

Epigenetic clocks in the pediatric population: when and why they tick?

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Abstract

Recent research efforts have provided compelling evidence of genome-wide DNA methylation alterations in pediatrics. It is currently well established that epigenetic clocks, composed of DNA methylation sites, can estimate the gestational and chronological age of cells and tissues from different ages. Also, extensive research is aimed at their correlation with early life exposure and pediatric diseases. This review aimed to systematically summarize the epigenetic clocks in the pediatric population. Publications were collected from PubMed and Web of Science databases up to Apr 2021. Epigenetic clocks, DNA methylation clocks, epigenetic age acceleration or deceleration, pediatric and the pediatric population were used as search criteria. Here, we first review the currently applicative pediatric epigenetic clocks. We then highlight the interpretation for epigenetic age deviations in the pediatric population and their association with external factors, developmental trajectories, and pediatric diseases. Considering the remaining unknown of pediatric clocks, research strategies into them are also discussed. In all, pediatric epigenetic clocks may act as potent tools to understand development, growth and diseases in early life.

Keywords: Epigenetic clocks; DNA methylation; Early life; Neonates; Children; Epigenetic age acceleration

Introduction

DNA methylation (DNAm), the addition of methyl group to the fifth position of cytosine, is the best-studied and most mechanistically understood epigenetic modification, which plays essential roles in development and growth.^[1,2] *In utero*, DNAm is involved in multiple vital processes including cell differentiation,^[3-5] X-chromosome inactivation,^[6] and fetal growth.^[7] Beyond birth, the role of DNAm includes maintaining cell-type identity and genome stability,^[8-10] responding to external exposures,^[11,12] and involvement in neural^[13] and immune^[14] development.

The epigenetic clock also referred to as the DNAm clock is used to estimate the age of any DNA source (cells, tissues, or organs) based on a relatively small set of cytosine-guanine dinucleotide (CpG) sites. The selection of clock-related CpGs is established on the precise knowledge of all methylated CpG dinucleotides in the whole genome, enabled by the incredible advent of DNAm array technology.^[15,16] Since 2011, the year epigenetic clock was first created,^[17] it has been regarded as a promising marker for studying development, cancer, and aging.^[18] Although comparisons of individual variables can address straightforward questions, such as which specific loci are

hyper- or hypo-methylated as a result of external stress factors like childhood maltreatment, they cannot answer more functional and generalized questions, such as whether childhood maltreatment affects early life developmental trajectories. To answer such questions, epigenetic clocks arise.

We are only beginning to understand the role of epigenetic clocks in the pediatric population. Early life and childhood are two of the major susceptibility windows during which epigenetic programming is sensitive to external influence.^[19] Epigenetic age is not linear throughout the lifespan.^[20] Evidence from human and mice have demonstrated that changes in DNAm early in life differ from those later in life.^[21] Since it is influenced by both genetic and environmental factors,^[22,23] DNAm has also emerged as a key mechanism of interest for understanding the gene-environment interplay in normal development and related diseases. Thereby, the pediatric epigenetic clock is a thriving topic in unraveling the biological magic behind development and growth for youth.

In this paper, we first begin by reviewing the most prominently applied types of epigenetic clocks for neonates (gestational age [GA]) and children (chronological age). We then discuss the connotation of the disturbed ticking of

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pediatric epigenetic clocks – that is, when estimated epigenetic age deviates from our expectation. We also review the potential accelerators or decelerators for epigenetic age deviations (EADs). Finally, we turn to the available methods in uncovering the mechanism of these clocks and also highlight the future perspectives of this emerging star.

Epigenetic Clocks for the Pediatric Population

During the development process of epigenetic clocks, early studies focused on adult-specific or all-age clocks.^[18,24] These clocks sacrificed their precision in predicting

pediatric chronological age so that these DNAm age estimators can be applied to a wider population. To better understand the age-related DNAm changes in pediatrics, clocks for neonates and children are being introduced [Figure 1]. The past 5 years have seen the progress of several pediatric estimators that use different sets of CpGs originating from different age spectra and tissue [Table 1]. For neonates, both preterm and term infants are covered; for children, those who are younger than 20 years are also included in the construction of pediatric epigenetic clocks.

Similar to those DNAm estimators for aging, pediatric clocks are built by regressing gestational or chronological

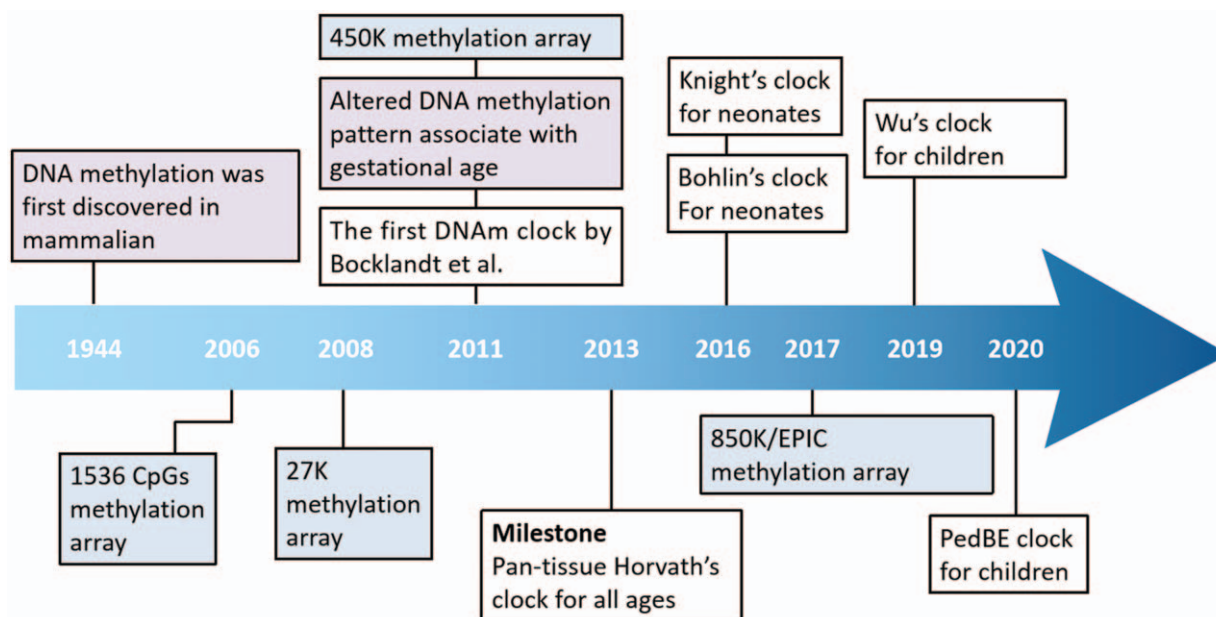


Figure 1: A brief history of pediatric epigenetic clocks. White boxes represent the pediatric-specific and -related epigenetic clocks, during which Horvath clock in 2013 was a landmark study. Purple boxes represent important events in our understanding of DNAm. Blue boxes mark the development of methylation array technologies. CpG: Cytosine-guanine dinucleotide; DNAm: DNA methylation; PedBE: Pediatric buccal epigenetic.

Table 1: A summary of pediatric epigenetic clocks.

Epigenetic clocks	Tissue	Age group of training sets	Size of training sets	CpG sites	Correlation in training	Training error	Correlation in testing	Test error
GA prediction								
Knight 2016 ^[28]	Cord blood	Neonates	207	148	0.99	0.35 weeks	0.91	1.24 weeks
Bohlin 2016 ^[29]	Cord blood	Neonates	1068	96	0.81	0.40 months	–	–
Falick Michaeli 2019 ^[30]	Cord blood	Neonates	41	743	0.77	–	–	–
Chronological age prediction								
Horvath 2013 ^[18]	Pan-tissue	Children and adults (0-centenarians)	3931	353	0.97	2.9 years	0.96	3.6 years
Skin and Blood 2018 ^[31]	Multi tissue: Blood, buccal, fibroblast, skin, epithelium	Children and adults (0–85 years)	896	391	–	–	0.91*, 0.98 [†]	2.6 years*, 2.5 years [†]
Wu 2019 ^[32]	Blood	Children (1–18 years)	716	111	0.98	5.9 months	0.98	6.7 months
PedBE 2020 ^[33]	Buccal	Children (0–20 years)	1032	94	0.99	0.47 years	0.98	0.35 years

Different correlation coefficients were presented during the application of the skin and blood clock to different tissues. * The corresponding performance of skin and blood clock in fibroblast. [†] Its performance in blood samples. –: Not reported; CpG: Cytosine-guanine dinucleotide; GA: Gestational age; PedBE: Pediatric buccal epigenetic.

age on tens to hundreds of CpGs using a supervised machine learning method, predominantly ElasticNet regression.^[25,26] The weight of each selected CpG is also decided by the penalized regression model. Therefore, both the CpGs and the corresponding mathematical algorithm are automatically yielded by the supervised machine learning analysis to transform DNAm levels into an estimated epigenetic age. The calculated age is not only a reflection of intended gestational and chronological age but also of the biological age of the DNA source. Since pediatric epigenetic clocks are mainly built on healthy cohorts, the deviation of predicted age from the chronological age can provide information on the individuals' psychological function.^[27]

Epigenetic clocks for GA estimation

Even before the development of the epigenetic GA clock, GA itself has been proven to be associated with methylation changes at various CpG sites.^[34-37] Back in 2011, Schroeder *et al*^[37] studied the association between neonatal methylation pattern and GA, suggesting that neonatal DNA methylated states vary with GA. Though the training database of Horvath's clock (2013) included DNA samples from cord blood, the age of all these subjects was set to 0.^[18] Because of this, Horvath and other adult-specific epigenetic clocks,^[17,18,24] as initially operationalized, are inappropriate for GA prediction. That is why in 2016 two epigenetic GA clocks, Knight and Bohlin, filled the gap for GA prediction, providing putative sight into predicting DNAm age in neonates both preterm and term.

The epigenetic clock designed by Knight *et al*^[28] for GA was based on cord blood and blood spot samples from 1434 neonates. This clock was derived by regressing early obstetric ultrasound or last menstrual period (LMP) on DNAm levels using a penalized regression model. Interestingly, the final selected 148 CpGs were uniformly distributed across the genome, without enrichment in any specific biological pathways. As a result of the relatively small training set (207 individuals) in Knight clock, overfitting may occur while being applied to other cohorts.^[38,39] In the same year, Bohlin *et al*^[29] developed another statistical model for GA prediction with DNA extracted only from cord blood, using data from 1753 newborns in the Norwegian Mother and Child Birth Cohort Study. The 96 CpG sites in the Bohlin prediction model and their associated genes were substantially different from the corresponding CpGs and genes in Horvath clock, partially supporting the idea that epigenetic clocks for GA and aging are based on different molecular mechanisms. With a five-time bigger training group than Knight clock, the clock by Bohlin performed much better in other cohorts.^[38] In both GA clocks, ultrasound-based regression models notably outperform LMP-based models in terms of model fit and standard error measured as days within a 95% prediction interval. Falick Michaeli *et al*^[30] performed reduced representation bisulfite sequencing (RRBS) to create another epigenetic GA model based on 41 cord blood and matching placenta samples from their own hospital in Israel. Their clock combined 332 differentially methylated regions (DMRs) that underwent demethylation and 411 DMRs that underwent *de novo* methylation in late GA. Since this

clock was developed on DNA extracted by RRBS, it might meet difficulties in the application process to other array-established cohorts. In research, a neonate epigenetic clock is a potent tool in exploring *in utero* gene-environment interplay; in the clinic, these clocks can be a substitute measurement of GA for those without full access to ultrasound in early pregnancy or precise reporting of LMP.

Epigenetic clocks for pediatric chronological age

Back in 2013, Horvath developed the first pan-tissue epigenetic clock, using 8000 samples from 82 Illumina (San Diego, CA, USA) DNAm array datasets, encompassing 51 healthy tissues and cell types.^[18] As a landmark age estimator, Horvath clock calculated DNAm age on the basis of 353 CpGs in almost all human cell types and tissues, excluding sperm. It was Horvath clock that opened a new era for DNAm age prediction and deepened our learning of the DNAm's relationship in aging, cancer, and development. Till today, Horvath clock is still the most accepted in estimating epigenetic ages for all ages, including the pediatric population.

Another wide-spectra DNAm age estimator available in the pediatric population is the skin and blood clock.^[31] Despite the numerous successful predictions made by Horvath clock, it performs suboptimally in fibroblast lines derived from the skin of patients with Hutchinson Gilford Progeria Syndrome (HGPS). Trying to develop an epigenetic clock that can capture the aging acceleration in HGPS, Horvath *et al*^[31] introduced another multi-tissue DNAm age prediction model for fibroblasts, keratinocytes, skin cells, endothelial cells, saliva samples, and blood. All cells used in this clock can be isolated from skin, except for blood, allowing DNA samples to be easily acquired from individuals. Another advantage of this clock is that it is valuable in studying the dynamics of DNAm in *ex vivo* experiments, helping to unravel the molecular process in pediatric DNAm variation.

Though correlations between pediatric chronological age and Horvath-measured DNAm age have been reported, a high degree of variability from chronological age has also been observed.^[40] Recent research reported poor performance of Horvath clock by tracking the developmental trajectories from birth to late adolescence.^[41] This is probably because of the dynamics of DNAm in early life and childhood. The two clocks mentioned above sacrifice their accuracy in the pediatric population to serve all ages. Therefore, clocks specific to pediatrics were required. It was not until 2019 and 2020 that Wu *et al*^[32] and McEwen *et al*^[33] designed two children-specific epigenetic clocks.

Wu *et al*^[32] first established a methylation-based age prediction model for children using data from 716 blood samples from children between 9 and 212 months. The elastic net model consists of 111 CpG sites, mostly located in genes associated with development and aging. Interestingly, researchers used 67 pairs of monozygotic twins to validate Wu's model, whose genetic background and environmental exposure are purposed to be extremely similar. The predicted DNAm ages of twins 1 and twins 2 did not differ significantly while using Wu clock, while

differences did occur while using Horvath clock. To this degree, Wu clock outperformed Horvath clock in estimating children's DNAm ages by blood samples. Pediatric buccal epigenetic (PedBE) clock is another pediatric-specific clock.^[33] This non-invasive clock, known as PedBE, is based on 1721 genome-wide DNAm profiles of typically developing individuals aged 0 to 20 years old. Elastic net penalized regression was used to select 94 CpGs from a training dataset of 1032 subjects. A simple swab of buccal epithelial cells (BECs) enables the construction of a highly accurate biological tool to estimate DNAm age-specific to the pediatric population. Non-invasiveness is the one of starring points of the PedBE clock, widening the potential usage in the clinic. Furthermore, the collection of BECs induces less cellular heterogeneity when compared with other accessible tissues, such as blood, and has a high degree of DNAm stability.^[42-44] Though not pan-tissue, the PedBE clock succeeded in achieving the highest accuracy in estimating DNAm age ever based on BECs with a 0.35-year median absolute error in the test cohort.^[45]

According to a comparative study,^[45] all epigenetic clocks for children are tested together in different types of pediatric tissues. Because they were trained on different types of tissue, their performances varied. The correlation to chronological age in blood samples using skin and blood clock was significantly better ($P_{adj} < 0.05$) than all of the other clocks, including those trained predominantly in adults. The PedBE clock is the best in buccal cell samples, totally surpassing Horvath's, due to its focus on a more homogeneous tissue, which reduces confounders when building clocks. This highlights the difference in methylation dynamics in children *vs.* adults and the importance of using methylation clocks trained on pediatric samples.

EAD in the Pediatric Population

The application of epigenetic clocks has been turning the blurred picture of methylated states pattern into a specific number. As would be expected, the application of epigenetic clocks to the general population invariably reveals outliers: individuals whose chronological and epigenetic ages are divergent. Two kinds of deviations are commonly used: the first one, known as raw deviation, is calculated by subtracting the chronological (gestational) age from the DNAm age; the second one, residual, is extracted from a linear regression of DNAm age on chronological (gestational) age.^[38] In simple operational terms, those with epigenetic age that is older than expected (the gestational/chronological age) are described as positive epigenetic age acceleration (PEAA), whereas the reverse situation would be described as negative epigenetic age acceleration (NEAA).^[25]

Investigating how the estimated epigenetic age differs across a group of subjects of the same chronological age could help determine the dynamic DNAm patterns in different life stages. For epigenetic aging clocks, positive deviation of DNAm age suggests that the underlying tissue ages faster than expected, whereas negative deviation suggests the tissue ages slower than expected. Several

studies concerning epigenetic aging clocks have reported the connections between epigenetic age acceleration (EAA) and premature aging disorders (such as Down syndrome^[46] and Werner syndrome^[47]), neuropathology in elderly individuals,^[48-50] all-cause mortality^[51-53] and the risk of developing certain types of cancers.^[54-56] Earlier studies of pediatric EAD tended to follow the model of epigenetic aging clocks, which focused more on EAA and interpreted it as an early sign of aging. DNAm age deviation in pediatrics, however, may not entirely follow the same pattern presented in later life. Epigenetic age in the early 20 years enjoys a more dynamic paradigm. In the paper Knight clock first published,^[28] researchers tried to explain the acceleration in the estimated GA. According to Knight theory, an accelerated GA may reflect differences in the physiological development of the newborn so that neonates with a higher DNAm GA are more developmentally mature than their chronological age suggested, reflecting the developmental trajectories of individuals. Another possibility is that the differences between DNAm GA and chronological GA reflect epigenetic programming by early life environmental exposure, such as maternal prenatal and perinatal stress or pregnancy disorders, which may affect neonatal outcomes and development. The clinical observations of patients with DNMT3A overgrowth syndrome support their hypothesis indirectly.^[57] Germline mutations in *DNMT3A* (a gene encoding a pivotal enzyme in DNAm) lead to widespread hypomethylation at specific genome sites enriched at locations annotated as genes involved in morphogenesis, development, and differentiation. At the same time, patients may present highly accelerated DNAm aging with faster growth than normal. Patients are also characterized by intellectual disability and subtle facial anomalies.^[57,58] Other common phenotypes include overweight in late childhood and features of autism spectrum disorder (ASD) with impaired communication and socialization skills.^[59]

Therefore, the interpretation of pediatric EAD should not entirely copy that for the elderly. Both PEAA and NEAA deserve attention while interpreting deviations. EADs at this stage caused by changes in methylated states are highly likely to be associated with developmental trajectories, developmental diseases, and certain environmental conditions that may accelerate or decelerate biological development in early life and childhood.

Biomarkers of developmental trajectories

In girls, pubertal timing is a milestone in development and growth. According to the calculation of Horvath clock, a 5-year PEAA on average was related to a significant decrease in time to menarche.^[60] Besides pubertal timing, measures of EAD are also appreciated by the fact that they are associated with a great number of developmental characteristics, including weight, body mass index (BMI), height, fat mass, bone density, subscapular skinfold, and upper-arm circumference. For instance, in a longitudinal analysis measuring DNAm by Horvath clock in 1018 children from the Avon Longitudinal Study of Parents and Children (ALSPC), researchers present evidence that children with higher age acceleration (AA) at birth had

faster growth in weight and BMI during childhood and adolescence (age 7 and age 17), while NEAA was associated with an increase in height and fat mass. Bright *et al*^[62] also analyzed ALSPC, used Bohlin clock to verify the connection between gestational age acceleration (GAA) and developmental characteristics. Slightly different from the result by Simpkin *et al*,^[61] they found that the association between GAA and increased birth weight and length only persisted to age 9 months. From age 5 years onward, the association of GAA and weight reversed such that by age 10 years, greater GAA was associated with lower childhood weight. Though confusing, one reasonable explanation for the variation is that different clocks may produce different results. Both the chosen clock and the chosen cohort are vital in understanding the role played by EAA in developmental trajectories. A minimal mistake can end up with completely unconvincing results.

Deviations under environmental exposure

There is a large body of research covering the effect of environmental exposure on the DNAm aging of children. Animal studies have confirmed DNAm is programmed by early life experience. The epigenetic response associated with early life stress has a broad footprint in DNAm in blood.^[63] These environmental factors can be divided into physical environmental effects and social ones. The association between external exposure and EAD is detailed in Table 2.

The exposures of the social and physical environment can be subdivided into exposures in prenatal periods and those that occur in childhood. The first kinds of exposures mainly affect newborns by maternal-fetal communications. The physical environment is the primary focus of the majority of *in utero* studies, examining measures such as air pollution, maternal alcohol assumption, maternal and neonatal nutrition, and other toxicant exposure. The majority of extant studies are based on retrospective data, despite the Tianjin GDM cohort.

For children, the association between external factors and EAD is mainly restricted to the social environment, such as violence, sexual abuse, low socioeconomic status, and cumulative exposure to sexual abuse, physical abuse, or neglect.^[67-70,75] Animal studies have identified the developmental programming of the hypothalamic-pituitary-adrenal stress axis as a target in epigenetics to pediatric health research. These studies should not be regarded as conclusive since they were mainly analyzed by Horvath clock, except for the recent publication by Nishitani *et al*^[70] using the PedBE clock. While findings generally support an association between childhood adversity and DNAm deviations, factors such as the lack of longitudinal data, low comparability across studies, and potential genetic and pre-environmental confounding currently limit the conclusions that can be drawn. The established associations between external factors and EAA only confirm the statistical significance, not endorsing the

Table 2: Epigenetic age acceleration and deceleration under external exposure.

Deviation	Conditions	Source of DNA	Studied population/cohort	Clock
Acceleration	Preterm ^[28]	Cord blood	Individual samples from neonatal care intensive unit	Knight
	Maternal low socioeconomic status ^[28]	Cord blood	CANDLE	Knight
	Maternal smoking ^[40]	Cord blood	ALSPAC	Horvath
	Maternal age >40 years ^[64]	Cord blood	PRDEO	Knight
	Preeclampsia and fetal demise in a previous pregnancy ^[64]	Cord blood	PRDEO	Knight
	Prenatal exposure to air pollution ^[65]	Cord blood	CHILD (n = 145)	Bohlin
	Exposure to lead in boys ^[32,66]	Peripheral blood	GES60598 (24 boys and 18 girls)	Wu
	Exposure to neighborhood violence ^[67]	Saliva samples	101 African American children (aged 6–13)	Horvath
	Threat-related early life adversity ^[68]	Saliva samples	262 children (aged 8–16)	Horvath
	Cumulative ACE exposure in girls ^[69]	Peripheral blood	ALSPAC	Horvath
Deceleration	Childhood maltreatment (ICD–10-CM Code T74) ^[70]	Buccal cell	25 cases with childhood maltreatment and 31 control	PedBE
	Insulin-treated GDM ^[64,71]	Cord blood	PRDEO	Knight
		Peripheral blood	Tianjin GDM Observational Study (pairs = 548)	Horvath
	Sjögren syndrome ^[64]	Cord blood	PRDEO	Knight
	Maternal antenatal depression ^[72]	Cord blood	PRDEO	Knight
	Decreased CRP in the third trimester of pregnancy ^[73]	Cord blood	32 monozygotic pregnancies from Spain	Knight
	Vitamin D3 supplementation in African American mothers ^[74]	Cord blood	Ninety-two pregnant women (21% African Americans, 28% Hispanics)	Knight and Bohlin

ACE: Adverse childhood experience; ALSPAC: Avon Longitudinal Study of Parents and Children; CANDLE: A socioeconomically diverse cohort; CHILD: Canadian Healthy Infant Longitudinal Development; CRP: Cerebroplacental ratio; GDM: Gestational diabetes mellitus; PedBE: Pediatric buccal epigenetic; PRDEO: Prediction and prevention of preeclampsia and intrauterine growth restriction.

biological association between ACE and EAA. A recent systematic review shed doubt on the association between childhood maltreatment and DNAm changes in the blood.^[76] The study analyzed found inconsistent associations of DNAm in blood with childhood maltreatment in several well-studied candidate genes (eg, *NR3C1* and *FKBP5*). This study compels us to replicate and revisit the clinical importance of EAA in pediatric populations.

Indicators for developmental diseases

Some work has been done in exploring the association between developmental diseases and EADs. While identifying genes linked to CpGs with DNAm trajectories, genes annotated to CpGs with overall decreasing DNAm levels were enriched in immune development, whereas those annotated to CpGs with increasing levels were enriched in neurodevelopmental functions.^[41] Several 1-year-old infants with a 1-day deviation in epigenetic GA were 8% more likely to be sensitized to allergens and thus develop an allergy.^[65] At mid-childhood, a 1-year increase in EAA is cross-sectionally associated with greater levels of total serum immunoglobulin E, as well as greater odds of asthma and atopic sensitization.^[77] AA in ASD, a typical neurodevelopmental spectrum disorder, has been studied by both Wu and PedBE clocks.^[32,33] Two independent cohorts (GSE27044 in Wu and GSE50795 in PedBE) were studied, respectively. Again, similar to the results of the association between EAD and weight, the correlation between deviation and autism conducted by two clocks was not the same. Individuals with ASD had increased PedBE age deviation compared with controls, consistent with their altered developmentally related phenotypes, such as increased body growth, head growth, and body weight, as well as accelerated postnatal cortical development.^[78] In Wu clock, however, no significance was found in the deviation between an individual with autism and their siblings. Therefore, it is still too early to conclude that AAs are predictors for developmental diseases in pediatrics. Only with more studies in different cohorts and clocks can we confirmed the connection between epigenetic clocks and risks of pediatric diseases.

Studies described above are mainly cross-sectional; however, longitudinal studies are also required. Because of the dynamics of pediatric methylated patterns, longitudinal following of children can look deeper into the long-term effects of external exposure on pediatric DNAm. Although the methylated states significantly change for environmental exposures and developmental diseases, these changes may not last for a long time and eventually resolve during development and growth. This phenomenon is partially due to the uneven aging pace in the pediatric population. Knowledge of longitudinal methylation dynamics helps us identify not only whom to interfere with but also when to take action.

Epigenetic clocks serve as mediators between physical and social factors and pediatric phenotypes,^[65,77] such as developmental trajectories and developmental diseases. In other words, epigenetic clocks are pivotal tools in connecting external factors and internal developmental programming, creating a novel study method in this area.

By testing additional pediatric datasets, as they become available, EAD will become important for evaluating the environmental and contextual factors shaping child development, chiefly through the DNA methylome, and how this in turn associates with health and disease.

Understanding the Mechanism Behind Pediatric Epigenetic Clocks

The use of machine learning methods to analyze large sets of methylated CpGs has generated various powerful epigenetic clocks. However, this data-driven approach has proposed challenges over understanding the underlying mechanism.^[25] When epigenetic early life clocks were first developed, the limited overlap between clocks for pediatrics and that for adults was interpreted as the difference in biological processes between development and aging. For example, only six CpG sites included in the DNAm GA predictor overlap with CpG sites in the predictor by Horvath, the chronological clocks for all ages.^[18,29] However, while comparing clocks trained on a similar age group with DNA derived from the same tissues (eg, Knight clock and Bohlin clock), the overlap between these two clocks is still limited.^[28] Though confusing at first glance, the desolation of overlap was due to the algorithm behind it. Machine learning selects only a relatively small number of CpGs to construct clocks, leaving numerous potential chronological-like CpGs behind, widely distributed across the genome. In the original publication by Bohlin *et al*,^[29] two entirely different epigenetic GA clocks were designed on the basis of the same training test. Therefore, it is inappropriate to take the minute overlap between aging clocks and pediatric clocks as evidence that the mechanism between these two sets of clocks is different, despite the fact that they do differ. Based on these considerations, we propose that the process of selecting specific CpGs for clocks is a combination of machine learning algorithms and biological programming.

Therefore, in the process of understanding this fresh idea, clock CpGs are usually analyzed in two ways: single by single or in clusters [Figure 2]. *In vivo* studies help to unravel the function of every single CpG and related gene. For instance, hypomethylation of insulin-like growth factor 2, one of the clock sites in Bohlin clock, was observed in preterm during the first year of life.^[79] Another way to understand the role of clock CpGs is to locate their positions in the genome. Clock CpGs (and/or other clock-like CpGs whose methylation changes in the pediatric population) were labeled by gene-associated regions, CpG island-associated regions, as well as enhancer elements. Clock-like CpGs located in gene bodies are more likely to get decreasing DNAm tended to have higher levels of DNAm; CpGs with increasing levels of DNAm are more often located in promoter regions and at enhancers to have low levels of DNAm.^[80,81]

Besides looking into the vital locus, cluster analyses can also be conducted to uncover the mechanism behind the ticking of epigenetic clocks. Analyses of gene ontology enrichment and Kyoto Encyclopedia of Genes and Genomes pathways of the clock or clock-like CpGs suggest that apparent links exist between epigenetic clocks

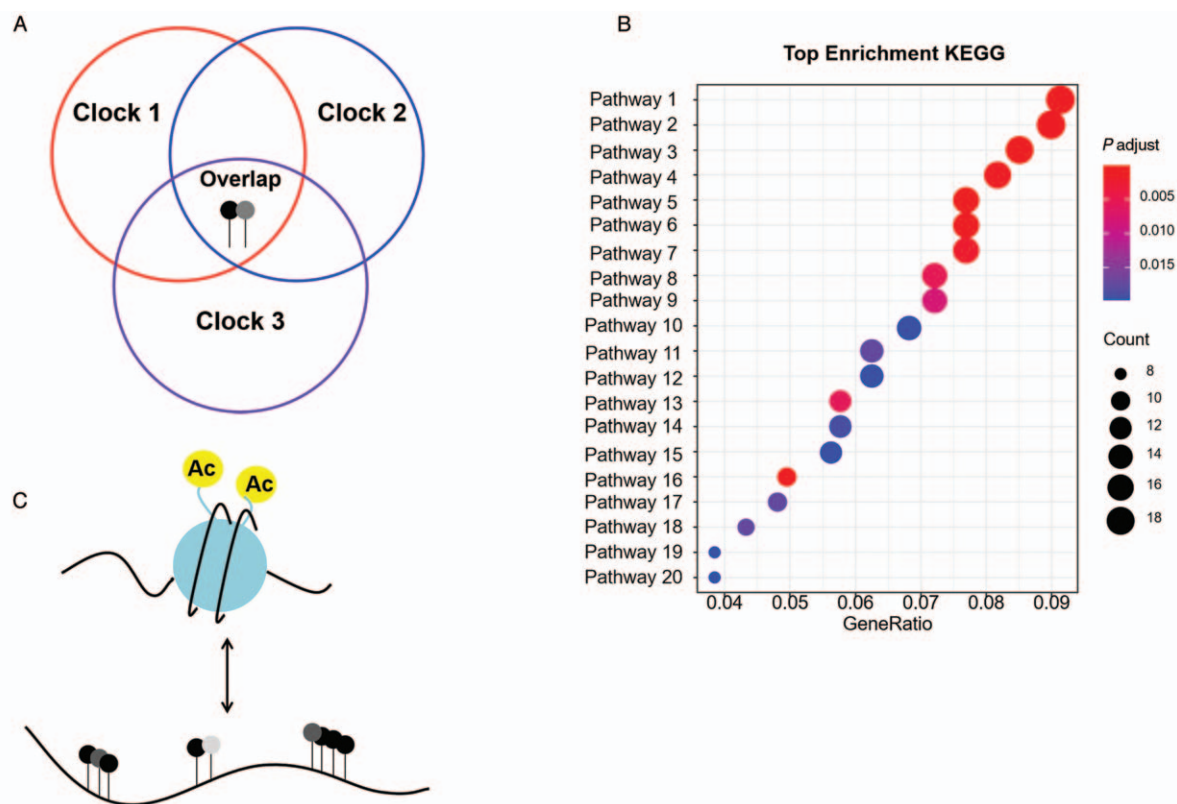


Figure 2: Approaches to understanding the mechanism underlying pediatric epigenetic clocks. (A) Focusing on the overlap of CpGs by different clocks and reveal the functions of their related gene. (B) In GO enrichment and KEGG pathways analysis, neurologic- and immune-related pathways are commonly enriched. (C) Other chromatin changes also affect the dynamics of epigenetic clocks. Ac: Acetylation; CpG: Cytosine-guanine dinucleotide; KEGG: Kyoto Encyclopedia of Genes and Genomes; GO: Gene ontology.

and cell and tissue development. In diverse clocks, genes linked to clock CpGs are mostly enriched in development, differentiation, cell aging, and cellular senescence. For instance, CpG sites associated with GAs were located in genes implicated in labor and delivery (eg, *AVP*, *OXT*, *CRHBP*, and *ESR1*) or that may influence the risk for adverse health outcomes later in life (eg, *DUOX2*, *TMEM176A*, and *CASP8*).^[37] The 353 methylation sites used to compute Horvath clock shows significant enrichment for immune cell trafficking, hematologic system development and function, organismal development, embryonic development, and tissue development, all of which are important life process in developmental trajectories. The obvious connections between the DNAm clock and development raise the possibility that the epigenetic clock reflects a programmed process.

The ticking of the epigenetic clocks appears to reflect a general progression of high- and low-methylated CpGs to an intermediate level near 50%.^[26] This suggests a smoothening with the development of the epigenetic landscape and a chronological clock driven by an increase in entropy. Entropy at one locus might be inconspicuous; the consequences for the functionality of the whole genome are substantial. Accordingly, both subtle changes at wide-range CpGs across the clock and intensive alterations at single key sites are responsible for the ticking of the epigenetic clock.

DNAm captured by epigenetic clocks may also be secondary to other chromatin modifications.^[82] Histone

modification affects DNAm. For example, DNAm is excluded from gene promoters by H3K4me3 (histone H3, lysine 4 trimethylation) but recruited to gene bodies and heterochromatin by H3K36me3 (histone H3, lysine 36 trimethylation) and H3K9me3 (histone H3, lysine 9 trimethylation), respectively.^[83] Conversely, DNA hypomethylation causes a redistribution of polycomb and H3K27me3 (histone H3, lysine 27 trimethylation).^[84] By this view, the DNAm clock can be depicted as an “epigenetic network clock,” and the epigenetic clock reviewed here may be secondary to other parts of the wider epigenetic networks,^[85] consisting of other epigenetic changes.

We are only at the primary stage of understanding the mechanism behind epigenetic clocks. To conclude, the ticking of pediatric epigenetic clocks is a blend of related mathematic algorithms and the biological process of development and growth. Our genomes have continued to undergo a programmed variation of methylation since the day we were born, serving as a memory device that could affect development and aging.^[86] Pediatric epigenetic clocks specifically reflect the entropic increase of the methylation landscape in the pediatric population linked to biochemical activity.

Concluding Remarks and Future Perspectives

Since the 1990s, researchers have documented age- and development-associated changes to DNAm. In the past

decade, identified collections of individual CpG sites whose aggregate methylation status provides an accurate measure of gestational and pediatric chronological age have been conducted through computational analysis. Ticking of this clock in pediatric, different from that in aging, most likely reflect the development-dependent entropic increase of the DNAm landscape. Although the epigenetic clock has initially been used as a molecular biomarker for chronological age and aging prediction, evidence suggests that DNAm may also be a valuable tool for a biomarker in the pediatric population. It can be used for evaluating the maturity of the preterm, assessing the impact of early exposure, and predicting developmental trajectories and diseases.

Future studies are recommended to be focused on the following areas. First, epigenetic clocks to date have been generated using methylation data from accessible cells and tissues. However, for those vital but inaccessible tissues, such as the brain and heart, the reliability of the pan-tissue clock or peripheral-tissue clock still requires testing. Only with a precise tissue-specific clock can we better understand the development and aging process in humans. Second, although animal epigenetic clocks have been established in various models, these clocks are mainly intended for aging models.^[51,87-89] To further uncover critical issues in the pediatric population, pediatric animal models are in need, especially in mice. Third, the molecular mechanisms underlying epigenetic clocks are still far from understood. Of special interest is the exact distinction between pediatric and aging clocks at the molecular level. Fourth, the consequences of epigenetic clocks are still uncertain. Controversy remains whether the pediatric epigenetic clock is a result of the pediatric phenotypes or a driving force of specific phenotypes. A deeper understanding of the molecular mechanism behind the clocks can help to solve these previous questions and find out the potential therapeutic targets for epigenetic modifications.

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Conflicts of interest

None.

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