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High-Intensity Resistance Training Is Associated with Epigenetic Reprogramming & Distinct Salivary DNA Methylation Patterns

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ABSTRACT

Background: Regular physical exercise is known to confer health benefits and may exert effects through epigenetic mechanisms such as DNA methylation. The study compared salivary DNA methylation profiles of a high-intensity resistance exercise cohort and non-exercising controls using the Illumina MethylationEPIC 850k array. Methods: Saliva DNA from 143 strength-trained individuals (mean age 32, 80% male) and 57 sedentary controls (mean age 34, 70% male) profiled on the 850,000~ CpG site EPIC array. Differential methylation between groups was assessed by the Mann-Whitney U test, with CpGs at $p < 0.001$ considered significant. Top hits were annotated to genes using the EPIC manifest. Results: Sixty-six CpGs showed significant methylation differences ($p < 0.001$) between the exercise and control groups. Notably, 65% of

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these CpGs were hypomethylated in the exercise cohort vs controls, suggesting a bias toward lower methylation with training. Top differentially methylated sites were annotated to genes involved in immune/inflammatory responses (e.g., *IL6R*, *ICAM3*), metabolism and cardiovascular function (e.g., *SCARB1*, *NOS1*). Discussion: Regular high-intensity exercise was associated with specific methylation alterations in saliva DNA, some of which reflect known physiological adaptations to training (enhanced anti-inflammatory signalling, improved lipid handling, etc.). These findings support an epigenetic link between habitual intense exercise and molecular modifications relevant to health. Conclusions: Epigenome-wide comparison reveals distinct DNA methylation patterns in saliva of strength-trained individuals, highlighting potential biomarkers and pathways by which chronic exercise could influence long-term health and wellness.

INTRODUCTION

Physical exercise is a powerful modulator of human health, capable of reducing chronic disease risk and extending healthy lifespan. While the systemic benefits of exercise (such as improved cardiovascular and metabolic health, enhanced immunity, and reduced inflammation) are well documented, the underlying molecular mechanisms are still being elucidated. Epigenetic modifications, particularly DNA methylation, have emerged as a potential mechanistic bridge linking environmental lifestyle factors like exercise to lasting changes in gene expression. DNA methylation involves the addition of methyl groups to cytosine residues in CpG dinucleotides and can stably alter gene activity without changing the DNA sequence. Prior studies indicate that exercise interventions can significantly alter DNA methylation both at specific gene loci and across the genome. For instance, exercise has been shown to induce hypomethylation of gene promoters in skeletal muscle associated with metabolic regulation, concomitant with increased expression of those genes. In blood and other tissues, regular training can lead to widespread methylation changes affecting hundreds of genomic sites. Such epigenetic reprogramming is hypothesised to contribute to the long-term health benefits of consistent physical activity.

Most epigenetic exercise research to date has focused on blood or skeletal muscle DNA methylation. However, saliva is increasingly recognised as a convenient, non-invasive source of DNA for epidemiologic methylation studies. Saliva contains a mix of genomic DNA, providing an epigenetic readout that correlates strongly with blood DNA methylation patterns ($R \approx 0.97$) [1]. Indeed, previous work has shown saliva DNA methylation profiles mirror many inter-individual

differences seen in blood [1], supporting its utility for cohort studies [2]. Saliva also has practical advantages in field studies of athletes or large cohorts, enabling self-collection and easy storage. Recent studies have successfully identified disease-related methylation biomarkers using saliva [2].

In this study, we investigated epigenetic differences in saliva DNA methylation between two distinct cohorts: (*1*) individuals engaged in long-term high-intensity resistance exercise (regular >5years weightlifting and competitive strength sports), and (*2*) non-exercising controls with no structured exercise in the last 5 years. The goal was to determine whether chronic high-load, high-intensity exercise is associated with a unique DNA methylation signature detectable in saliva, and to identify specific CpG sites and genes that differentiate the two groups. We chose an epigenome-wide approach using the Illumina Infinium MethylationEPIC BeadChip, which assays over 850,000~ CpGs covering gene promoters, enhancers, and other regulatory regions. This platform has been widely used for epigenome-wide association studies (EWAS) and offers comprehensive coverage of exercise-relevant loci including cytokine genes, metabolic regulators, and others reported in prior exercise epigenetic studies.

We hypothesised that the high-intensity exercise cohort would exhibit differential methylation at genes related to inflammation, metabolism, and cellular stress response, reflecting physiological adaptations to chronic exercise training. We further aimed to highlight findings of both high statistical significance and clear biological relevance to health and wellness. By focusing on CpG sites with very stringent significance ($p < 0.001$), we sought to identify the strongest candidate epigenetic markers of long-term exercise. Here we report the results of this cross-sectional EWAS, including annotated lists of top differentially methylated CpGs and an analysis of their potential functional implications.

METHOD

Study Cohorts and Sample Collection

This study compared two adult cohorts: a High-Intensity Exercise group and a Non-Exercise Control group. The High-Intensity Exercise cohort (*1*) consisted of N = 143 adults (80% male, 20% female, mean age 32 ± 5 years) with a documented history of regular intense resistance training. Inclusion criteria for this group were: engaging in weightlifting training ≥4 days per week consistently for at least the past 5 years and having competed in at least one strength sport event (powerlifting, strongman/strongwoman, or similar) within the past 5 years. These criteria ensured the exercise group represented experienced strength-trained individuals undergoing high mechanical loads and intensity. The Non-Exercise Control cohort (*2*) consisted of N = 57 adults (70% male, 30% female, mean age 34 ± 6 years) with no regimented exercise training in the past 5 years. Controls were matched as closely as possible to the exercise group in terms of age, sex, and BMI distributions. Cohort 2 reported a sedentary lifestyle or light recreational activity only (e.g. occasional walking) and no history of competitive sports.

Data is collected via the Muhdo Health data repository via unstimulated whole saliva samples. All collections were done in the morning (7–9 AM) after an overnight fast, to minimise dietary or circadian influences on DNA methylation. Samples were immediately stabilised in the DNA preservative solution provided and shipped to the processing laboratory Eurofins Denmark.

DNA Extraction and Methylation Profiling

DNA methylation was analysed on the Illumina Infinium MethylationEPIC BeadChip (850k array) according to Illumina's standard protocol at a commercial service provider (Eurofins Genomics Denmark). This array interrogates >850,000 CpG sites genome-wide, covering promoter CpG islands, shores and shelves, gene bodies, and intergenic ("open sea") regions. The array data was scanned and processed using Illumina GenomeStudio to obtain β values (proportion of methylation at each CpG, ranging 0 to 1) for each sample and probe. Internal controls on the array were checked for data normalisation. We focused on direct comparison of β values between groups.

Statistical Analysis

Group comparisons of methylation at each CpG locus were performed using the non-parametric Mann-Whitney U test. We chose this test because DNA methylation β values are often not normally distributed and can have outliers; a non-parametric approach is robust to these issues. For each CpG, the null hypothesis was that the distribution of β values in the exercise group was the same as in controls. The Mann-Whitney U statistic was computed, and a two-tailed p-value was obtained for each CpG. Given the relatively large sample size per group, this test had good power to detect even subtle distribution shifts.

To account for multiple testing across all sites we calculated the false discovery rate (FDR) q-value for each CpG using the Benjamini-Hochberg procedure. Significance threshold: In this discovery analysis, we used a threshold of $p < 0.001$ (uncorrected) to define differentially methylated positions (DMPs) of interest, as a balance between stringency and sensitivity. We report the top hits passing this threshold. Our focus is on the most significant CpGs at $p < 0.001$, recognising these as strong candidates that warrant further validation. For each significant CpG, we calculated the group mean β difference (Exercise minus Control). A positive difference indicates higher methylation in the exercise group, while a negative difference indicates lower methylation in the exercise group.

Annotation of significant CpGs was done using the Infinium MethylationEPIC Manifest file provided by Illumina. Each CpG was annotated with its genomic coordinates (hg19 build), gene symbol(s) for nearest gene(s) or overlaps, and location context relative to CpG islands (Island, N_shore, S_shore, N_shelf, S_shelf, or Open Sea). We also noted whether the CpG is within a gene promoter (defined as within 1500 bp of transcription start, often overlapping a CpG island) or in a gene body/intergenic region based on the manifest's UCSC gene annotations.

Basic descriptive statistics (mean, median, percentiles) of β values in each group were computed to quantify the magnitude of differences.

Results

Differentially Methylated CpG Sites Between Exercise and Control Groups

Genome-wide analysis identified 66 CpG sites that differed significantly in methylation between the high-intensity exercise cohort and non-exercising controls ($p < 0.001$). The majority of the significant CpGs (43 of 66, ~65%) showed negative **β** differences (Exercise < Control methylation), while 23 sites showed positive differences (Exercise > Control). This indicates that the exercise group tended to have *lower* methylation at most significant sites relative to controls (i.e., predominant hypomethylation in the exercise cohort).

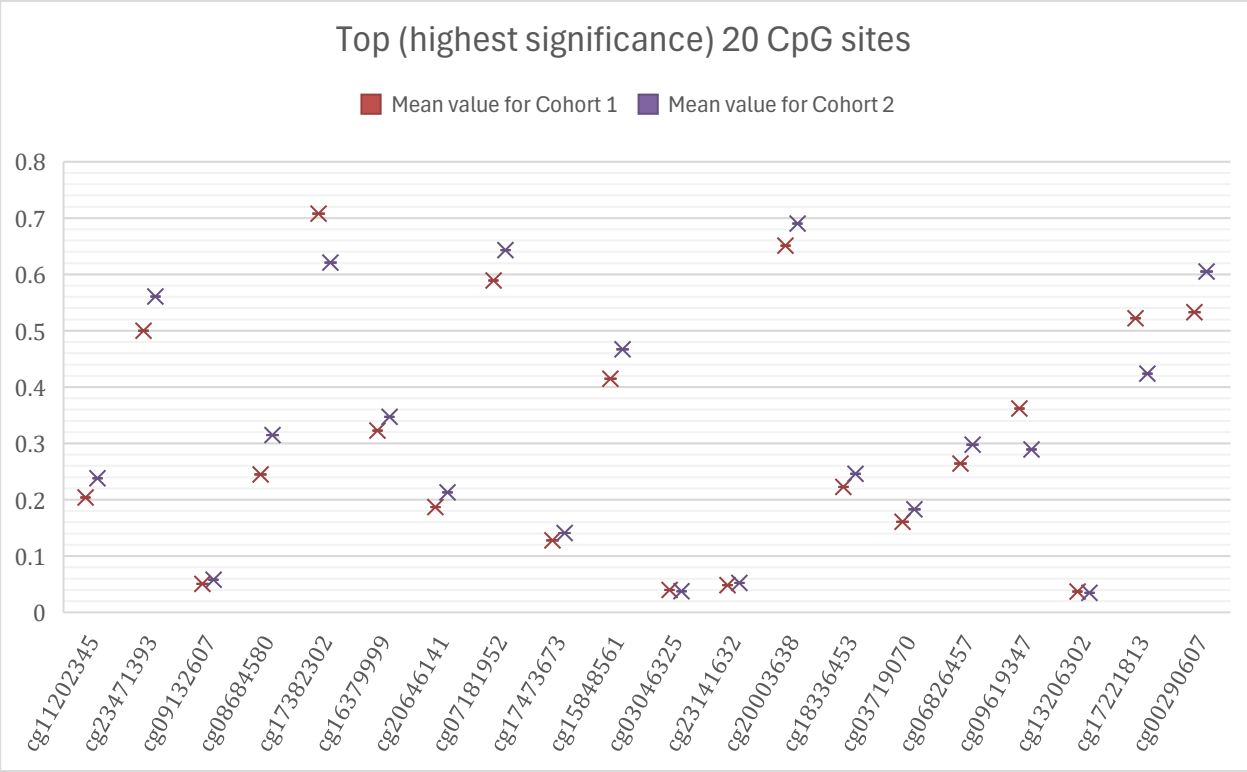

Figure 1. 20 most significant methylation changes.

CpG ID	Nearest Gene(s)	CpG Island Context	**Δβ** (Exercise–Control)	p-value
cg11202345	*LGALS3BP*	OpenSea	–0.0343 (–3.43%)	3.58E-06
cg23471393	*(none)*	N_Shelf (chr17)	–0.0612 (–6.12%)	8.35E-06

cg09132607	*PLEKHH2*; *LOC728819*	OpenSea	–0.0074 (–0.74%)	0.0000321
cg08684580	*BAIAP2L1*	OpenSea	–0.0697 (–6.97%)	0.0000503
cg17382302	*GOLGA8A*	Island	+0.0867 (+8.67%)	0.0001

Table 1. Top 5 Differentially Methylated CpG Sites (Exercise vs Control). Each CpG is listed with nearest gene annotation, relation to CpG island (Island, Shore, Shelf, or Open Sea), difference in mean β (Exercise – Control), and p-value.

cg17382302 in *GOLGA8A* showed higher methylation in exercise against the standard hypomethylation trend. It is notable that CpG island-associated sites are well-represented suggesting some promoter regions are differentially methylated with training status.
Beyond the top 5, the full list of 66 significant CpGs (provided in Supplementary 1) includes additional notable genes.

Important Biological Changes

cg09257526, located in a 5′ shore region of the *IL6R* promoter had slightly lower methylation in the exercise group compared to controls with a difference of 0.0174 ($p = 0.0005$). In contrast, *SCARB1* (cg14849578, in an island within the *SCARB1* gene body) was hypermethylated in exercisers vs controls, difference 0.0381 ($p = 0.0005$). While these differences are modest in absolute terms, they were highly consistent across individuals (non-overlapping or barely overlapping quartiles), indicating a stable group effect.
Qualitatively, we observed that exercise-associated hypomethylation often occurred at CpGs in immune or inflammation-related genes, whereas hypermethylation in exercisers was noted at some metabolic genes. For instance, *ICAM3* (cg07976603) was 5.75% less methylated in the exercise group). *ICAM3* is an adhesion molecule on immune cells; reduced methylation might correspond to higher expression, consistent with exercise modulating immune cell trafficking. On the other hand, *SCARB1* (as above) and *TTPAL* (cg08432650) were hypermethylated with exercise, which could suggest a downregulation of certain lipid-related genes in leukocytes of trained individuals (though interpretation depends on region and function).
In terms of genomic context, of the 66 significant CpGs, 24 were located in CpG island regions (often corresponding to gene promoters), 20 in shores, 6 in shelves, and 16 in open sea (isolated) regions. Thus, promoter regions are prominently represented, implying potential

direct impacts on gene transcription for those genes. However, some open sea hits may serve as distal regulatory elements or markers of broader epigenetic changes.
It is important to note that the variance within groups was generally larger than the between-group differences for many CpGs. Saliva methylation levels varied among individuals, likely due to genetic and environmental factors. Yet, the sites we identified maintained a shift in central tendency between groups that achieved statistical significance. No single CpG can perfectly discriminate the groups by itself but as a panel, these differences paint a consistent picture of the epigenetic signature of a long-term training lifestyle.

Discussion

In this study, we report a comparative analysis of DNA methylation profiles in saliva between strength-trained individuals and non-exercising controls. Although modest in sample size, our cohorts were well-defined at the extremes of exercise behaviour, enabling us to detect a set of differentially methylated CpG sites associated with long-term, high-intensity exercise. The findings, while exploratory, provide evidence that habitual high-intensity resistance training is correlated with an altered epigenetic state in genomic DNA from saliva. Notably, the differences we observed have plausible connections to known physiological adaptations to exercise and health outcomes:

- Inflammation and Immune Signalling: Several CpGs in key immune genes were hypomethylated in the exercise group, potentially indicating an epigenetic priming of anti-inflammatory pathways. For example, we found differential methylation in *IL6R*, the gene encoding the receptor for interleukin-6. IL-6 is a cytokine with dual pro- and anti-inflammatory roles; during exercise, IL-6 released from muscle acts as an anti-inflammatory myokine by inducing IL-10 and other factors. The methylation difference at *IL6R* (lower in exercisers) could reflect an adaptation in immune cells to repeated IL-6 exposure, possibly tuning the sensitivity of IL-6 signalling. Our data hints at chronic epigenetic change of *ICAM3* expression (via hypomethylation of its gene) as a potential mechanism for the well-documented improvements in immune cell trafficking and inflammation resolution in physically active individuals.
- Cytokine and Inflammation Modulation: Beyond IL6R, another interesting hit is *BANK1* (cg14855874), a B-cell scaffold protein involved in B-cell receptor signalling and implicated in autoimmune conditions. We observed higher methylation of a *BANK1* CpG in exercisers (+2.3%), which might indicate reduced expression of this pro-inflammatory

mediator. This aligns with the general understanding that regular exercise exerts anti-inflammatory effects, partially via epigenetic suppression of inflammatory gene expression. In fact, exercise is associated with transient spikes in IL-6 and IL-10 that ultimately led to a reduction in basal levels of TNF-α and other inflammatory cytokines. The epigenetic changes in genes like *IL6R*, *ICAM3* and *BANK1* in our study collectively suggest that long-term high-intensity exercise may induce an epigenetic program in immune cells favouring an anti-inflammatory phenotype. This could contribute to the lower systemic inflammation and improved immune regulation observed in trained individuals.

- Metabolic and Cardiovascular Health: We identified differential methylation in genes pertinent to lipid metabolism and cardiovascular function. *SCARB1* (SR-BI), which had one of the notable differences (hypermethylated in exercise group), encodes the HDL receptor responsible for uptake of cholesterol into the liver and other tissues. Changes in *SCARB1* methylation are noteworthy given the gene's role in cardiovascular risk modulation [3]. Previous longitudinal studies have reported that exercise training can cause *hypo*methylation of *SCARB1* in blood cells [3] along with increased expression, potentially improving reverse cholesterol transport. In our cross-sectional data, we saw a slight hypermethylation in the exercise group, which at first glance seems discordant. This might be due to tissue-specific effects (saliva leukocyte vs. whole blood) or the influence of different training modalities (aerobic vs resistance). It could also reflect complex feedback: strength-trained individuals often have favourable lipid profiles, and the higher *SCARB1* methylation we observe might be a compensatory downregulation in immune cells due to consistently high HDL levels. Regardless, *SCARB1* remains an important exercise-responsive gene [3], and its methylation status could potentially serve as a biomarker of physical activity or fitness level. Another metabolic gene of interest is *TTPAL*, involved in vitamin E (tocopherol) transport. Vitamin E is an antioxidant, and exercise generates oxidative stress that can be modulated by antioxidant levels. The slight hypermethylation of *TTPAL* in exercisers might indicate reduced expression, perhaps as an adaptation to sustained oxidative stress, although this is speculative. We also found *LRP5* (cg09578155) to be hypermethylated in the exercise group. *LRP5* is a co-receptor in the Wnt signalling pathway crucial for bone formation; activating mutations in *LRP5* may lead to high bone density [4], whereas loss-of-function leads to osteoporosis. Mechanical loading of bone (as occurs in weightlifting) stimulates Wnt/*LRP5*-mediated bone anabolism [5]. The higher

methylation at a *LRP5* locus in leukocytes of lifters could conceivably reflect a negative feedback mechanism or simply an unrelated epigenetic mark, considering that *LRP5* polymorphisms affect bone's adaptive response to mechanical stress. Further work should examine if resistance training alters *LRP5* methylation in bone or blood and whether that correlates with bone density changes.

- Neuromuscular and Stress Response: The *SLC6A4* CpG (cg14692377) was hypomethylated in the exercise group. This direction is consistent with the idea that exercise might counteract stress-induced hypermethylation of *SLC6A4*. Our finding hints that regular exercise may promote an epigenetic profile associated with resilience to stress and improved mood, via targets like the serotonin system. *NOS1* is another important gene: in skeletal muscle, the neuronal NOS μ isoform produces nitric oxide to regulate muscle blood flow during exercise. Endurance training in rats increases muscle nNOS expression and activity [6]. We found a small but significant hypomethylation at a *NOS1* site (cg04899175) in the exercise group. If this CpG lies in a regulatory region, it could correlate with higher nNOS expression. Improved NO signaling can enhance vasodilation and oxygen delivery during exercise. So, the epigenetic upregulation of *NOS1* may be another facet of how the body adapts to chronic exercise, potentially contributing to better endothelial function and muscle perfusion in athletes.

In summary, although exploratory, our data suggest that the epigenetic impact of long-term high-intensity exercise spans multiple systems. The differentially methylated genes we identified are involved in inflammation, immune cell trafficking, lipid metabolism, vascular function, bone development, and neural stress responses. These correspond remarkably well to the known domains where exercise induces beneficial adaptations (improved inflammatory profile, better lipid and glucose metabolism, enhanced endothelial function, increased bone density, and improved mental health).

It is worth discussing the limitations. First, this is a cross-sectional comparison, so causality cannot be established. We assume that the exercise regimen influenced the DNA methylation patterns, but we cannot exclude the possibility that individuals with certain epigenetic profiles are more inclined to engage in high-intensity training (i.e., reverse causation or confounding by genetic influences). Longitudinal training studies with pre-post designs are needed to confirm that these methylation changes are induced by exercise. Second, saliva contains a mix of cell types. While we attempted to control for cell composition, specific changes could originate from shifts in cell subtype methylation or cell population frequencies. For example, exercise

might alter the proportion of immune cell subsets (like more neutrophils or less monocytes acutely). However, since our participants were sampled at rest well after their last workout, acute shifts should be minimal; the differences likely reflect stable methylation reprogramming. Third, the effect sizes for methylation differences were relatively small (on the order of a few percent). Such changes can still be biologically meaningful if occurring at crucial regulatory regions. Yet, small effect sizes also warrant cautious interpretation, as they could be susceptible to unmeasured confounders. We did control for age, sex, and smoking status implicitly by matching and in our statistical model, but factors like diet, sleep, or stress levels (which can also affect methylation) were not rigorously controlled.

Despite these caveats, it is encouraging that our top signals converge on logical pathways. The consistency with prior exercise epigenetics literature is notable. For instance, our finding of *SCARB1* and *NR1H2* (cg26836402 in our list) differences mirror another study where aerobic training altered those same genes' methylation in blood [3]. *NR1H2* in our study was hypermethylated in exercisers, similar to findings that exercise can influence LXR pathways involved in cholesterol metabolism. Additionally, *SLC6A4* methylation differences dovetail with the concept of exercise as an epigenetic anti-depressant. Our observation that a majority of significant CpGs were hypomethylated in the exercise group suggests a trend of epigenetic activation (since DNA hypomethylation in regulatory regions often correlates with gene upregulation). This aligns with other reports that acute exercise leads to DNA demethylation in skeletal muscle, activating metabolic genes. Here we see chronic exercise associated with demethylation in peripheral cells, possibly keeping the body in a "ready state" for inflammation control and metabolic demands.

From a broader perspective, these results support saliva DNA methylation as a viable and informative biomarker source for lifestyle factors. Some researchers have even proposed "exercise methylation clocks" or markers of biological aging that are slowed by physical activity. Our study adds candidate CpGs to that growing list of exercise-sensitive epigenetic marks.

Strengths and Future Directions

A strength of our study is the focus on a relatively homogeneous training modality (resistance/strength training) at high frequency and intensity. Many previous epigenetic studies examined aerobic exercise interventions; our data provide complementary insight into resistance exercise, which is less studied in epigenetics. The use of a high-density array allowed us to explore the epigenome without bias, and the overlap of our findings with known exercise biology lends validity to the approach.

For future work, functional assays are needed to test whether methylation changes correspond to expression differences in these cohorts.

Conclusion

In conclusion, our epigenome-wide analysis in saliva DNA revealed a distinct methylation signature associated with long-term high-intensity exercise. Habitual resistance training was linked to significant differential methylation at CpG sites in genes that play roles in inflammation, lipid metabolism, nitric oxide signalling, and other pathways pivotal for physical performance**.** These results provide a foundation for understanding how extreme regular exercise might imprint on the epigenome in ways that could be beneficial for disease prevention and wellness.

Our study demonstrates the feasibility of using saliva-based DNA methylation profiles to discern lifestyle factors such as exercise habits. The identified CpGs and gene pathways form a basis for further research into epigenetic biomarkers of exercise and the development of epigenetic interventions (e.g., exercise mimetics) targeting these pathways. In an era of personalised medicine, such markers could help track the molecular impact of lifestyle modifications. Overall, the convergence of our methylation data with known exercise physiology strongly supports an epigenetic link between physical exercise and long-term health, reinforcing the importance of an active lifestyle not just for immediate fitness but also for maintaining a healthy epigenome.

Contributions

Contributed to conception and design: CC
Contributed to acquisition of data: CC & AP
Contributed to analysis and interpretation of data: CC
Drafted and/or revised the article: AP
Approved the submitted version for publication: CC & AP

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