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Original Paper

Associations of DNA Methylation Mortality Risk Markers with Congenital Microcephaly from Zika Virus: A Study of Brazilian Children Less than 4 Years of Age

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ABSTRACT

Background: Zika virus (ZIKV)-associated congenital microcephaly is an important contributor to pediatric death, and more robust pediatric mortality risk metrics are needed to help guide life plans and clinical decision making for these patients. Although common etiologies of pediatric and adult mortality differ, early life health can impact adult outcomes—potentially through DNA methylation. Hence, in this pilot study, we take an early step in identifying pediatric mortality risk metrics by examining associations of ZIKV infection and associated congenital microcephaly with existing adult DNA methylation-based mortality biomarkers: GrimAge and Zhang's mortality score (ZMS).
Methods: Mortality measures were calculated from previously published HumanMethylationEPIC BeadChip data from 44 Brazilian children aged 5–40 months (18 with ZIKV-associated microcephaly; 7 normocephalic, exposed to ZIKV *in utero*; and 19 unexposed controls). We used linear models adjusted for chronological age, sex, methylation batch and white blood cell proportions to evaluate ZIKV and mortality marker relationships.
Results: We observed significant decreases in GrimAge-component plasminogen activator inhibitor-1 [PAI-1; $\beta = -2453.06$ pg/ml, 95% confidence interval (CI) −3652.96, −1253.16, $p = 0.0002$], and ZMS-site cg14975410 methylation ($\beta = -0.06$, 95% CI −0.09, −0.03, $p = 0.0003$) among

children with microcephaly compared to controls. PAI-1 ($\beta = -2448.70$ pg/ml, 95% CI −4384.45, −512.95, $p = 0.01$) and cg14975410 ($\beta = 0.01$, 95% CI −0.04, 0.06, $p = 0.64$) results in comparisons of normocephalic, ZIKV-exposed children to controls were not statistically significant.

Conclusion: Our results suggest that elements of previously-identified adult epigenetic markers of mortality risk are associated with ZIKV-associated microcephaly, a known contributor to pediatric mortality risk. These findings may provide insights for efforts aimed at developing pediatric mortality markers.

KEYWORDS: GrimAge, Zika, congenital microcephaly, mortality score, PAI-1, cg14975410, epigenetics

INTRODUCTION

Prenatal Zika virus (ZIKV) infection and ZIKV-associated congenital microcephaly are important contributors to fetal/child death with an estimated perinatal mortality rate of 27.3% [1]. At present, severity of neurologic impairment is a major risk factor for pediatric deaths in this population, but the degree of neurological impairment remains insufficient for predicting timing and likelihood of death [2]. More robust predictions of mortality risk may be useful for families and healthcare providers in order to make decisions regarding medical interventions and overall life plans that involve parental work and childcare. One early step in helping to actualize such markers involves determining if existing adult mortality measures have any relationships with ZIKV infection and associated congenital microcephaly.

Emerging evidence demonstrating associations of ZIKV infection with DNA methylation provides meaningful motivation for evaluating DNA methylation-based biomarkers as tools for assessing ZIKV relationships with mortality risk [3–5]. DNA methylation is a biological process where methyl groups are transferred to cytosine nucleotides on DNA, and can subsequently influence DNA expression [6]. Many scientists have studied the relationships of DNA methylation with mortality, and two of the most robust methylation-based mortality predictors from these studies are GrimAge and Zhang's mortality score (ZMS) [7, 8]. Epigenetic GrimAge acceleration predicts all-cause time-to-death (HR = 1.10, $p = 2.0 \times 10^{-75}$), and was built using a DNA methylation surrogate of cigarette pack-years, and DNA methylation surrogates for seven plasma protein markers [adrenomedullin (ADM), beta-2-microglobulin (B2M), cystatin C, growth differentiation factor-15 (GDF-15), leptin, plasminogen activator inhibitor-1 (PAI-1), and tissue inhibitor metalloproteinases 1 (TIMP-1)] [7]. The ZMS epigenetic predictor was built using data from 10 DNA methylation sites and demonstrates significant hazard ratios ranging from 2.16 to 7.36 for all-cause mortality [8].

In this study, we examine the relationships of *in utero* ZIKV exposure and ZIKV-associated microcephaly at birth with mortality risk biomarkers assessed via GrimAge and ZMS. Although it is well appreciated that the common etiologies of adult and pediatric mortality differ, it is also appreciated that childhood health status can play a consequential role in shaping adult morbidity and mortality [9]. For instance, early-life hepatitis B infections can result in chronic liver disease and liver cancer later in life [10]. Low birth weight is associated with cardiovascular disease in adulthood [11] and altered methylation is believed to play a role in this disease risk [12]. Congenital microcephaly represents one specific instance where a pediatric condition may have more acute impacts on lifespan [13]. Hence, although both mortality measures were developed in adults, we hypothesized that the measures and/or their component proteins/methylation sites would be associated with ZIKV exposure and microcephaly. Additionally, we also evaluate relationships of ZIKV exposure and microcephaly with two DNA methylation-based chronological age predictors that are known to perform well in pediatric cohorts: Horvath's DNA methylation age (DNAmAge) and the Pediatric–Buccal–Epigenetic age (PedBEage) [14–16].

METHODS

Study population

We performed analyses using a publicly available NCBI GEO MethylationEPIC BeadChip dataset

(Series GSE145233, uploaded to 'https://www.ncbi.nlm.nih.gov/geo' on 20 July 2020) initially collected for a previously published epigenome-wide association study (EWAS) [3]. The dataset contained information on chronological age, child sex (i.e. male or female) and peripheral blood DNA methylation from 45 children born between 2015 and 2018 in Brazil. The DNA samples were collected between 0.4 and 3.7 years of age. Data were incomplete for one subject, so the study sample was comprised of the remaining 44 subjects. Children were classified into three groups: (i) children diagnosed with ZIKV-associated microcephaly at birth ($N = 18$), (ii) *in utero* ZIKV-exposed, normocephalic children (i.e. mothers had positive ZIKV serologies during pregnancy; $N = 7$) and (iii) unexposed, normocephalic control children ($N = 19$). Informed consent and ethical conduct of study information regarding the collection of this data along with additional details about study participants can be found in the initial publication [3].

DNA methylation data, DNA methylation age and mortality measures

Processed and normalized methylation data were downloaded from NCBI. Full descriptions of DNA processing for the downloaded dataset can be found in the initial publication [17]. DNA methylation data were then uploaded to Horvath's publicly available online calculator (http://dnamage.genetics.ucla.edu). The online calculator provided downloadable output which included values for DNAmAge, GrimAge, the GrimAge component proteins, and white blood cell abundance/proportions (CD8 naïve T cells, CD8pCD28nCD45Ra-T cells, plasmablasts, CD4T cells, NK cells, monocytes, and granulocytes). ZMS was calculated from the same DNA methylation dataset using CpG probe cutoffs presented in Supplementary Table S4 of its original manuscript [8]. Importantly, ZMS is calculated from 10 CpG probes and usually ranges from 0 to 10 with a greater score indicating a higher risk of mortality. However, two of these probes (cg06126421 and cg23665802) were missing in our downloaded data—likely removed during preprocessing and/or quality control. Hence, ZMS used in the present study has a potential maximum value of 8. PedBEage was also calculated from the same DNA methylation dataset using R code available at https://github.com/kobor-lab/Public-Scripts/ [15]. No probes for PedBEage were missing in the dataset.

Statistical analysis

We examined the relationships of *in utero* ZIKV exposure and microcephaly with our mortality risk markers using linear regression models adjusted for chronological age, sex, white blood cell abundance/proportions and methylation batch. All covariates were determined *a priori* based on available data and the previous study using this dataset [3]. In secondary analyses, using the same fully-adjusted models, we evaluated ZIKV relationships with the methylation sites or component proteins of the mortality markers. Given that we performed 17 independent tests, we adjusted for multiple hypotheses testing using the Bonferroni cutoff of $p < 0.003$ for statistical significance.

To interrogate the robustness of our findings, we also evaluated relationships of ZIKV status with methylation-based chronological age predictors: DNAmAge and PedBEage. These chronological age predictors served as imperfect internal controls that may be less related to ZIKV status given that they capture biological aging not frank mortality risk. Finally, given that ZIKV infection involves the immune system (with white blood cells being potential mediators of any observed relationships), we performed a sensitivity analysis with models without adjustments for white blood cell abundance/proportions. All statistical analyses were performed using R Version 3.6.3 (R Core Team, Vienna, Austria).

RESULTS

Table 1 presents the demographic and age characteristics for all study participants. The majority of participants were male (57%), 43% had no *in utero* exposure to ZIKV, 16% were exposed to ZIKV but did not have a diagnosis of microcephaly, and 41% were exposed to ZIKV and also had a diagnosis of microcephaly. Participants had a mean (SD) chronological age of 1.8 (1.0) years at blood sample collection. With respect to DNA methylation-based chronological age predictors, participants had a mean (SD) DNAmAge and PedBEage of 5.5 (2.2) and 4.8 (0.5) years, respectively. Mean (SD)

Table 1. Study participant characteristics

	All participants ($N = 44$)	Unexposed to ZIKV ($N = 19$)	Exposed to ZIKV ($N = 7$)	ZIKV microcephaly ($N = 18$)
Chronological age (years), mean (SD), (range)	1.8 (1.0), (0.4–3.7)	2.0 (1.0), (0.6–3.7)	0.6 (0.04), (0.5–0.6)	2.1 (0.7), (0.4–3.3)
DNAmAge (years), mean (SD), (range)	5.5 (2.2), (1.6–11.5)	5.8 (2.4), (2.5–11.5)	2.5 (0.9), (1.6–4.3)	6.2 (1.3), (3.4–8.0)
PedBEage (years), mean (SD), (range)	4.8 (0.5), (4.0–6.1)	4.9 (0.5), (4.0–6.1)	4.2 (0.2), (4.0–4.5)	4.7 (0.4), (4.1–5.5)
GrimAge (years), mean (SD), (range)	16.9 (2.5), (11.8–22.7)	17.1 (2.7), (12.8–21.3)	15.2 (2.0), (11.8–17.7)	17.6 (2.2), (13.3–22.7)
GrimAge component protein surrogates (pg/ml), mean (SD)				
DNAm ADM	362.3 (14.6)	362.8 (16.0)	348.0 (9.0)	367.4 (11.4)
DNAm B2M	2 320 269 (72 642.9)	2 313 736 (65 551.1)	2 269 677 (83 794.7)	2 346 841 (66 862.4)
DNAm Cystatin C	392 380 (19 356.9)	388 781 (20 019.3)	376 907.3 (13 101.3)	402 196.1 (15 739.6)
DNAm GDF15	−91.9 (93.4)	−84.6 (72.5)	−117.3 (78.4)	−89.8 (118.4)
DNAm Leptin	29 026.8 (1848.1)	29 507.9 (2236.4)	29 363.9 (1581.4)	28 387.8 (1312.6)
DNAm TIMP1	21 149.8 (491.4)	21 211.5 (544.5)	20 710.0 (192.9)	21 255.7 (433.5)
DNAm PAI1	13 776.7 (2097.0)	15 039.8 (1499.2)	12 855.3 (1499.2)	12 801.7 (2175.1)
ZMS, mean (SD), (range)	2.0 (1.0), (0–5)	1.7 (0.7), (1–3)	1.7 (1.0), (1–3)	2.3 (1.2), (0–5)
ZMS Component CpGs, mean (SD)				
cg01612140	0.44 (0.07)	0.44 (0.07)	0.47 (0.04)	0.42 (0.07)
cg05575921	0.88 (0.02)	0.88 (0.02)	0.89 (0.02)	0.88 (0.03)
cg08362785	0.49 (0.04)	0.50 (0.04)	0.46 (0.02)	0.50 (0.03)
cg10321156	0.38 (0.06)	0.40 (0.05)	0.36 (0.02)	0.38 (0.07)
cg14975410	0.44 (0.06)	0.45 (0.05)	0.47 (0.05)	0.40 (0.04)
cg19572487	0.48 (0.06)	0.48 (0.07)	0.50 (0.07)	0.48 (0.05)
cg24704287	0.46 (0.04)	0.45 (0.04)	0.49 (0.03)	0.45 (0.04)
cg25983901	0.64 (0.08)	0.67 (0.07)	0.68 (0.05)	0.61 (0.09)
Sex, *N* (%)				
Female	19 (43)	10 (53)	2 (29)	7 (39)
Male	25 (57)	9 (47)	5 (71)	11 (61)

GrimAge and ZMS for participants were 16.9 (2.5) years and 2.0 (1.0), respectively.

Supplemenatry Table S1 depicts a Spearman correlation matrix for the age variables and major mortality measures used in the analyses. Both DNAmAge ($r = 0.85$, $p < 0.001$) and PedBEage ($r = 0.67$, $p < 0.001$) were highly correlated with chronological age. PedBEage and DNAmAge had median absolute errors of 2.9 years and 3.6 years, respectively. GrimAge and ZMS were not significantly correlated ($r = 0.28$, $p = 0.07$). GrimAge was significantly correlated with chronological age ($r = 0.39$, $p = 0.009$), but ZMS was not ($r = 0.08$, $p = 0.62$).

Table 2 describes the results from fully-adjusted linear models examining the relationships of *in utero* ZIKV exposure and microcephaly with the DNA methylation chronological age predictors and mortality risk markers. Children exposed to ZIKV or diagnosed with microcephaly demonstrated no significant differences in DNAmAge or PedBEage when compared to unexposed children. Similarly, children exposed to ZIKV or diagnosed with microcephaly demonstrated no significant differences in GrimAge or ZMS when compared to unexposed children. Nevertheless, secondary analyses of GrimAge and ZMS component measures did demonstrate statistically significant findings. In particular, one GrimAge component measure—DNAm PAI-1—met the Bonferroni threshold for statistical significance. Compared to unexposed children, children with microcephaly had lower levels of DNAm PAI-1 ($\beta = -2453.06$ pg/ml, 95% CI -3652.96, -1253.16, $p = 0.0002$). Results for exposed children without microcephaly demonstrated similar trends but did not meet the threshold for statistical significance ($\beta = -2448.70$ pg/ml, 95% CI -4384.45, -512.95, $p = 0.01$). One ZMS CpG component—cg14975410—met the threshold for statistical significance. Compared to unexposed children, children with microcephaly had lower methylation at the cg14975410 site ($\beta = -0.06$, 95% CI -0.09, -0.03, $p = 0.0003$). Results for exposed children without microcephaly did not demonstrate similar trends ($\beta = 0.01$, 95% CI -0.04, 0.06, $p = 0.64$). Comparable results were observed in the sensitivity analysis models not adjusting for white blood cell abundance/proportions (Supplemenatry Table S2). Furthermore, neither sex nor having a chronological age less than 1-year significantly modified the associations of ZIKV exposure or microcephaly with any mortality measure.

DISCUSSION

In this pilot study of Brazilian children aged 0.4–3.7 years, we did not observe statistically significant changes in GrimAge or ZMS when comparing *in utero* ZIKV-exposed children and children with microcephaly to unexposed, normocephalic children. Nevertheless, we did observe statistically significant decreases in one GrimAge component (DNAm PAI-1) and one ZMS methylation site (cg14975410).

Importantly, PedBEage and DNAmAge both were highly correlated with chronological age in our study sample. PedBEage has a previously reported median absolute error (MedAE) in blood cells of 3.3 years [15]. In our study, PedBEage had a MedAE of 2.9 years. Likewise, previously reported MedAE for DNAmAge was 3.6 years and a MedAE of 3.6 years was observed in the present study [14]. Furthermore, keeping with our hypothesis that PedBEage and DNAmAge are predominantly predictors of biological aging rather than mortality, we observed no associations between these measures and ZIKV exposure or microcephaly. Despite the comparable age prediction performance observed in our study sample, it is important to note that DNAmAge and PedBEage are good but imperfect internal controls in this study sample. DNAmAge was trained and tested in adults (although it performs well in children), while PedBEage was trained and tested in pediatric samples of buccal epithelial cells and is known to perform worse in blood when compared to buccal epithelium [14, 15]. These details in DNAmAge and PedBEage biomarker development could also play a role in our lack of observed associations with ZIKV exposure or microcephaly.

We observed no significant relationships between the main mortality measures and *in utero* ZIKV exposure or microcephaly. This is again may be because the mortality measures were developed in adults and common etiologies for adult mortality (e.g. ischemic heart disease, chronic obstructive

Table 2. Relationships of *in utero* ZIKV exposure and congenital microcephaly with DNA methylation chronological age and mortality predictors

Models[a]	Difference in DNA methylation marker (95% CI)	*p*-value
DNAm chronological age predictor models		
DNAmAge (years)		
Unexposed	Reference	–
Exposed	−0.69 (−2.00, 0.63)	0.30
Microcephaly	0.30 (−0.51, 1.12)	0.45
PedBEage (years)		
Unexposed	Reference	–
Exposed	−0.30 (−0.67, 0.06)	0.10
Microcephaly	−0.10 (−0.31, 0.12)	0.37
GrimAge and components models		
GrimAge (years)		
Unexposed	Reference	–
Exposed	−1.25 (−3.61, 1.11)	0.29
Microcephaly	−0.19 (−1.66, 1.27)	0.79
DNAm ADM (pg/ml)		
Unexposed	Reference	–
Exposed	−8.17 (−22.27, 5.93)	0.25
Microcephaly	4.14 (−4.60, 12.88)	0.34
DNAm B2M (pg/ml)		
Unexposed	Reference	–
Exposed	−10 418.7 (−65 452.32, 44 614.96)	0.70
Microcephaly	28 108.9 (−6004.44, 62 222.22)	0.10
DNAm cystatin C (pg/ml)		
Unexposed	Reference	–
Exposed	782.45 (−13 579.48, 15 144.39)	0.91
Microcephaly	9539.63 (637.20, 18 442.07)	0.04
DNAm GDF15 (pg/ml)		
Unexposed	Reference	–
Exposed	22.94 (−73.54, 119.41)	0.63
Microcephaly	−10.81 (−70.61, 49.00)	0.72
DNAm leptin (pg/ml)		
Unexposed	Reference	–
Exposed	487.14 (−1686.29, 2660.56)	0.65
Microcephaly	−1077.17 (−2424.40, 270.06)	0.11
DNAm TIMP1 (pg/ml)		
Unexposed	Reference	–
Exposed	−73.69 (−511.30, 363.93)	0.73
Microcephaly	8.57 (−262.69, 279.83)	0.95
DNAm PAI1 (pg/ml)		
Unexposed	Reference	–
Exposed	−2448.70 (−4384.45, −512.95)	0.01
Microcephaly	−2453.06 (−3652.96, −1253.16)	*0.0002*

(continued)

Table 2. (continued)

Models[a]	Difference in DNA methylation marker (95% CI)	*p*-value
ZMS and components models		
ZMS		
Unexposed	Reference	–
Exposed	−0.25 (−1.32, 0.82)	0.64
Microcephaly	0.63 (−0.03, 1.29)	0.06
cg01612140		
Unexposed	Reference	–
Exposed	0.03 (−0.02, 0.08)	0.25
Microcephaly	−0.01 (−0.04, 0.02)	0.55
cg05575921		
Unexposed	Reference	–
Exposed	0.007 (−0.02, 0.04)	0.64
Microcephaly	0.002 (−0.02, 0.02)	0.81
cg08362785		
Unexposed	Reference	–
Exposed	−0.02 (−0.04, 0.004)	0.10
Microcephaly	−0.009 (−0.02, 0.004)	0.18
cg10321156		
Unexposed	Reference	–
Exposed	−0.007 (−0.06, 0.04)	0.77
Microcephaly	0.0002 (−0.03, 0.03)	0.99
cg14975410		
Unexposed	Reference	–
Exposed	0.01 (−0.04, 0.06)	0.64
Microcephaly	−0.06 (−0.09, −0.03)	*0.0003*
cg19572487		
Unexposed	Reference	–
Exposed	0.02 (−0.03, 0.08)	0.40
Microcephaly	−0.02 (−0.05, 0.02)	0.27
cg24704287		
Unexposed	Reference	–
Exposed	−0.001 (−0.03, 0.02)	0.92
Microcephaly	0.004 (−0.01, 0.02)	0.56
cg25983901		
Unexposed	Reference	–
Exposed	−0.02 (−0.08, 0.04)	0.51
Microcephaly	−0.05 (−0.09, −0.01)	0.009

[a]Models adjusted for chronological age, sex, white blood cell abundance/proportions (CD8 naïve T cells, CD8pCD28nCD45Ra-T cells, plasmablasts, CD4T cells, NK cells, monocytes and granulocytes) and batch.
p-values < 0.003 are italicized and considered statistically significant in these analyses.

pulmonary disease) are less prevalent in children. Nevertheless, we hoped that our mortality risk markers or at least components of the markers would be associated with ZIKV exposure and microcephaly. This appeared to be the case with our cg14975410 and DNAm PAI-1 results.

Beyond inclusion in the ZMS, there is not much literature on cg14975410. The cg14975410 site is found on chromosome 3 and lower methylation values are associated with an increased risk of mortality (specifically the cutoff of cg14975410 methylation ≤ 0.44 gives a person an additional point in the ZMS) [8]. Our findings are in accord with this existing work given that when compared to control children, children born with ZIKV-associated microcephaly had statistically significantly lower cg14975410 methylation levels. In an effort to learn more about the methylation site, we queried the ARIES database for any reported genetic influences in the pregnancy and birth time periods on methylation at cg14975410. A number of reported associations of single-nucleotide polymorphisms (SNPs) with cg14975410 methylation are indeed listed in the database (http://www.mqtldb.org/) [18]. Nevertheless, we do no more than mention these findings because we have no genetic information on our study subjects. Specifically, much caution should be taken when drawing any conclusions from this query given that the data in the database is from populations of European descent and there may be significant differences in the SNPs and minor allele frequencies present in our Brazilian study population. Future studies including genetic information will be better positioned to elucidate population-specific relationships of cg14975410 methylation with ZIKV exposure and microcephaly.

Plasminogen activator inhibitor-1 (PAI-1) is an inhibitor of blood clot breakdown in the body, and increased levels of PAI-1 have been associated with hypertension, time to heart disease and diabetes [7]. Increased second trimester DNAm PAI-1 measured in mothers from Los Angeles, CA, USA, has been associated with shorter gestational length and lower birth weight [19]. Increased levels of PAI-1 have also been observed in *in vitro* studies of cells infected with ZIKV [20]. However, in the present analysis, we observe that children with microcephaly have a lower PAI-1 when compared to controls. Our data also suggest that ZIKV exposure independent of microcephaly is also associated with lower PAI-1. Although the implication of PAI-1 in our study appears promising and there are some rare, reported instances of increased bleeding risk with ZIKV infection (increased bleeding risk would be observed with lower PAI-1 levels) [21], additional work is necessary to understand why our results demonstrate directionality that is the opposite of most existing studies. Moreover, correlations between measured and DNA methylation estimated PAI-1 range from $r = 0.69$ (training data) to $r = 0.36$ (testing data) [7]. Therefore DNAm PAI-1 may not fully reflect clinical levels. Given the known elevated prevalence of low birth weight in children born with Congenital Zika Syndrome [22], associations of low birth weight with increased pediatric mortality [23], and reported strong correlations of DNAm PAI-1 with computed tomography imaging-derived measures of adiposity [7], having birth weight or weight at blood draw data on these children would be helpful to better understand the relationships of microcephaly with PAI-1. Future expanded studies should be sure to include data on weight and body habitus.

It is also important to consider our findings in the context of the original EWAS. In that differential methylation analysis of 747 178 sites, the authors identified no significant differentially methylated sites when comparing control children to ZIKV-exposed, normocephalic children. In comparisons of control and microcephalic children, 42 differentially methylated sites were identified with the top hits belonging to genes *RABGAP1L*, *ISG15* and *MX1* [3]. This pattern of more robust findings comparing microcephalic and control children mirror the significant findings in our present study and suggests that the observed methylation differences are related to microcephaly and not just ZIKV exposure. Furthermore, cg14975410 and PAI-1 were not among the differentially methylated probes/genes identified in the EWAS of our study participants. Given that EWAS and analyses of composite CpG biomarkers like mortality scores often provide different information on methylation relationships [24], to better understand how the two sets of findings may be related, we searched the literature, for instances, where *RABGAP1L*, *ISG15* or *MX1* had relationships with adult mortality directly or indirectly through associations with diseases that are major contributors to adult mortality. We did not identify human study relationships for *RABGAP1L*. However, there were some interesting findings for

ISG15 and *MX1*. Although ISG15 is known for its role in viral processes, it has also been implicated as a clinical prognosticator in nasopharyngeal carcinoma [25] and esophageal squamous cell cancers [26]. Both MX1 and ISG15 levels may be useful for predicting outcomes in acute respiratory distress syndrome [27]. Although our analysis is a pilot study aimed at directing future research efforts, it is important to explicitly state that there still remains a need for a pediatric mortality risk marker for ZIKV-associated microcephaly. More specifically, we were not able to ascertain whether mortality markers developed in adults directly predict mortality in children since there are no data as to whether these children died. We hope that the significant and robust associations with adult-defined mortality risk measures that we present, when combined with the previously published EWAS findings, may offer insights for the development of this future marker.

Strengths of the present study include the utilization of novel DNA methylation-based mortality markers, and adjustments of significance thresholds for multiple testing to avoid Type I error. Furthermore, our results are comparable in sensitivity analyses unadjusted for white blood cell proportions. Still, this study does possess some limitations in addition to not being able to directly predict child mortality. In particular, we had limited information on important covariates including demographic, clinical and lifestyle factors. Hence, we cannot rule out the impact of the aforementioned unknowns and residual confounding in our analyses. Furthermore, we utilized a modified version of the ZMS given that two of the component CpG probes were missing in our dataset. This may have limited the utility of our score, and there is a possibility that the relationships we observed would be different from the full ZMS. Nevertheless, given that each individual probe has been associated with mortality [8], we performed probe level analyses and report those in this manuscript. Future studies remain necessary to characterize the relationships of ZIKV exposure and associated microcephaly with the full ZMS score and the two probes (cg06126421 and cg23665802) missing in our data. Finally, our findings are from a modestly-sized sample of Brazilian children and merit additional studies to establish their generalizability.

In conclusion, our findings suggest that some elements of existing DNA methylation-based mortality markers built from adult data are associated with ZIKV-associated microcephaly, a known contributor to pediatric mortality risk. Nevertheless, if our findings prove to be generalizable, research efforts will need to be taken to develop measures that more closely mirror and predict pediatric mortality.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Tropical Pediatrics* online.

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CONFLICT OF INTEREST STATEMENT

None declared.

DATA AVAILABILITY STATEMENT

The dataset used in this study (Series GSE145233) is publicly available at: https://www.ncbi.nlm.nih.gov/geo.

REFERENCES

1. Melo ASDO, Aguiar RS, Amorim MMR, *et al.* Congenital Zika virus infection: beyond neonatal microcephaly. JAMA Neurol 2016;73:1407–16.
2. Wheeler AC. Development of infants with congenital Zika syndrome: what do we know and what can we expect? Pediatrics 2018;141:S154–60.
3. Anderson D, Neri JICF, Souza CRM, *et al.* Zika virus changes methylation of genes involved in immune response and neural development in Brazilian babies born with congenital microcephaly. J Infect Dis 2020
4. Kandilya D, Maskomani S, Shyamasundar S, *et al.* Zika virus alters DNA methylation status of genes involved in Hippo signaling pathway in human neural progenitor cells. Epigenomics 2019;11:1143–61.
5. Janssens S, Schotsaert M, Karnik R, *et al.* Zika virus alters DNA methylation of neural genes in an organoid model of the developing human brain. mSystems 2018;3.

6. Moore LD, Le T, Fan G. DNA methylation and its basic function. Neuropsychopharmacology 2013;38:23–38.
7. Lu AT, Quach A, Wilson JG, *et al.* DNA methylation GrimAge strongly predicts lifespan and healthspan. Aging (Albany NY) 2019;11:303–27.
8. Zhang Y, Wilson R, Heiss J, *et al.* DNA methylation signatures in peripheral blood strongly predict all-cause mortality. Nat Commun 2017;8:14617.
9. Gribble JN, Preston SH, Population NRC (US) C onThe Epidemiological Transition: Policy and Planning Implications for Developing Countries: Workshop Proceedings Childhood Precursors of Adult Morbidity and Mortality in Developing Countries: implications for Health Programs [Internet]. National Academies Press (US); 1993. https://www.ncbi.nlm.nih.gov/books/NBK236451/ (5 January 2021, date last accessed).
10. Chang M-H, You S-L, Chen C-J, *et al.* Long-term effects of hepatitis B immunization of infants in preventing liver cancer. Gastroenterology 2016;151:472–80.e1.
11. Smith CJ, Ryckman KK, Barnabei VM, *et al.* The impact of birth weight on cardiovascular disease risk in the Women's Health Initiative. Nutr Metab Cardiovasc Dis 2016;26:239–45.
12. Wehkalampi K, Muurinen M, Wirta SB, *et al.* Altered methylation of IGF2 locus 20 years after preterm birth at very low birth weight. PLoS One 2013;8:e67379.
13. Hussain A, Ali F, Latiwesh OB, *et al.* A comprehensive review of the manifestations and pathogenesis of Zika virus in neonates and adults. Cureus 2008;10:e3290. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6235632/ (5 January 2021, date last accessed).
14. Horvath S. DNA methylation age of human tissues and cell types. Genome Biol 2013;14:R115.
15. McEwen LM, O'Donnell KJ, McGill MG, *et al.* The PedBE clock accurately estimates DNA methylation age in pediatric buccal cells. Proc Natl Acad Sci USA 2020;117:23329–35.
16. Tollefsbol T. Environmental Epigenetics in Toxicology and Public Health, Vol. 22, 1st edn. In: Fry R (ed). Waltham: Academic Press, 2020. https://www.elsevier.com/books/environmental-epigenetics-in-toxicology-and-public-health/fry/978-0-12-819968-8
17. Ovejero-Benito MC, Cabaleiro T, Sanz-García A, *et al.* Epigenetic biomarkers associated with antitumour necrosis factor drug response in moderate-to-severe psoriasis. Br J Dermatol 2018;178:798–800.
18. Gaunt TR, Shihab HA, Hemani G, *et al.* Systematic identification of genetic influences on methylation across the human life course. Genome Biol 2016;17:61.
19. Ross KM, Carroll JE, Horvath S, *et al.* Epigenetic age and pregnancy outcomes: grimAge acceleration is associated with shorter gestational length and lower birthweight. Clin Epigenet 2020;12:120.
20. Bayless NL, Greenberg RS, Swigut T, *et al.* Zika virus infection induces cranial neural crest cells to produce cytokines at levels detrimental for neurogenesis. Cell Host Microbe 2016;20:423–8.
21. Karimi O, Goorhuis A, Schinkel J, *et al.* Thrombocytopenia and subcutaneous bleedings in a patient with Zika virus infection. Lancet 2016;387:939–40.
22. Carvalho-Sauer R, Costa M da CN, Barreto FR, *et al.* Congenital Zika syndrome: prevalence of low birth weight and associated factors. Bahia, 2015-2017. Int J Infect Dis 2019;82:44–50.
23. Vilanova CS, Hirakata VN, Souza Buriol VD, *et al.* The relationship between the different low birth weight strata of newborns with infant mortality and the influence of the main health determinants in the extreme south of Brazil. Popul Health Metrics 2019;17:15.
24. Madden RA, McCartney DL, Walker RM, *et al.* Birth weight associations with DNA methylation differences in an adult population. Epigenetics 2020;1–14.
25. Chen R-H, Du Y, Han P, *et al.* ISG15 predicts poor prognosis and promotes cancer stem cell phenotype in nasopharyngeal carcinoma. Oncotarget 2016;7:16910–22.
26. Tao J, Hua P, Wen J, *et al.* Prognostic value of ISG15 mRNA level in drinkers with esophageal squamous cell cancers. Int J Clin Exp Pathol 2015;8:10975–84.
27. Nick JA, Caceres SM, Kret JE, *et al.* Extremes of interferon-stimulated gene expression associate with worse outcomes in the acute respiratory distress syndrome. PLoS One 2016;11:e0162490.