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Within-individual changes in mitochondrial DNA copy number across the life course and links to individual performance

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Ageing is characterized by complex biological processes reflected in cellular and molecular changes. Mitochondria, which are crucial for energy production and cellular homeostasis, are particularly vulnerable to age-related deterioration. The number of copies of mitochondrial DNA (mtDNAcn) varies across and within tissues in response to energetic activity and mtDNA integrity, and is related to health and physical performance. Age-related changes in mtDNAcn can be difficult to study due to differential survival of phenotypes into older ages, but studies of changes in mtDNAcn within individuals are very limited. In this study, we investigated changes in red blood cell mtDNAcn across the life course within individual zebra finches (*Taeniopygia guttata*), a well-established avian model, from the nestling stage into old age. Our findings revealed a pronounced decline in relative mtDNAcn during post-natal development, followed by comparative stability throughout adulthood. This pattern was remarkably consistent among individuals. We found no significant relationship between variation in mtDNAcn and growth during the nestling period. However, based on measurements of disturbed take-off speed in late adulthood, we found that individuals with higher physical performance at that stage had higher relative mtDNAcn, suggesting a link between variation in individual bioenergetics and biological state.

1. Introduction

Ageing is a complex biological process driven by multiple molecular and cellular changes, including mitochondrial dysfunction [1]. Eukaryote cells generally contain variable numbers of mitochondria, which in turn contain multiple copies of their mitochondrial genome (mtDNA). The mtDNA works in concert with the nuclear genome to enable the mitochondria to play an important role in energy generation, biosynthesis and intercellular signalling. Declines in mtDNA integrity with age reflect the broader decline in mitochondrial health observed across numerous tissues [2]. mtDNA replication is not tied to cell division and differences in mtDNA copy number (mtDNAcn) across and within the cell types of an individual arise for multiple reasons, including variation in energetic demands and an increased requirement to compensate for damage to mtDNA due to exposure to adverse environmental conditions or replication errors [3–5]. However, while individuals may be able to compensate for damage to mtDNA to a degree by increasing

mtDNAcn, the capacity to do this appears itself to decline with age [4,6]. As a consequence, reduced cellular mtDNAcn in adulthood has been associated with a number of adverse health outcomes [7]. In samples of human blood, for example, reductions in cellular mtDNAcn with age are associated with reduced haematopoietic activity, mitochondrial efficiency and physical performance (e.g. [4]).

Comparison of mtDNAcn in different age groups might, therefore, provide valuable insights into how cellular energetics are linked to frailty, performance, ageing and mortality risk (e.g. [7]). However, an important problem in most studies to date is that they have been cross-sectional. A detailed study of mtDNAcn in the cellular fraction of peripheral blood in a large sample of humans aged 18–93 years [8] found evidence of a decline from *ca* 48 years of age onwards. This study found evidence of a consistent negative association between mtDNAcn and health and physical performance; the risk of death was also higher in those with relatively low mtDNAcn. However, as the authors note in their paper, this sample lacked data from early life. Furthermore, in cross-sectional studies, patterns of within-individual change can be masked in such cross-sectional designs due to changes in the phenotypic and genotypic composition of the individuals still alive at different ages. In a sub-sample of individuals in the Mengel-From *et al.* study that were older than 73 years, within-individual changes were examined in a 10 year follow-up. Interestingly, the rate of decline within this relatively elderly age group was twice that seen in the overall cross-sectional study. As the authors again point out, whether the rate of decline in the cross-sectional study appeared slower due to early death of those with the fastest declines or, as seems most likely, there is an acceleration in the rate of decline in mtDNAcn in the elderly is unclear. Within-individual studies covering a wider age group including early life are needed to examine this.

There is little information on age-related variation in mtDNAcn outside of mammalian models [9,10]. Birds offer a particular advantage in mitochondrial studies, since, as in other non-mammalian vertebrates, their nucleated red blood cells (RBCs) contain functional mitochondria [11]. While avian RBCs contain relatively low numbers of mitochondria, oxygen consumption in RBCs is a good predictor of whole organism oxygen consumption [12], and certain aspects of RBC mitochondrial function have been found to be positively associated with physical performance in at least one study [13]. By isolating the RBC fraction of the blood, it is also possible to minimize the influence of variation in blood cell composition on mtDNAcn, a common problem in mammalian studies. Moreover, using the RBC fraction in birds also largely excludes circulating cell-free mtDNAcn, which can act as a different type of biomarker [14,15]. In birds, repeated sampling of individuals throughout life is possible using minimally invasive small blood samples. We, therefore, used previously stored blood samples taken from a group of zebra finches at set age points from hatching to 4 years in order to examine within-individual changes in mtDNAcn across their life course.

Age-related changes in mtDNAcn may also reflect strategic shifts in physiological demand. During early life, for example, high metabolic demands for growth and rapid cell proliferation require increased mitochondrial activity, which can influence cellular mtDNAcn, as has been observed in some bird species [16,17]. In adulthood, mitochondrial biogenesis and degradation are typically balanced. However, in late adulthood, a decline in mtDNAcn is associated with senescence (e.g., [8]), and the extent of this decline could contribute to the variation in frailty levels later in life [18]. As a first step in examining how mtDNAcn relates to performance at the organismal level, we examine the relationship between mtDNAcn in RBCs and growth during early life, and with their physical performance measured in a disturbed take-off flight at the time of their last blood sample in old age. Parameters recorded during the explosive burst of activity in disturbed take-off flight have proved useful measures of physical performance in birds, since the need to become airborne very rapidly is energetically demanding [19]. Performance during this escape response is maximized to increase survival chances and has been shown to decline with age in the zebra finch [20,21].

2. Material and methods

(a) Animal housing and blood sampling

Our subjects were captive male zebra finches ($n = 24$) hatched in 2018 from 15 randomly paired families from an outbred captive population at the University of Glasgow. They were reared and subsequently maintained under standardized conditions [22]. Zebra finches are altricial and require parental care until they become independent from their parents at around 30 days. They reach sexual maturity around 100 days and begin to show signs of senescence by 2.5-3 years [22,23]. Blood samples (approx. 50–100 µl) were obtained from the birds at various time points through their immature and adult lives. During the nestling and fledging period, they were sampled around one week after hatching (mean, median and range in days); (6.9, 7 and 6–8), 15 days (14.5, 15 and 12–16) and again at 30 days (30.2, 30 and 28–32); as adults they were sampled at 120 days (121.0, 121 and 119–124), just over 1 year (483.0, 487 and 456–497) and 4 years old (1444.0, 1467 and 1313–1485). Blood was collected in heparinized capillary tubes, centrifuged at 3000*g* for 10 min at 4°C to separate RBCs from plasma and stored at −80°C. At each sampling point, body mass (± 0.01 g) and wing length (1 mm) were recorded. Body mass was not measured on the day of the flight trials; therefore, wing length was used to take into account the variation in body size. Since the individuals were housed in aviaries in a standardized way throughout their lives, there were no marked differences in activity levels among individuals.

(b) DNA extraction and relative mitochondrial copy number assay

DNA was extracted from the RBC pellets using the Puregene Blood Core Kit B following manufacturer's specifications (Qiagen, Manchester, UK) and then frozen at −80°C until analysis. White blood cells and thrombocytes are located on the top of the blood

cell pellet after centrifugation [24]; therefore, RBCs were collected from the bottom of the pellet to minimize contamination by other cell types. DNA integrity was assessed in a random subsample using a TapeStation (Agilent Technologies), showing high DNA integrity values in all cases (n = 30; DIN > 9.3; mean ± s.d.: 0.5). Relative mtDNAcn in RBCs was measured as described in [10]; see also [25] for sperm in the study species and the electronic supplementary materials for details. Briefly, real-time quantitative PCR (qPCR) was used to estimate the ratio between one mitochondrial gene (*NDS2*) to a nuclear single copy gene (*RAG1*) and expressed relative to a reference sample [17]. All reactions were run in triplicates, and samples from different individuals were distributed across seven plates per set of primers such that each plate contained multiple age classes. When possible, repeated samples from the same individual at different ages were included on the same plate to minimize plate effects, since we were interested in within-individual changes. qPCR efficiencies (mean ± s.d.) of *NDS2* and *RAG1* were 95.8 ± 3.13 and 90.7 ± 5.33, respectively. The assay repeatability was high, both at the intra-plate (*NDS2*: R = 0.99, 95% CI [0.992, 0.996], $p < 0.001$; *RAG1*: R = 0.99, 95% CI [0.990, 0.994], $p < 0.001$) and inter-plate level (mtDNAcn based on three samples run in each plate: R = 0.96, 95% CI [0.639, 0.991], $p < 0.001$).

(c) Flight performance methodology and analysis

At 4 years, flight parameters in the initial take-off stages of a disturbed flight were assessed for 20 birds, of which 18 successfully completed the trials, and their flight parameters were measured as follows (see e.g. [26]). Briefly, each bird was placed within a small rectangular container (18 × 15 × 9.5 cm; L × W × H) inside a vertical flight chamber (52 × 48 × 132 cm; L × W × H) and allowed to rest for approximately 30 s with the lid closed. After this time elapsed, the recording of the flight trial was initiated using the slow-motion function on an iPhone SE which was set to record at 240 frames per second (fps). This was mounted to a tripod 95 cm from the vertical flight chamber. The container was then opened remotely using a motor servo arm connected to an Arduino microprocessor. The sudden opening of the lid was sufficient to trigger the escape response of the bird, which then flew to the perch 110 cm directly above the flight container. The trial was repeated three times for each bird, and two flight traits were extracted in each trial, wingbeat frequency and flight speed when airborne (i.e. between the heights of 50 and 100 cm). Flight performance during take-off involves an initial anaerobic phase followed by aerobic metabolism, with most of the muscle fibres in small passerines dominated by fast oxidative glycolytic fibres [19].

Each flight trait was analysed separately. Briefly, for wingbeat frequency, we counted the number of wingbeats from take-off to perch (beat s^{-1}). For flight speed, the slow-motion videos were converted back to 60 fps using the Apple Photos app. Then we used EthoVision XT 17 [27] to track the birds once they were fully airborne during their respective trials, travelling upwards between 50 and 100 cm from the take-off point. The resulting tracked path was checked and subsequently adjusted to remove any anomalous objects that were picked up in error and to ensure that the path accurately followed the bird to the required height. Flight speed (mean movement of the centre point of the bird in cm s^{-1}) was then calculated from these paths. Both flight metrics were repeatable among individuals, wingbeat frequency (R = 0.73, 95% CI [0.510, 0.930], $p < 0.001$), flight speed (R = 0.44, 95% CI [0.070, 0.760], p = 0.004).

(d) Statistical analyses

We first examined the variation in mtDNAcn with age using a linear mixed model with mtDNAcn (log- and z-transformed for normalization) as a dependent variable and age as a six-level factor (7, 15, 30, 120, 483 and 1444 days; approx. 4 years). The nest ID (family) and individual unique ID were included as random effects, as well as the plate ID, although the latter was excluded to facilitate convergence as the explained variance was negligible. Given the results from this model and the nonlinear pattern (figure 1), we explored the variance explained by family and individual unique ID in two separate models ('nestling': 7–30 days; and 'adulthood': 120 days to 4 years) using the above-described structure.

We then investigated whether and how mtDNAcn covaried with (i) body mass growth during the 'nestling' stage (7–30 days measurements) and (ii) the two flight performance metrics at 4 years. To do so, we fitted two bivariate mixed models. (i) For early life body mass changes, we treated RBC mtDNAcn and body mass as joint response variables, with age as covariate and family and individual unique ID as random effects. This approach allowed us to test whether variation in RBC mtDNAcn was associated with body mass change while accounting for repeated measures and clutch effects. (ii) For disturbed flight performance, we modelled flight speed and wingbeat frequency as response variables, including mtDNAcn at age 4 years and wing length (as a proxy for body size) as covariates and individual unique ID as random effect. Here, mtDNAcn was treated as a predictor to test whether it explained variation in performance later in life. In both cases, the response variables were assuming a Gaussian error distribution.

All models were fitted using a Bayesian framework with MCMCglmm [28] in R 4.3.2 [29]; we used parameter-expanded priors [28], and the number of iterations and thinning interval were chosen to ensure that the minimum MCMC effective sample sizes for all parameters equalled 1000. Burn-in was set to a minimum of 5000 iterations. The retained effective sample sizes yielded absolute autocorrelation values lower than 0.1 and satisfied convergence criteria based on the Heidelberger & Welch convergence diagnostic [30]. In all cases, we drew inferences from posterior means and 95% credible intervals (CIs). For bivariate models, we extracted posterior distributions of covariance matrices and computed correlations between traits by standardizing covariances by the square root of the product of the respective trait variances. We also report the pMCMC values, representing the probabilities that posterior estimates include zero, to test for the significance of the fixed effects. The random effect variance in each model was obtained by dividing the variance in the traits due to differences among individuals (individual ID; individual repeatability) or families (family ID; family repeatability) by the total phenotypic variance after accounting for the fixed effects. All continuous variables were z-transformed (mean-centred and variance standardized).

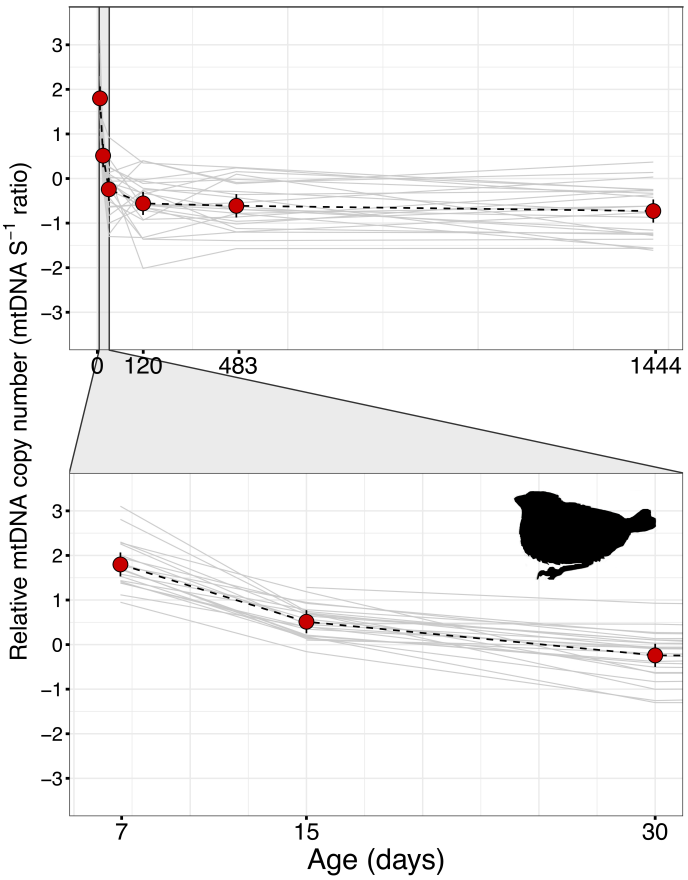

Figure 1. Age-related variation in RBCs relative (i.e. relative to the reference sample) mtDNAcn in male zebra finch. Data are longitudinal ($n = 24$), although not all individuals have been sampled at each sampling point: 7 days = 20 individuals, 15 days = 24 individuals, 30 days = 24 individuals, 120 days = 24 individuals, 483 days = 23 individuals and 1444 days = 24 individuals. Points represent model mean ± 95% CI per age group and lines individual raw data. mtDNAcn is z-transformed and separated in nestling and adult period to facilitate visualization.

3. Results

The pattern of change in RBC mtDNAcn from early life onwards was clear and consistent among individuals. mtDNAcn declined markedly in all individuals during the nestling period. The decline was most rapid between the first measurement (at 7 days) and the second (at 15 days) and continued to day 30 (figure 1; electronic supplementary material, table S1). Thereafter, the mtDNAcn was relatively stable during adulthood, i.e. 120–1444 days (figure 1). Indeed, during adulthood, there was evidence for a certain degree of individual repeatability (0.30, 95% CI [0.004, 0.781]), but this was lower in the nestling period (0.14, 95% CI [0.003, 0.403]) when there was more heterogeneity in the pattern of change; see also figure 1 and electronic supplementary material, figure S1.

The decline in mtDNAcn across the nestling period between 7 and 30 days was not related to growth (i.e. changes in body mass between 7 and 30 days; correlation: 0.21×10^{-4}, 95% CI [−0.017, 0.022]; electronic supplementary material, table S2a). However, the speed during take-off in a disturbed flight of individuals measured at 4 years was significantly related to their mtDNAcn measured at the same age (0.867, 95% CI [0.154, 1.559]; figure 2; electronic supplementary material, table S2b). Wing length was negatively related to flight speed, indicating that larger birds were slower (−0.626, 95% CI [−0.983, −0.286]; electronic supplementary material, table S2b). There was no relationship between wingbeat frequency and mtDNAcn (−0.281, 95% CI [−1.411, 0.728]; figure 2; electronic supplementary material, table S2b). Flight speed and wingbeat frequency were not correlated (−0.037, 95% CI [−0.588, 0.779]; electronic supplementary material, table S2b).

4. Discussion

We found very marked declines in mtDNAcn in RBC within individual male zebra finches during the nestling period. The consistency in the pattern of change across individuals is remarkable. RBC mtDNAcn was highest at 7 days for all individuals, presumably reflecting high mitochondrial biogenesis during rapid early growth; a marked drop in mtDNAcn had occurred by 15 days. The decline in mtDNAcn continued, albeit more slowly, between 15 and 30 days, by which time individuals are independent of their parents and have almost reached adult size.

It is interesting that the decrease in mtDNAcn is not uniform across an individual's life course. Most of the decline occurs during the nestling period. After this, mtDNAcn remains relatively stable up to 4 years, i.e. at least two thirds of the species' normal lifespan [31]. The decline in mtDNAcn after early life may reflect lower DNA replication errors or reduced energetic

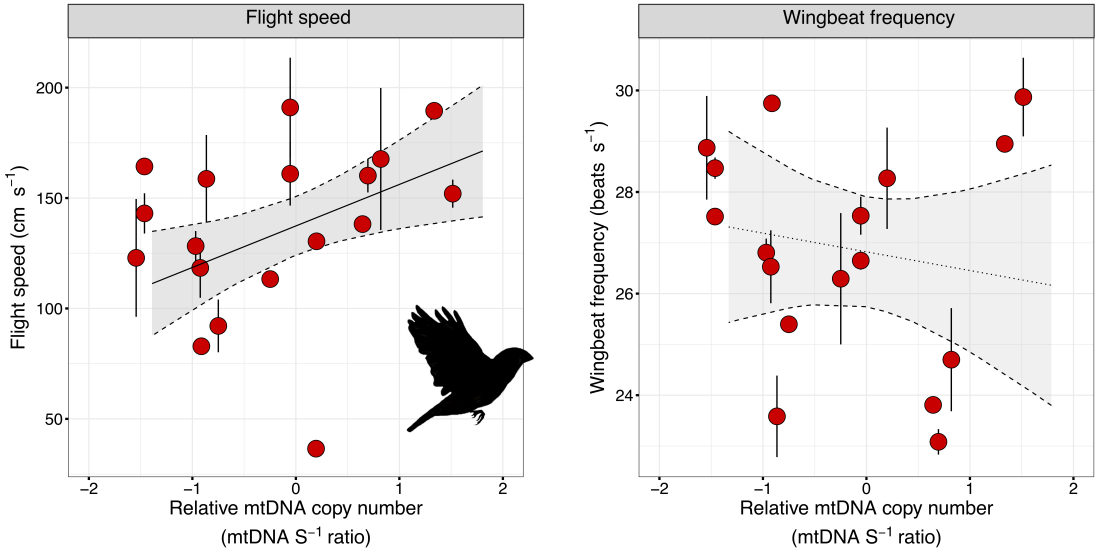

Figure 2. Adulthood (age 1444 days; approx. 4 years) correlations between flight performance traits and RBC relative mtDNAcn in male zebra finch. Each dot represents an individual's flight trial mean ± s.e., black lines represent the model regression together with the 95% CI (shadowed area), solid line: pMCMC < 0.05; dotted line: pMCMC > 0.05. $n = 18$ individuals.

requirements associated with rapid growth during development. The variation in the magnitude of the within-individual decline in mtDNAcn during the nestling stage was, however, not influenced by the degree of body mass gain individuals showed during the same period. Importantly, this study used within-individual measurements in a single cell type (RBCs), which means that the pattern observed was not confounded by differential longevity of certain individuals with varying mtDNAcn phenotypes [9], changes in the composition of cell types in the samples, cell-free mtDNA or major differences in exercise levels. It would be interesting to examine changes in mtDNAcn prior to 7 days to get a more complete picture of the dynamics in early development. It is important to bear in mind, however, that all the individuals used in this study lived to at least 4 years, and thus 'missing' from this dataset are individuals that died before reaching 4 years.

A rapid decline in blood mitochondrial content during nestling development has previously been observed in wild collared flycatchers (*Ficedula albicollis*) between 7 and 15 days old [32] and between 2 and 14 days old in wild great tits (*Parus major*) [33,34]. As in our study, this decline was not related to individuals' size or nestling growth, inferred by changes in body mass (but see [17]). During development, haematocrit often increases as the result of the acceleration of erythropoiesis, as documented in zebra finch nestlings (reviewed in [35]), decreasing later with age during adulthood [36]. Therefore, it is also plausible that the decrease in mtDNAcn is related to the maturation of the haematopoietic system due to an overall lower proportion of immature RBCs, as these are expected to exhibit a higher metabolic activity than mature RBCs [37] and might contain more mitochondria. To our knowledge, however, there is no quantitative information on how the proportion of immature RBCs changes across development in birds. Outside of the nestling and fledging period, we found no changes in RBC mtDNAcn under the relatively stable environmental conditions of our study. However, we cannot rule out potential effects of environmental variation, which were not explicitly tested in this study as all the birds were in the same captive environment. This contrasts with a cross-sectional study in collared flycatchers where a continued decline was found [10], probably reflecting differences in sampling design (longitudinal versus cross sectional approaches) or changes in activity. It would be interesting to see whether variable environmental conditions influence the pattern of change in mtDNAcn, and how changes in exercise levels, for example in migratory species, might affect mtDNAcn patterns.

Interestingly, we found that during late adulthood, *ca* 4 years old, an individual's mtDNAcn positively correlates with its flight speed attained on becoming airborne (between 50 and 100 cm), i.e. after the initial upward thrust that partly comes from the legs. While both immediate take-off and upward vertical flight will involve a combination of aerobic and anaerobic metabolism, it is likely that flight becomes more aerobically intensive once the bird is fully airborne [19]. mtDNAcn content in RBC probably reflects that in muscle, enabling individuals with higher mtDNAcn to fly upwards faster, which could directly benefit their survival [38]. In humans, higher mtDNAcn in peripheral blood has also been associated with higher physical performance (e.g. [8,39]), although other studies suggest no link between skeletal muscle function and blood cell bioenergetics [40]. The studies in human blood, however, do not involve RBCs since they do not contain mitochondria in mammals. While avian RBC haemoglobin levels have been suggested to positively correlate with mitochondrial efficiency [41], we did not measure haemoglobin concentration in this study. Nonetheless, it seems likely that the relationship between RBC mtDNAcn and flight speed reflects variation in overall individual quality, with mtDNAcn likely correlated across tissues, rather than being mediated primarily by muscular bioenergetics or haemoglobin concentration, but this warrants further investigation. There is limited information on cross-tissue correlations in mtDNAcn in birds, and to our knowledge, such correlations have only been studied in species with limited capacity for active flight, i.e. poultry (e.g. [42]).

In summary, our within-individual data spanning a large age range show a consistent pattern of loss in mtDNAcn during early life, with relative stability into late adulthood. More investigation is needed to examine whether variation in early life conditions can modify these trajectories and their fitness consequences and also the effect of variation in ageing rates and physical activity. The correlation between RBC mtDNAcn in older individuals with flight speed highlights a potential link

between cellular bioenergetics and ecologically relevant behaviours, as has also been shown with respect to RBC mitochondrial functionality [13], and this also warrants more detailed investigation.

Ethics. All housing conditions and protocols were approved by and carried out under UK Home Office Project Licence (70/8335) and following local ethical review.

Data accessibility. All data and code required to reproduce the analyses in this study are publicly available from Zenodo [43].

Supplementary material is available online [44].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. P.S.: conceptualization, formal analysis, investigation, methodology, visualization, writing—original draft; M.H.-G.: conceptualization, data curation, writing—review and editing; W.B.: conceptualization, methodology, writing—review and editing; E.I.-C.: conceptualization, formal analysis, methodology, writing—review and editing; P.M.: conceptualization, project administration, resources, writing—original draft.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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