Clinical Epigenetics

BRIEF REPORT

Open Access

Technical considerations for placental tissue processing and the subsequent impact on genome-wide DNA methylation analysis

Kristen M. Kocher[1,4], Julius Ngwa[1], Kushal J. Kapse[1], Surajit Bhattacharya[3], Christopher Rossi[2], Emmanuele Delot[3,4], Adre duPlessis[4,5], Catherine Limperopoulos[1,4] and Nickie Andescavage[1,4,6*]

Abstract

To assess the impact of postnatal processing on placental DNA methylation, array data from flash-frozen placental tissue was compared to perfluorocarbon-immersed and formalin-fixed paraffin-embedded placental tissue. We observed that tissue exposed to perfluorocarbon showed no significant DNA methylation differences when compared to unprocessed tissue, while formalin processing altered the quality and reliability of the data produced on the DNA methylation array platform. Placental DNA methylation allows for the study of gene–environment interactions that influence the fetal environment and development. Our study highlights that placental post-processing techniques must be considered in the evaluation and interpretation of epigenetic studies.

Keywords Placenta, DNA methylation, Epigenetic aging, FFPE

Introduction

The placenta is a complex organ that serves as a maternal–fetal interface, supporting fetal development and, on a molecular level, reveals critical insight into the intrauterine environment throughout gestation [1, 2]. The unique structural composition of the placenta allows for the close approximation of both maternal and fetal circulations, allowing for the exchange of nutrients, oxygen, and waste, which are integral for gestation [2]. Adverse intrauterine exposures, mediated by the placenta, are correlated with short- and long-term outcomes in offspring [3] allowing placental studies to provide a rare lens into maternal–fetal dyads and intergenerational environmental effects on offspring development [4].

A proposed key mechanism for the intergenerational impact of such fetal programming occurs through epigenetic modifications that happen early in development [5–7]. The epigenome is known to be influenced by disease, age, and both physical (e.g., fetal growth restriction and preterm birth) and psychosocial stressors (e.g., stress, anxiety, and trauma), providing insight into the molecular underpinnings of intrauterine stressors [5, 8–15]. Many recent epigenetic studies leverage placental DNA methylation and the concept of the "methylation clock," which identifies age-related CpG sites to determine molecular aging, to study accelerated placental age by revealing age-related biomarkers of disease or prognostic outcome [16, 17]. Collectively, these studies offer

*Correspondence:
Nickie Andescavage
[1] Developing Brain Institute, Center for Prenatal, Neonatal and Maternal Health Research, Children's Research Institute, Children's National Hospital, Washington, DC, USA
[2] Department of Pathology and Laboratory Medicine, Children's National Hospital, Washington, DC, USA
[3] Center for Genetic Medicine Research, Children's Research Institute, Children's National Hospital, Washington, DC, USA
[4] Department of Pediatrics, The George Washington University, Washington, DC, USA
[5] Zickler Family Prenatal Pediatrics Institute, Children's National Hospital, Washington, DC, USA
[6] Division of Neonatology, Children's National Hospital, Washington, DC, USA

novel insights into adverse intrauterine exposures, the response of the placenta to these stimuli, the impact on fetal programming during development, and how these may impact or predict later in life, pediatric outcomes.

Few studies to date have focused on the potential impact of placental processing techniques on the underlying epigenome to ensure reputable data generation and analysis among the larger scientific community. Largely, these studies focus on the viability of DNA extracted from formalin fixation, paraffin-embedded (FFPE) tissue on the methylation array platform and, in some instances, comparison of output data with that of fresh and flash-frozen specimens [18–21]. These studies suggest that FFPE tissue may be leverageable on this platform but with distinct limitations that require specific downstream technical considerations. Placental tissue, however, may be exposed to perfluorocarbon media, in addition to FFPE, prior to biobanking, yet this exposure has not been evaluated relative to subsequent epigenetic studies. Therefore, the scope of this work will elucidate the potential effects of the aforementioned processing techniques on placental DNA methylation through direct comparison of matched placental tissue that has been immediately flash frozen, exposed to perfluorocarbon immersion liquid prior to ex vivo MRI, and FFPE processing.

Methods

Study participants

The biospecimens used in this study were obtained as part of a prospective, observational study to assess the impact of prenatal maternal COVID-19 infection and associated maternal distress on offspring neurodevelopment. For the present analysis, six term placentas from COVID-negative pregnancies were collected for ex vivo magnetic resonance imaging and biobanking. This study protocol (Pro00014257) was registered to and approved by the Children's National Institutional Review Board, and all participants provided written, informed consent. Maternal, infant, and placental metrics are found in Table 1 and Supplementary file 2: Table 1.

Specimen collection & processing

Placentas were couriered immediately after delivery on ice from local hospitals and remained at 4 °C until biopsied and flash frozen, underwent MRI scan, followed by histopathological processing. All placentas were scanned, biopsied, and histologically processed within 1–6 h (no more than 24 h) of delivery. Full-thickness placental biopsies were collected at three distinct steps during tissue post-processing: flash frozen, full-thickness placental biopsy after delivery (F1), after exposure to in

Table 1 Maternal–infant demographic and clinical information associated with placenta specimens

Maternal information	**Mean**	**SD**
Maternal age at delivery, in years	34.2	4.5
	n	**%**
Self-reported race		
Asian	1	16.7
Black	1	16.7
White	4	66.7
Self-reported COVID-19 infection during pregnancy, negative	6	100
Smoking, no	6	100
Infant information	**Mean**	**SD**
Gestational age at delivery, in weeks	39.4	1.4
Weight at delivery, in g	3274.2	573.3
Length at delivery, in cm	51.1	2.1
Head circumference, in cm	33.8	1.3
	n	**%**
Sex, female	4	67
Placental pathology	**Mean**	**SD**
Weight, in g	434.0	78.1
	n	**%**
Maternal–placental diagnosis, none	3	50

proton-free perfluorocarbon immersion liquid (Fluorinert FC-40) (F2), and part of standard histopathological, FFPE processing (F3). A portion of F3 DNA was additionally "restored" using the Illumina Infinium HD FFPE Restore Protocol (F4) prior to downstream analysis. Please refer to Supplementary Methods (file 3)and Supplementary Figures S1A-B & 2 (file 1) for detailed methodology.

Bioinformatic analysis of DNA methylation data

Raw intensity values (idat) from the Illumina Infinium EPIC BeadChip array (version 1) were imported into R (version 4.3.1) for normalization, quality assessment, statistical analysis, and placental epigenetic age estimation (Lee clock) [16]. Pipeline and package details regarding statistical tests, methylation aging calculations, and relevant references can be found in Supplementary file 3: Methods.

Results & discussion

Description of sample characteristics

Six placental samples were couriered to Children's National Hospital from regional birthing hospitals for immediate MRI visualization, biobanking, and histopathology. The average maternal age at time of delivery was 34.2 years (± 4.5 years). Selected placentas were from pregnancies without reported smoking, alcohol, or drug use; additional maternal–neonatal characteristics are found in Table 1. Pregnancy and placental pathologies were unremarkable in 3 of the pregnancies; relevant placental pathologies for the other three placentas include intrauterine growth restriction, early term pregnancy, uterine fibroids, Group B streptococcal infection, and subchorionic bleed during delivery (specific details can be found in Supplementary file 2: Table 1).

FFPE processing is associated with greater loss of probes during informatic correction and decreased correlation, when compared to flash-frozen tissue

Overall, correlation analysis between processing methods suggests that there is a significant effect from FFPE processing on sample quality, as neither FFPE (F3, F4) group is correlated significantly with flash-frozen (F1) or perfluorocarbon exposed (F2) samples (Fig. 1A). We do, however, note significant correlation between F1 and F2 ($p < 0.0001$, $r = 0.99$), as well as F3 and F4 ($p = 0.014$, $r = 0.90$) (Fig. 1A). Visually, matched flash-frozen samples (F1, F2) and FFPE samples (F3, F4) largely cluster by processing method on principal component analysis (PCA), with the exception of 1 sample ID (ID 50, Fig. 1B). We hypothesize that a contributing factor to this variation may be due to the courier time of this specific placenta (24 h), compared to the other samples (1–6 h), potentially impacting the tissue and resultant DNA integrity. Additionally, we noted a distinct percentage difference in the cell-type composition of this particular sample, which may contribute to its unique behavior in our analyses (Supplementary file 1: Figure s3).

Regarding overall cell-type composition analysis, we investigated functionally normalized data using the PlaNET package with the cibersort (CBS) method, producing an output of placental cell (nRBC, Endothelial, Hofbauer, Stromal, Trophoblast, and Syncytiotrophoblast) estimates per sample (Supplementary file 2: Table 2) [22]. Because we extracted full-section biopsies from term placentas (Supplementary file 1: Figure s2), as anticipated, we note the largest population for each sample to be syncytiotrophoblasts. One-way repeated measures ANOVA of the estimates between processing methods revealed no significant differences in the major cell population, syncytiotrophoblast, which make up an average of 67% of the total cell-type composition among all samples. Further, among all assayed cell types, only stromal cells, which make up an average of 11% of the total cell-type composition among all samples, exhibited significant differences (p-value = 0.042) between processing methods. While the present samples appeared to have nearly uniform cell-type composition among all processing methods ensuring comparable results for the ensuring processing method comparisons, future studies may benefit from employing cell-type deconvolution to these types of data to further correct and account for widespread sample–sample variability.

To evaluate the association between principal component 1 (PC1, Fig. 1B) and processing method types, we performed a linear mixed effects model with random intercept specification and observed an r-squared of 0.885 and p-value of 4.0e-10, suggesting a strong, significant association between PC1 and processing type on the axis that accounts for the most variation among the samples. Further, we performed a correlation analysis using the values of PC1 and correlation coefficients, generated by Pearson's correlation, for each sample type and determine significant associations on PC1 between F3 and F4, but no other groups (Supplementary file 2: Table 3). Finally, using unsupervised hierarchical clustering and a correlation heatmap approach to visually assess the similarity of samples, we noted that, once again, with the exception of one sample (F1-ID50), all samples clustered together by either FFPE (F3, F4) or flash-frozen processing (F1, F2) (Fig. 1C). Notably, F3 and F4 samples appeared to subcluster by ID, while F1 and F2 samples did not. Together, these findings emphasize that F3 and F4, both groups that undergo FFPE processing, have been significantly impacted by processing methodology and that the restoration

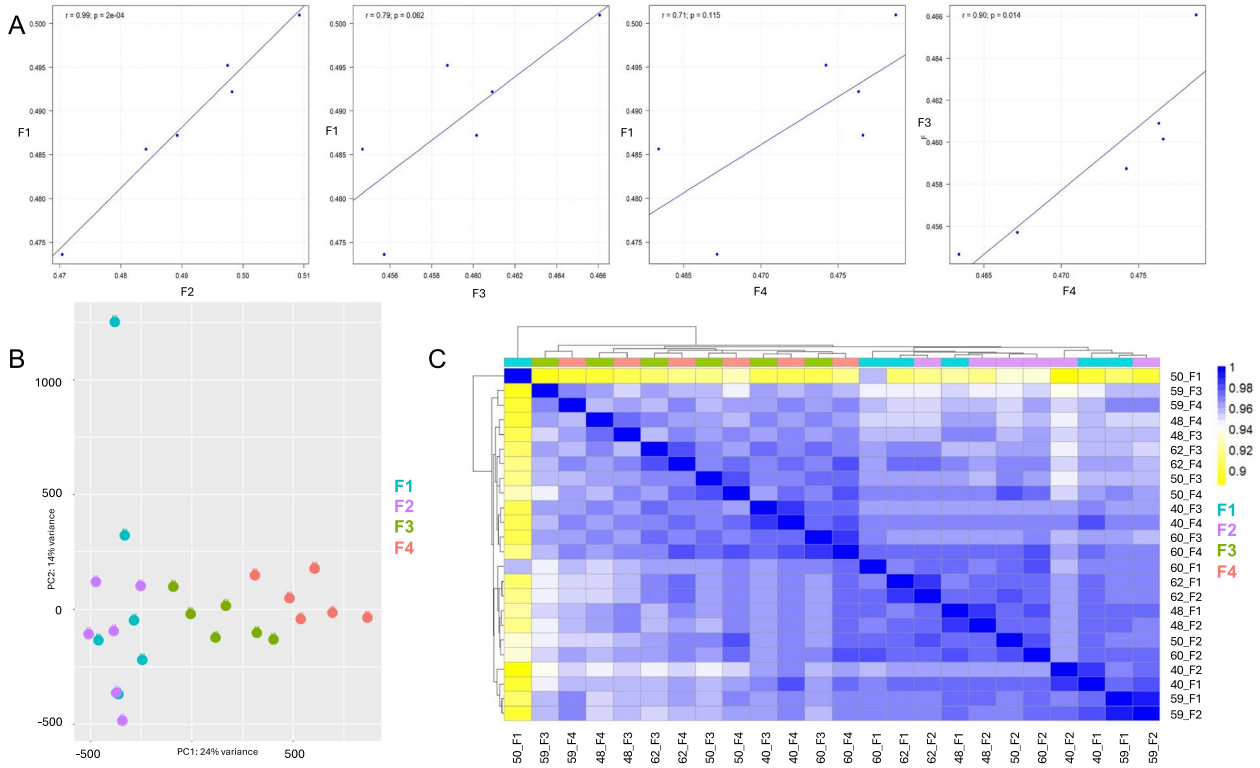

Fig. 1 The impact of post-processing on the placental epigenome. (**A**) Correlation between placental processing methods and normalized and filtered beta value intensities for all processing groups: fresh, flash-frozen tissue (F1), Fluorinert FC-40 exposed tissue (F2), FFPE processed tissue (F3), and FFPE processed and restored tissue (F4). All normalized and filtered beta values were averaged per sample in each processing group (F1-F4) and correlated by processing method. (**B**) Principal component analysis of variance and (**C**) correlation heatmap of beta values from each sample using unsupervised hierarchical clustering to reveal significant differences cluster by processing type

protocol does not appear to significantly improve FFPE DNA quality. Notably, flash-frozen (F1) and perfluorocarbon exposed (F2) samples are significantly correlated with each other and not FFPE processed sample types (F3, F4).

To assess the impact of each tissue processing technique on resultant data, we utilized standard filtering and normalization criteria that are generally used with methylation array data quality control during computational processing. The total number of probes detected from an EPIC array is 866,238. The samples that were fresh and flash frozen, either unexposed (F1) or exposed (F2) to Fluorinert FC-40, had the least number of total probes removed during filtering and normalization (F1: 76,919 and F2: 76,721) and had a final probe count of 789,319 and 789,517, respectively (Supplementary file 2: Table 4). This was followed by the FFPE processed samples (F3), with a loss of 90,908 probes, and a total final probe count of 775,330. Finally, the FFPE restored (F4) samples had the greatest number of filtered probes, 92,716, with a total final probe count of 773,522, the lowest total probes of all four sample types (Supplementary file 2: Table 4).

Visually, normalization of beta value intensities from all samples appeared to improve the overall beta value distributions; however, F3 and F4 do appear to have slightly weaker distributions, suggesting impaired quality of DNA and hybridization of probes and thus impacting output intensities (Supplementary file 1: Figure s4A-D) [23]. To evaluate the overall differences in intensity values generated by processing group, we computed the total fluorescence intensity per sample (methylated and unmethylated channels) of non-normalized probes and performed a paired t-test to identify significant differences between the processing methods. We observed that there was no statistical difference between FFPE (F3) and FFPE restored (F4) samples but did note significant differences between F3 and flash-frozen samples (F1), F3 and perfluorocarbon exposed samples (F2), and even between F1 and F2 (Supplementary file 2: Table 5).

Of interest, the removal of all probes with detection p-value of ≥ 0.05 was notably where the greatest loss occurred between the flash-frozen (F1, F2) and archival (F3, F4) specimens (Supplementary file 2: Table 4). This quality control step, used in our previously published

informatic pipeline [11, 24], is performed using *minfi* and ensures only beta values that have passed this statistical threshold to proceed to downstream analysis [25, 26]. Among our data, we compared the different proportions of probes with detection p-value ≥ 0.05 between each group. Using the raw data, a total of 866,238 probes were assessed for all four groups. We compared the different proportions of probes with a detection p-value ≥ 0.05 among the different groups. Using a 2-proportion z-test to compare the probes with detection in F1 (0.26%) and F2 (0.25%), there was no significant difference between the two groups ($p = 0.083$). However, when assessing F1 and F3 (2.13%), there was a significant difference ($p < 0.0001$), which was also noted when comparing F1 and F4 (2.23%) ($p < 0.0001$). Finally, when comparing F3 (2.13%) and F4 (2.23%), we did observe a significant difference ($p < 0.0001$), which taken together, suggests that F1 and F2 processing methods (flash freezing and perfluorocarbon immersion) do not significantly alter probe inputs into the bioinformatic pipeline, while F3 and F4 (FFPE and FFPE restored) processing methods do. FFPE processing, even with the restoration protocol, may diminish the quality of intensity signal generated by the EPIC array probes and reduce the number of leverageable probes for downstream analysis that cannot be rescued by downstream bioinformatic correction. Additionally, the lack of differences between F1 and F2 here may assuage concerns that perfluorocarbon exposure during ex vivo imaging has an impact on probe intensity quality, especially once data have been normalized and passed the probe detection p-value ≥ 0.05 step.

Among all four groups (F1, F2, F3, and F4), there was an intersection total of 760,439 CpG sites for the six participants across all groups. Using the beta values of the CpG sites, we performed the Mann–Whitney Wilcoxon paired test on each row of the CpG sites matrix for the four different methods to identify the proportion of CpG sites that showed significant differences when comparing F1 and the remaining three groups, including F3 vs. F4 comparisons within each processing group. With a threshold set at $p < 0.05$ to identify differences in the number of CpG sites among processing methods, a total of 46,125 CpG sites (6.07%) showed significant differences between F1 and F2. Interestingly, a total of 270,929 CpG sites (35.63%) showed significant differences between F1 and F3, and a total of 244,177 CpG sites (32.11%) showed significant differences between F1 and F4. When comparing F3 and F4, a total of 206,503 CpG sites (27.16%) showed significant differences. Together, this further emphasizes the impact of FFPE processing on output DNA methylation data on an individual probe level between processing types.

Gestational age is accurately estimated in flash-frozen and archival samples using the Lee methylation clock

While the present data vary greatly in probe intensity quality between fresh-frozen and archival placental samples for downstream applications like epigenome-wide association studies, we further tested the efficacy in output data from differentially processed tissue in predicting epigenetic age. All samples, regardless of processing technique, achieved the *methylclock* default threshold of < 20% missing CpG sites, with only a maximum of 0.8% missing sites per sample (Supplementary file 2: Table 6) [27]. We tested all three Lee clock subsets and found that regardless of processing method or clock used, the GA for each sample was accurately predicted and not significantly different than chronological age in any instance of processing type using a two-tailed paired t-test (Supplementary file 2: Table 6). There was a trend to underestimate predicted age among F3/F4 when compared to F1/F2 specimens, although this did not reach statistical significance (Supplementary file 2: Table 6). When comparing just the estimated GAs using Lee.refRPC for each processing method, we noted no significant differences between the F1 and F2, F3 and F4, or, interestingly, F2 and F4 (Supplementary file 2: Table 6). There were, however, significant differences between F1 and F3, F1 and F4, and F2 and F3 (Supplementary file 2: Table 7). This may suggest that while chronological GA is accurately predicting by the tool, the estimated outputs between processing methods do show significant differences between FFPE processed (F3, F4) and flash frozen (F1) or perfluorocarbon exposed (F2), which is notable and worth reporting as a potential limitation when using this tool. It is, therefore, possible that F1 and F2 processed samples provide more accurate age estimation and are more similar to each other then perhaps FFPE processed samples (F3 and F4). This may be attributed to reduced quality of the DNA extracted from FFPE tissue and diminished beta value intensities, which we have explored in this study, but further evaluation and a greater sample size are needed.

Conclusion

Together, these findings indicate that FFPE processing has a significant impact on DNA methylation array data, a factor that should be considered when choosing to analyze this type of tissue, especially if the intended use is as a proxy for matched fresh, flash-frozen specimens. Analyses with the Illumina FFPE Restore protocol suggest that this restoration step on resultant output data and probe quality relative to fresh-frozen tissue is non-significant and even impaired, and thus, future studies may want to

consider this finding when utilizing this additional step [21]. The quality of data that is used in the analysis and interpretation of FFPE specimens should be cautioned as a distinct limitation. In contrast, our findings suggest that biospecimen exposure to perfluorocarbon stabilization media and time spent performing ex vivo imaging does not significantly differ from matched fresh, unexposed tissue. This finding allows for ease and flexibility of sample selection when considering specimens that may undergo ex vivo MRI prior to biobanking and downstream DNA methylation array analysis.

We also noted all samples, regardless of processing technique, accurately predict gestational age using the Lee placental methylation clocks, allowing increased utility of compromised samples in the realm of epigenetic research. In the context of epigenetic research, compromised tissue specimens may not be suitable for large-scale genomic analyses due to impaired output intensities; however, epigenetic aging analyses were similar regardless of processing method.

In the scope of this work, the following limitations should be noted to give increased context and clarity to future research. Our sample size is notably small ($n=6$); however, we believe that use of multiple, matched specimens from the same placenta, exposed to a variety of processing techniques provides increased granularity into the impact of these post-processing methods on placental DNA methylation. This sample size provides an accurate depiction of potential impacts of perfluorocarbon and FFPE processing on the placental epigenome compared to fresh, flash-frozen tissue from the same placenta; however, expanding the sample size may allow for improved assessment of the normalization and filtering of beta value intensities and downstream analysis. Additionally, individual placental biopsies were collected for each processing technique, thus introducing the possibility that specimens were extracted from slightly different locations and reflective of different pathologies. Based on cell-type composition analysis, variability in percentage distributions of cell-types may be attributed to human error in collection, and thus, it is worth noting as a potential confounder. Lastly, while precautions were in place to reduce DNA and tissue degradation, such as ensuring timely placental processing, it is possible that slight variations in time between each step could have impacted overall DNA quality and thus data output, potentially confounding the impact of processing method on this analysis. One sample did experience a 24hour time interval between delivery and processing, and as a result, while preserved at 4 °C, we observed that this sample (ID 50_F1) has a smaller percentage of syncytiotrophoblasts and trophoblasts relative to other samples, and a greater composition of nucleated red blood cells (nRBCs) (Supplementary file 2: Table 1, Fig. 1B-C, Supplementary file 1: Figure s3). Due to the extended courier time of this placenta, it is possible that cell degradation via hypoxia is responsible for the diminished syncytiotrophoblast/trophoblast population and elevated nRBC population, which are commonly observed in hypoxic conditions [28]. Further, a recent study by Haftorn et al. suggests that the presence of nRBCs impacts DNA methylation analyses, specifically in the context of assessing placental GA [29]. Together, this provides compelling evidence that both courier time and subsequent cell-type composition analysis are necessary to consider in epigenomic analysis placental tissue.

This work provides a foundational, proof-of-concept model for studies aiming to assess the human placental epigenome, especially in the context of leveraging archival or post-processed biospecimens. Epigenetic age acceleration is a rapidly emerging field, and this study highlights the ability of both fresh and archival placental samples to estimate epigenetic age that closely reflects chronologic age using a "methylation clock" approach. These results will allow for greater confidence in the future work focusing the use of placental epigenetic aging to determine or predict neonatal and long-term pediatric outcomes.

Abbreviations

FFPE	Formalin-fixed, paraffin-embedded
MRI	Magnetic resonance imaging
GA	Gestational age
RPC	Robust placental clock
CPC	Control placental clock
refRPC	Refined robust placental clock

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-024-01741-8.

Supplementary Material 1. Figure S1. Graphical overview of placental tissue post-processing and subsequent epigenomic analysis. Figure was generated using BioRender.com. Supplemental Figure 2. Figure S2. Graphical representation of biopsy sites used for downstream DNA methylation array analysis. Approximation of biopsy sites for each sample are indicated by dashed yellow circles. Cord insertion site is denoted as a landmark with reference to the biopsy sites. Image was created using BioRender.com. Figure S3. Placental biopsy cell-type estimation using PlaNET package and cibersort estimation. The percentage of each cell population per sample is indicated as follows: syncytiotrophoblasts, trophoblasts, stromal cells, Hofbauer cells, endothelial cells, and nucleated red blood cells. Samples are labeled by ID and processing method. Figure S4. Supplemental Figure 3. Raw beta value intensities generated from Infinium MethylationEPIC platform for each placental post-processing method: flash-frozen, Fluorinert FC-40 exposed, FFPE processed, FFPE "restored." Benchmarked values and informatic steps used for QC, normalization, and filtering can be found in Supplemental Table 3.

Supplementary Material 2. Table S1. Detailed breakdown of maternal–infant demographic and clinical information associated with placenta specimens by case. Table S2. Placental cell-type estimates per sample and placental processing method generated using PlaNET package and cibersort estimation. Table S3. Correlation Matrix of PC1 and Processing

Methods. Table S4. Total probes removed at each filtering step by tissue processing type. Table S5. Association of total fluorescence intensity and processing methods. Table S6. Epigenetic age estimation by Lee methylation clock is not significantly different from chronological gestational age. Table S7. Epigenetic age estimation by LeeRPC varies based on processing method.

Supplementary Material 3. Supplemental Methods.

Acknowledgements
First and foremost, the authors would like to sincerely thank the participating patients and their families for their time and contribution to this work. We would like to additionally acknowledge Dr. Eric Vilain and the Children's Research Institute Genomics Core for the invaluable support and training that helped to ensure this work could be performed.

Author contributions
KMK assisted in funding acquisition and study design, ran all experiments, analyzed the data generated, and was a major contributor writing of manuscript. KJK acquired and banked placental tissue and obtained magnetic resonance imaging for ex vivo scans. JN and SB performed statistical testing and assisted with data analysis. CR performed the histological examination of placenta after imaging. AP was involved in study design and data interpretation. ED, CL, and NA were all involved in funding acquisition, study design, and data interpretation. NA was also a major contributor of writing the manuscript. All authors have read, revised, and approved the final manuscript.

Funding
This project was supported by NIH National Center for Advancing Translational Sciences through the Clinical and Translational Science Institute at Children's National (UL1TR001876), the NIH Eunice Kennedy Shriver National Institute of Child Health and Human Development (T32HD098066), and the A. James & Alice B. Clark Foundation.

Availability of data and materials
The datasets used in the present study are available on Gene Expression Omnibus (GEO) under the accession number GSE269799.

Declarations

Ethics approval and consent to participate
This study was approved by Children's National Hospital Institutional Review Board (approval: Pro00014257). Informed written consent was acquired prior to study enrollment by all study participants or their legal guardian.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 26 March 2024 Accepted: 7 September 2024
Published online: 16 September 2024

References

1. Nelissen ECM, van Montfoort APA, Dumoulin JCM, Evers JLH. Epigenetics and the placenta. Hum Reprod Update. 2011;17(3).
2. Baergen RN, Burton GJ, Kaplan CG. Benirschke's Pathology of the Human Placenta: Seventh Edition. Benirschke's Pathology of the Human Placenta: Seventh Edition. 2021.
3. Rosenfeld CS. The placenta-brain-axis. Vol. 99, Journal of Neuroscience Research. 2021.
4. Gonçalves BM, Graceli JB, da Rocha PB, Tilli HP, Vieira EM, de Sibio MT, et al. Placental model as an important tool to study maternal-fetal interface. Reproductive Toxicology. 2022;112.
5. Harary D, Akinyemi A, Charron MJ, Fuloria M. Fetal growth and intrauterine epigenetic programming of obesity and cardiometabolic disease. Vol. 23, NeoReviews. 2022.
6. Zhu Z, Cao F, Li X. Epigenetic programming and fetal metabolic programming. Vol. 10, Frontiers in Endocrinology. 2019.
7. Buss C, Entringer S, Wadhwa PD. Fetal programming of brain development: Intrauterine stress and susceptibility to psychopathology. In: Science Signaling. 2012.
8. Moore LD, Le T, Fan G. DNA methylation and its basic function. Vol. 38, Neuropsychopharmacology. 2013.
9. Gomaa N, Konwar C, Gladish N, Au-Young SH, Guo T, Sheng M, et al. Association of pediatric buccal epigenetic age acceleration with adverse neonatal brain growth and neurodevelopmental outcomes among children born very preterm with a neonatal infection. JAMA Netw Open. 2022;5(11).
10. Provenzi L, Mambretti F, Villa M, Grumi S, Citterio A, Bertazzoli E, et al. Hidden pandemic: COVID-19-related stress, SLC6A4 methylation, and infants' temperament at 3 months. Sci Rep. 2021;11(1).
11. Kocher K, Bhattacharya S, Niforatos-Andescavage N, Almalvez M, Henderson D, Vilain E, et al. Genome-wide neonatal epigenetic changes associated with maternal exposure to the COVID-19 pandemic. BMC Med Genomics. 2023;16(1).
12. Cardenas A, Faleschini S, Cortes Hidalgo A, Rifas-Shiman SL, Baccarelli AA, Demeo DL, et al. Prenatal maternal antidepressants, anxiety, and depression and offspring DNA methylation: Epigenome-wide associations at birth and persistence into early childhood. Clin Epigenetics. 2019;11(1).
13. Oberlander TF, Weinberg J, Papsdorf M, Grunau R, Misri S, Devlin AM. Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. Epigenetics. 2008;3(2).
14. Snijders C, Maihofer AX, Ratanatharathorn A, Baker DG, Boks MP, Geuze E, et al. Longitudinal epigenome-wide association studies of three male military cohorts reveal multiple CpG sites associated with post-traumatic stress disorder. Clin Epigenetics. 2020;12(1).
15. McGill MG, Pokhvisneva I, Clappison AS, McEwen LM, Beijers R, Tollenaar MS, et al. Maternal prenatal anxiety and the fetal origins of epigenetic aging. Biol Psychiatry. 2022;91(3).
16. Lee Y, Choufani S, Weksberg R, Wilson SL, Yuan V, Burt A, et al. Placental epigenetic clocks: estimating gestational age using placental DNA methylation levels. Aging. 2019;11(12).
17. Mayne BT, Leemaqz SY, Smith AK, Breen J, Roberts CT, Bianco-Miotto T. Accelerated placental aging in early onset preeclampsia pregnancies identified by DNA methylation. Epigenomics. 2017;9(3).
18. Moran S, Vizoso M, Martinez-Cardús A, Gomez A, Matías-Guiu X, Chiavenna SM, et al. Validation of DNA methylation profiling in formalin-fixed paraffin-embedded samples using the infinium human Methylation450 Microarray. Epigenetics. 2014;9(6).
19. Oliveira DVNP, Hentze J, O'Rourke CJ, Andersen JB, Høgdall C, Høgdall E V. DNA methylation in ovarian tumors—a comparison between fresh tissue and FFPE samples. Reproductive Sciences. 2021;28(11).
20. Dupont ME, Jacobsen SB, Christiansen SNN, Tfelt-Hansen J, Smerup MH, Andersen JD, et al. Fresh and frozen cardiac tissue are comparable in DNA methylation array β-values, but formalin-fixed, paraffin-embedded tissue may overestimate DNA methylation levels. Sci Rep. 2023;13(1):16381.
21. Dupont ME, Christiansen SN, Jacobsen SB, Kampmann ML, Olsen KB, Tfelt-Hansen J, et al. DNA quality evaluation of formalin-fixed paraffin-embedded heart tissue for DNA methylation array analysis. Sci Rep. 2023;13(1).
22. Yuan V, Price EM, Del Gobbo G, Mostafavi S, Cox B, Binder AM, et al. Accurate ethnicity prediction from placental DNA methylation data. Epigenetics Chromatin. 2019;12(1).
23. Maksimovic J, Gordon L, Oshlack A. SWAN: Subset-quantile within array normalization for illumina infinium HumanMethylation450 BeadChips. Genome Biol. 2012;13(6).
24. Kocher K, Bhattacharya S, Bramble MS, Okitundu-Luwa D, Ngoyi DM, Tshala-Katumbay D, et al. DNA methylation patterns associated with konzo in Sub-Saharan Africa. Clin Epigenetics. 2022;14(1).
25. Wilhelm-Benartzi CS, Koestler DC, Karagas MR, Flanagan JM, Christensen BC, Kelsey KT, et al. Review of processing and analysis methods for DNA methylation array data. Vol. 109, Br J Cancer. 2013.

26. Mansell G, Gorrie-Stone TJ, Bao Y, Kumari M, Schalkwyk LS, Mill J, et al. Guidance for DNA methylation studies: statistical insights from the Illumina EPIC array. BMC Genomics. 2019;20(1).
27. Pelegi-Siso D, De Prado P, Ronkainen J, Bustamante M, Gonzalez JR. Methylclock: A Bioconductor package to estimate DNA methylation age methylclock: A Bioconductor package to estimate DNA methylation age. Bioinformatics. 2021;37(12).
28. Cohen MC, Peres LC, Al-Adnani M, Zapata-Vázquez R. Increased number of fetal nucleated red blood cells in the placentas of term or near-term stillborn and neonates correlates with the presence of diffuse intradural hemorrhage in the perinatal period. Pediatric and Dev Pathol. 2014;17(1).
29. Haftorn KL, Denault WRP, Lee Y, Page CM, Romanowska J, Lyle R, et al. Nucleated red blood cells explain most of the association between DNA methylation and gestational age. Commun Biol. 2023;6(1).

Publisher's Note