/m <sup>2</sup> )	25.6 (3.2)	27.8 (5.5)
Total cholesterol (mg/dl)	202.5 (44.3)	215.7 (49.0)
Triglycerides (mg/dl)	63.9 (1.7)	73.8 (45.4)
SBP (mm Hg)	121.5 (12.6)	121.4 (16.9)
DBP (mm Hg)	77.6 (8.8)	75.8 (8.4)
Diabetes (%)	0.9	1.0
Current smokers (%) <sup>a</sup>	20.0	0.0
Lipid-lowering meds (%) <sup>b</sup>	1.0	1.0
Antihypertensive meds (%) <sup>b</sup>	0.2	0.3

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; HAPI, Hereditary and Phenotype Intervention; meds, medication; SBP, systolic blood pressure.

<sup>a</sup>Indicates use of cigarettes, cigars, and pipes.

<sup>b</sup>Medication use assessed at the time of recruitment, before participants were asked to discontinue use, per our study protocol.

equilibrium was noted. The GLUT9 Val253Ile genotype was strongly associated with serum UA levels (Table 2), and accounted for 4.3% of the variance in our population. Among homozygotes for the Ile allele (Ile/Ile), serum UA levels were nearly 1 mg/dl lower than for those with the wild-type (Val/Val) genotype; each copy of the Ile allele conferred an  $\sim 0.45$  mg/dl reduction in UA ( $P = 3.2 \times 10^{-11}$ ). Consistent with previous reports,<sup>11,12,16,21,22</sup> this effect was more pronounced in women than in men (data not shown). This strong association between genotype and phenotype is consistent with previous evidence and supports the first condition for the use of Mendelian randomization, as a weak genotype effect would otherwise yield low power to detect any effect.

### Association of serum UA levels with BP

Consistent with previous studies, we found serum UA levels to be correlated with multiple measurements of BP, metabolic syndrome factors, and renal function. Both our BP and serum UA measurements were approximately normally distributed and did not require normalization. Regression coefficients and *P*-values from this analysis are shown in Table 2. After standardized BP was taken on the initial clinic visit, subjects began 6 days of a high-salt diet, followed by an 8- to 14-day washout period, and then 6 days of a low-salt diet. Serum UA levels were positively correlated with multiple measurements of baseline BP (taken during clinic visit one and a home visit following the washout period) and 24-h ambulatory BP measurements. However, it is important to remember that these associations may not represent valid estimates of true causal effects given that they are subject to both confounding and reverse causality.

### Association of the GLUT9 Val253Ile genotype with BP

Mean values and regression coefficients of baseline and 24-h BP measurements by genotype are presented in Table 2. The right-most column of Table 2 shows the two-stage estimate of

**Table 2 | Observational estimates for uric acid adjusted for age and gender with individual phenotypes compared with corresponding genotype-based mean phenotype values and additive model point estimates for each Ile allele and corresponding P-value**

Trait (n=868)	Serum UA correlations		GLUT9 genotype-based approach				
	Estimate (s.e.)	P-value	Ile/Ile mean (s.d.)	Val/Ile mean (s.d.)	Val/Val mean (s.d.)	Effect estimate	Additive P-value
Uric acid, mg/dl	—	—	3.39 (1.46)	3.90 (0.98)	4.29 (1.00)	0.44 (0.06)	$3.2 \times 10^{-11}$
Estimated GFR (ml/min)	-4.5 (0.58)	$1.38 \times 10^{-14}$	89.0 (15.8)	95.7 (18.2)	96.0 (18.7)	0.42 (1.12)	0.706
Body mass index (kg/m <sup>2</sup> )	1.71 (0.14)	$1.52 \times 10^{-31}$	28.4 (5.0)	26.3 (4.3)	26.7 (4.6)	0.24 (0.29)	0.393
Triglycerides	14.2 (1.35)	$2.25 \times 10^{-24}$	71.8 (40.6)	67.3 (42.9)	69.4 (42.9)	2.38 (2.68)	0.347
Glucose	2.47 (0.44)	$2.48 \times 10^{-8}$	87.2 (5.7)	85.4 (7.5)	86.6 (10.3)	0.78 (0.84)	0.36
Clinic visit 1 SBP	1.91 (0.48)	$7.63 \times 10^{-5}$	115.5 (14.5)	122.2 (14.0)	121.4 (15.0)	0.08 (0.91)	0.378
Clinic visit 1 DBP	1.36 (0.3)	$7.71 \times 10^{-6}$	75.7 (7.2)	76.4 (8.9)	76.9 (8.7)	0.52 (0.58)	0.364
High-salt 24-hr SBP <sup>a</sup>	2.28 (0.51)	$8.73 \times 10^{-6}$	112.2 (8.2)	115.3 (9.0)	117.6 (10.8)	2.2 (0.79)	<b>0.006</b>
High-salt 24-h DBP <sup>a</sup>	1.53 (0.38)	$6.41 \times 10^{-5}$	69.4 (5.8)	70.0 (6.2)	70.6 (6.6)	0.42 (0.5)	0.406
Low-salt 24-h SBP <sup>a</sup>	1.87 (0.49)	$1.38 \times 10^{-4}$	110.5 (7.0)	115.1 (8.7)	116.5 (9.0)	1.48 (0.71)	<b>0.038</b>
Low-salt 24-h DBP <sup>a</sup>	0.88 (0.35)	0.012	69.7 (6.0)	71.3 (6.3)	71.8 (6.0)	0.19 (0.48)	0.689
Salt sensitivity 24-h SBP	-0.01 (0.17)	0.954	1.71 (4.3)	0.48 (4.6)	1.05 (5.6)	0.62 (0.49)	0.212
Salt sensitivity 24-h DBP	0.04 (0.24)	0.843	-0.29 (2.3)	-1.22 (3.5)	-1.16 (3.8)	-0.01 (0.31)	0.986

Abbreviations: CI, confidence interval; DBP, diastolic blood pressure; GFR, glomerular filtration rate; SBP, systolic blood pressure; UA, uric acid.

Estimates of serum UA correlations are based on 1 mg/dl change in UA. No significant difference was noted between high- and low-salt diet SBP measures. All blood pressure measurements are given in mm Hg.

<sup>a</sup>The 24-h blood pressure measurements were available in a subset of 516 of 868 participants.

Bold indicates P-value < 0.05.

the serum UA-BP association derived from the Mendelian randomization approach that uses the GLUT9 Val253Ile genotype. It is important to note that our clinic visit one BP measurements (average of three repeated BP measurements at a single visit) were obtained when the individuals were on a liberal diet, and these were not associated with genotype under an additive model (Table 2). However, when the same participants were on standardized prepared high-salt diets (delivered to the home of each subject), we noted a significant additive genotype-mediated association with our 24-h mean BP measurements. Moreover, this was internally replicated when the same individuals were on standardized low-salt diets where each Ile allele was associated with a decrease in BP. Our genotype-mediated effect estimate went from null effect (-0.08 mm Hg) per Ile allele on liberal diet to -1.5 mm Hg on low-salt diet and -2.2 mm Hg on high-salt diet (Table 2). Our power is over 90% to identify the observed effect with our given sample size. The potentially confounded associations between serum UA and renal function, body mass index, triglycerides, and glucose were nullified under the genotype-based approach (Table 2).

#### Association of serum UA with salt sensitivity

As with BP readings, mean values and regression coefficients of measurements of salt sensitivity are also presented in Table 2. Genotype was not associated with salt sensitivity, defined as BP on high-salt diet minus BP on low-salt diet ( $P = 0.446$ ). It should be noted that our relatively healthy and young Caucasian population were mostly non-salt sensitive, further reducing our power for this secondary analysis. We did, however, note an attenuation of the effect of genotype on systolic BP when on a low-sodium vs. high-sodium diet (absolute effect estimate of 2.2 vs. 1.48 mm Hg per Ile allele).

These results suggest potential effect modification or interaction between dietary salt intake and serum UA effect on BP. A formal test of interaction did not yield significant results; however, we must again acknowledge limited power to detect a statistically significant interaction given the sample size and modest difference in effect estimate size, as related to dietary modification of salt intake.

#### DISCUSSION

In this study, we used the Mendelian randomization principle to show that decreases in serum UA concentration due to a missense SNP in the GLUT9 gene are directly associated with lower level of BP. The strength of the association between change in serum UA level and systolic BP was significant, with a 1 mg/dl change in serum UA being associated with an ~3-5 mm Hg change in systolic BP, depending on salt intake. Considering that the population distribution of serum UA is much wider than that conferred solely by GLUT9 variability, the population effect of UA on BP and CV disease outcomes may be quite substantial.

The full spectrum of mechanisms by which GLUT9 affects serum UA level continues to be elucidated. It has recently been shown that GLUT9 is a urate transporter that localizes to both the apical and basolateral membranes of human renal proximal tubular cells *in vitro*; different splice variants affect trafficking of the transporter and dictate into which membrane it will be inserted.<sup>23</sup> Splice variant 1 localizes to the basolateral membrane and functions as an efflux transporter for UA out of the tubular cell, whereas splice variant 2 is located in the apical membrane and is involved in the rapid influx of UA into the proximal tubular cell.<sup>20,24</sup> Given the location of these two splice variants, it is reasonable to postulate that GLUT9 may alter serum UA concentration

via regulation of proximal urate reabsorption. The finding that different SNPs in the *GLUT9* gene modify fractional excretion of UA strengthens this theory.<sup>13,24,25</sup> Another possible mechanism for the effect of *GLUT9* on serum UA concentration is via its role in fructose homeostasis. It has been well described that increased fructose levels are associated with increased production of UA.<sup>26–29</sup> Given that UA transport by *GLUT9* is facilitated by fructose, it is plausible to suggest that *GLUT9* may also have a role in calibrating urate concentration in response to fructose.<sup>20,30</sup>

Although the exact pathway by which serum UA is associated with higher levels of BP is yet to be established, previous studies provide several potential mechanisms such as renal vasoconstriction with microvascular disease,<sup>31,32</sup> impaired vascular endothelial function or compliance,<sup>33–35</sup> and disturbances in sodium and volume homeostasis.<sup>36,37</sup> In an animal model, rats were rendered hyperuricemic by administration of the uricase inhibitor oxonic acid for 7 weeks while on a low-salt diet; control animals were maintained on the same low-salt diet but did not receive oxonic acid. While still on a low-salt diet, the oxonic acid was then discontinued for 2 weeks to allow the serum UA levels to return to normal. The rats were then randomized to a high- or low-salt diet to assess for salt sensitivity. Only the rats that were previously hyperuricemic demonstrated an increase in BP on the high-salt diet (that is, salt sensitivity).<sup>37</sup> In agreement with these findings, we observed suggestive evidence that a high-salt diet may accentuate the relationship of serum UA on BP. Further investigation in humans will need to be performed in order to determine whether elevations in serum UA increase are associated with salt sensitivity in various populations.

Although the number of subjects studied here is relatively modest, there are several notable strengths to our study that should be discussed. Foremost, all participants were on a standardized prepared diet before obtaining BP measurements, including an equivalent amount of dietary protein and carbohydrates. As the daily variability in serum UA in a noncontrolled setting is on the same order as that of the *GLUT9* allele, an uncontrolled dietary setting could significantly attenuate findings associated with a modest increase in serum UA.<sup>38</sup> Second, we used 24-h averaged ambulatory BP measurements on high- and low-salt diets. In contrast, our regular standardized clinic BP measurements on a liberal diet did not demonstrate a statistically significant association between genotype. Moreover, we have repeated 24-h BP measurements in all participants, showing internal replication. Third, our culturally isolated founder population is quite homogeneous and not susceptible to population stratification bias. Fourth, potential gene-by-environment interactions that are not controlled for in standard population-based studies (for example, salt and fructose) are accounted for in our experimental setting through our fixed-diet protocol. Fifth, our population is relatively young and healthy, which may be more sensitive to vascular effects of serum UA compared with older population with more vascular disease burden.<sup>6,39</sup> Finally, we used direct genotyp-

ing of *GLUT9* as opposed to imputation, further minimizing potential measurement error. In short, limitations related to potential uncontrolled effect modifiers, interactions, and measurement error as noted in our liberal diet and single-visit clinic-based BP measurements have been minimized by the nature of our selected population and controlled setting. Not surprisingly, combining large number of heterogeneous population cohorts in an uncontrolled setting to explore the association between *GLUT9* genotype and BP, where these limitations could not be mitigated, yielded null results, comparable to our findings when the participants were on a liberal diet along with similar single-visit standardized BP measurement.<sup>15,20</sup>

In summary, a genetic variant strongly associated with lower levels of serum UA is also associated with decreases in BP, when tested in an experimental setting. The evidence from our Mendelian randomization approach implies that this association is causal in nature, corroborating all the previous studies that strongly inferred a causal association between serum UA and BP. This study also highlights the potential importance of a controlled setting and homogenous population when examining secondarily mediated gene effects (for example, effect of genotype on UA and then secondarily on BP). These findings add impetus to the possible benefit of reduced serum UA control in hyperuricemic patients with CV disease risk factors, or established disease, and suggest that larger clinical trials of serum UA reduction are well warranted.

## MATERIALS AND METHODS

### Study sample

The HAPI Heart Study began recruitment in 2003 with the goal of identifying genes that interact with environmental exposure to alter risk of CV disease. The study was carried out in an Old Order Amish community in Lancaster County, Pennsylvania, and the full HAPI study included 868 individuals aged  $\geq 20$  years who were relatively healthy. Details of the study aims and recruitment procedures have been previously described.<sup>40</sup> In brief, exclusion criteria included severe hypertension (BP  $> 180/105$  mm Hg), malignancy, and kidney, liver, or untreated thyroid disease. The study protocol was approved by the Institutional Review Board at the University of Maryland School of Medicine, and informed consent was obtained from each study participant.

Baseline clinical and serological measurements were obtained during an initial clinic visit at the Amish Research Clinic in Strasburg, Pennsylvania. Baseline clinic BP was measured in triplicate using a standard sphygmomanometer in the sitting position after 5 min of rest, and the average of the three measurements was used for analysis. Fasting blood samples were also drawn at this time. Serum UA levels drawn at the screening exam were assayed by Quest Diagnostics (Baltimore, MD) and measured to the nearest 0.1 mg/dl. Biochemical measurements relevant to this study include basic metabolic and lipid panels (Quest Laboratory, Horsham, PA).

**Dietary salt intervention.** The dietary salt intervention study began immediately following the clinic visit and was completed in a subsample of 516 individuals. Study subjects were put on a high-sodium diet (280 meq per day) for 6 days, which is close to their customary diet, and then after a 6- to 14-day washout period,

were put on a low-sodium diet (40 meq per day) for 6 days. The potassium level was held constant at 140 meq per day during both diets, as was the daily protein content at 114 g and the total carbohydrate content at 411 g. All meals were prepared in a specially outfitted kitchen and delivered to the home of the subjects.

**Measurement of 24-h ambulatory BP.** The 24-h BP measurements were recorded at 30-min intervals by an ambulatory BP monitor worn by the subjects on the last day of each diet. Salt sensitivity was expressed on a continuous scale as the absolute difference between the average 24-h BP during the high- and low-salt diet. We used the mean of all recorded BP measurements during each 24-h period as our outcome measure. Specific protocols for the HAPI Heart study measurements have been published.<sup>40</sup>

**Genotyping of the GLUT9 gene.** Participants were genotyped for the rs16890979 SNP (Val253Ile) using TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) according to the standardized manufacturer's protocol. Replicate genotyping in 8% of the samples resulted in a >98% concordance rate.

### Analytical approach

**Mendelian randomization.** The Mendelian randomization approach exploits the fact that genotype precedes life events and is therefore not affected by lifestyle factors. As previously reviewed, Mendelian randomization is an application of instrumental variable analysis, and with certain assumptions the genotype-phenotype relation can be used to attain unconfounded estimates of the relationship between the gene product and outcomes of interest.<sup>41</sup> These assumptions include an adequately strong relationship between genotype and phenotype and the absence of alternate pathways from genotype to the outcome of interest (for example, pleiotropy, population stratification, linkage disequilibrium). The first assumption, a strong relation between genotype and phenotype, has been previously demonstrated for the SNP rs16890979 on the *GLUT9* gene (GLUT9 Val253Ile) and serum UA levels.<sup>10–12,16</sup> With regard to population stratification, our sample population of Old Order Amish individuals is extremely homogenous with no evidence of population stratification. We also considered the possibility of pleiotropic effect of GLUT9 on hexose (that is, glucose/fructose) transport as a potential source of confounding. However, given that GLUT9 is one of the principle high-capacity UA transporters and a very low-capacity fructose transporter (45–60-fold increase in UA transport capacity compared with glucose/fructose), it seems highly unlikely that fructose could account for these findings.<sup>13,23</sup> Considering the low capacity for fructose transport, the effect of fructose would have to be unrealistically strong to mediate a noticeable change in phenotype. A more plausible scenario is that fructose, by altering the transport rate of UA by GLUT9,<sup>20,30</sup> may account for some of the noted association between fructose intake and elevations in UA.<sup>28,42</sup> Given the missense nature of this highly conserved SNP, predicted exonic splicing enhancer function (<http://compbio.cs.queensu.ca/F-SNP/>), consistent strong linear association of GLUT9 SNPs with serum UA in numerous cohorts and ethnicities, and demonstrated role of GLUT9 as a major urate transporter, we are comfortable assuming that the associations noted in our result section are not secondary to linkage disequilibrium with another gene or other violations of the assumptions underlying Mendelian randomization.

### Statistical methods

The principle of Mendelian randomization is illustrated by contrasting the correlations observed between serum UA levels and BP

risk factors without consideration for the GLUT9 (Val253Ile) genotype with those obtained between serum UA levels and BP risk factors using a two-stage approach that uses the genotype-outcome and genotype-UA regressions. The simple (potentially confounded) estimates were obtained by regressing serum UA levels (the independent variable) on each BP risk factor (the dependent variable) separately, after adjusting for age and gender.

Many of the participants of the HAPI Heart Study are related. To address the correlations potentially existing in phenotype by virtue of the fact that subjects are related, we accounted for residual familial correlations in phenotype using a variance component regression framework. Specifically, we modeled variation in the trait as a function of fixed covariates, a polygenic component, and a normally distributed error component. The polygenic component was derived from the kinship coefficient matrix, which describes the relationship of each pair of individuals in the sample. The software packages SOLAR (Southwest Foundation for Biomedical Research, San Antonio, TX) and MMAP (University of Maryland School of Medicine, Baltimore, MD) were used for the analyses.

### DISCLOSURE

All the authors declared no competing interests.

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