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Improved reporting of DNA methylation data derived from studies of the human placenta

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Epigenetic variation is increasingly hypothesized as a mechanism underlying the effect of the in utero environment on long-term postnatal health; however, there is currently little clear data to support this in humans. A number of biological and technical factors provide challenges for the design of clinical epigenetic studies: from the type of cells or tissues that are available to the large range of predicted confounders that may impact findings. The human placenta, in addition to other neonatal tissues and whole blood, is commonly sampled for the study of epigenetic modifications. However there is little conformity for the most appropriate methods for study design, data analysis, and importantly, data interpretation. Here we present general recommendations for the reporting of DNA methylation in biological samples, with specific focus on the placenta. We outline key guidelines for: (1) placental sampling, (2) data analysis and presentation, and (3) interpretation of DNA methylation data. We emphasize the need to consider methodological noise, increase statistical power and to ensure appropriate adjustment for biological covariates. Finally, we highlight that epigenetic changes may be non-pathological and not necessarily translate into disease-associated changes. Improved reporting of DNA methylation data will be critical to identify epigenetic-based effects and to better understand the full phenotypic impact of these widely-reported epigenomic changes.

Keywords: DNA methylation, epigenetics, placenta, DOHAD, analysis, guidelines

Abbreviations: DOHAD, developmental origins of health and disease; EWAS, epigenome-wide association studies

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The study of the developmental origins of health and disease has recently intensified due to the advent of high throughput technologies to precisely measure epigenetic marks with increasing genomic coverage.[1,2] The number of publications that measure DNA methylation in this context has continued to increase year on year, generating excitement in the field of epigenetic epidemiology and providing promising insights into the mechanistic basis of some disease phenotypes.[3,4] However, inconsistent study design, analyses and interpretation have resulted in the presentation of conclusions that may not be well-supported by the data.

Several investigators have indicated the critical need for uniform reporting of epigenomic studies. Irizarry and colleagues made insightful recommendations for design, analysis, and validation of epigenome-wide association studies (EWAS) with focus on high throughput array and sequencing-based technologies.[5] Importantly, they highlighted the limitations of using blood samples as a surrogate for inaccessible but disease-relevant tissue and advised cautionary interpretation of the biological relevance of data obtained in such studies. Likewise, Heijmans and Mill recently emphasized the need for a framework to guide researchers in their EWAS and point out biological, technical, and methodological issues facing epidemiological epigeneticists.[6] They also highlight that unlike the genome, which is static, the epigenome is "malleable" and researchers should keep in mind that it can be influenced by stochastic events, unrelated to pathology.[7]

In this commentary, we wish to add to these EWAS recommendations but with focus on the use of placental samples in epigenetic studies. The placenta is an easily accessible tissue and is commonly sampled for studies of DNA methylation in addition to whole blood and cord blood samples.[8,9] The placenta is a multifunctional organ that mediates the exchange of nutrients and waste between the mother and fetus and produces hormones important for pregnancy maintenance. It can also act as a barrier to inactivate or block factors, including maternally-derived hormones or exogenous toxic chemicals that enter the maternal environment, from reaching the fetus. For example, fetal glucocorticoid exposure is regulated by the placental expression of 11β-hydroxysteroid dehydrogenase, which is highly expressed in the placenta and inactivates cortisol.[10] Therefore, it is important during study design to consider how different exposures affect the placenta, and in turn the developmental trajectory of the fetus.[11,12]

Important Biological Confounders

In an effort to improve reporting standards in studies of the human placenta, Nelson and Burton previously outlined the importance of uniform and comprehensive reporting of placental sampling methods and patient characteristics.[13] Their report made recommendations for the consistent provision of standardized information, where available, on potential study confounders, including patient ethnicity, prenatal medication use and type of delivery.[13] To complement these guidelines, we propose several methodological recommendations in the context of DNA methylation studies:

Tissue sampling

Use a consistent sampling location

The placenta is a complex organ composed of a collection of 60–70 villous trees that grow outwards from the chorionic plate (fetal surface of the placenta) into the basal plate (maternal decidua).[14] Sampling chorionic villi from the fetal side of the placenta (below the chorionic plate) is often preferred to minimize any risk of maternal decidual contamination. Nonetheless, due to variation in perfusion a subset of genes may be differentially expressed depending on the location (e.g., distance to the umbilical cord and to basal or chorionic plate) of the sampled region.[15-17] If the study question necessitates sampling from the maternal side (basal plate), these samples should be assessed for contamination from maternally-derived decidual cells. Areas of localized pathology, such as infarcts or chorioangiomas, should be avoided unless part of the study question. For example, in cases of placental mesenchymal dysplasia, cystic areas with abnormal vasculature exhibit abnormal DNA methylation (due to high levels of androgenetic cells), while other regions will appear normal.[18]

Sample more than one site across the placental surface

While some variation in gene expression across the placenta may be systematic, localized damage, or variation in villous maturation can cause stochastic variation in gene expression from site-to-site.[15,17] Stochastic variability has also been observed for DNA methylation and tends to exceed the potential variation due to anatomical location.[19,20] This spatial variability in placental DNA methylation may arise due to the clonal tree structure, such that programming events in one villus progenitor may be propagated through that tree independently of events in another.[20] Therefore DNA extracted from chorionic villi sampled at multiple sites (i.e., each from a different cotyledon) can be pooled to reduce sampling variability and provide a more representative biological measure from each individual placenta.[21]

Consider focusing on a specific cell type

Placental chorionic villi are composed of cell types of differing developmental origins, with trophoblast cells derived from the trophectoderm of the blastocyst, and various types of mesenchymal cells derived from the inner cell mass. DNA methylation levels are known to vary considerably between trophoblast and mesenchymal components of the placenta[22] and presumably vary between the differing types of trophoblast cells and components of mesenchyme, including fibroblasts, endothelial cells, macrophages (Hofbauer cells) and hematopoietic cells.[14] These heterogeneous placental cell populations have not been well characterized in terms of DNA methylation; thus correction for multiple cellular contributions in placental samples, as proposed for blood,[23] is not currently feasible. Furthermore, the major contributor to DNA derived from a term placenta is syncytiotrophoblast, a multinucleated syncytium that cannot be isolated by cell-sorting techniques. The ability to epigenetically profile the main placental cell types would be an undeniably useful step toward controlling for the effects of a heterogeneous cell population. Depending on the question being asked, it may be practical to limit the study to an isolated cell type, either through micro-dissection or antibody purification methods.

Gestational age

Evaluate gestational age as a potential confounder and correct if necessary

In the placenta, numerous DNA methylation changes of a large magnitude occur over gestation,[24] even within the third trimester itself.[25] This may be related to changes in cell composition. For example, trophoblast composition varies over gestation in which the proportions of cytotrophoblast cells shift from 85% in the first trimester to just 15% in the term placenta,[26] due to the differentiation of these progenitor cells into syncytiotrophoblast cells. DNA methylation may also be associated with gestational age due to "normal" programmed gene expression and epigenetic changes that occur throughout development within each cell type. Thus, if samples cannot be gestational-age matched, the effect of gestational age should be evaluated and corrected for if significant.

Fetal sex

Evaluate sex as a potential confounder and correct if necessary

In female cells, DNA methylation plays a major role in the random inactivation of one X chromosome. While CpG island promoters subject to X inactivation exhibit approximately 35% and 15% lower DNA methylation in males than females in blood and placenta respectively, this difference is not observed on autosomal chromosomes.[27] Due to the sex chromosome imbalance, ideally study samples should be matched for sex within the gestational age ranges studied. However, given that sex differences in DNA methylation do not largely extend to autosomal

chromosomes,[28-30] analyses through genome-wide platforms may proceed without sex matching by excluding any sex chromosomes targets, and importantly autosomal assays that potentially cross-hybridize to the X or Y chromosomes.[29,31] Despite these recommendations, analyses should still evaluate sex as a potential confounder.

Other biological confounders

Evaluate biological confounders after correction for the largest modifiers

A number of maternal characteristics can affect placental morphology, function, and molecular expression profiles.[21] Recording of clinical data such as maternal ethnicity, age, smoking status, alcohol intake, body mass index, pre-gestational and prenatal medication-use, and pregnancy complications such as preeclampsia or gestational diabetes, is important as they may influence placental DNA methylation. However, the useful assessment of such potential confounders can only be achieved when accounting for the known larger modifiers of DNA methylation, such as gestational age. This is particularly true if assessing the association of fetal birth weight on DNA methylation: birth weights should be normalized by conversion to Z-scores based on average normal birth weight ranges that account for gestational age and sex.

DNA Methylation Data Analysis and Presentation

Data reduction

Consider the genomic context of CpG sites

In some genomic regions, particularly promoters, CpG islands and CpG island shores, DNA methylation at adjacent CpG sites is often correlated.[2,32,33] Furthermore, for both biological and technical reasons, DNA methylation at individual CpG sites is not always independent. Presenting DNA methylation values over genomic regions may increase statistical power and reduce technical noise. For example, a methodology known as "bump hunting" can be applied to EWAS data to identify regions of differential methylation, and help to account for the non-independence of neighboring CpGs.[34] At the single gene level, correlation of adjacent CpG sites can be tested for when the study question permits. If significantly correlated, DNA methylation results may be presented as an assay average rather than multiple single CpG site values. It should be noted that when assessing DNA methylation at multiple loci (whether within a single gene or across multiple genes), adjustment for multiple comparisons is essential to assess the false positive rate. Alternatively, statistical modeling may be used to manage the issue of correlated CpG sites within an assay without loss of information at individual sites. For example, the use of linear mixed model analyses[35] can account for heterogeneous effects of DNA methylation at individual but correlated sites of the same gene region.[36]

Data presentation

Present raw values rather than fold changes

The mean DNA methylation level in each comparison group is best reported using a value appropriate to the analysis method, for example, a β-value for Illumina Infinium DNA methylation array platforms, or a percentage value for bisulfite pyrosequencing or reduced representation bisulfite sequencing data. While β-values correlate to relative percent methylation, there can be considerable differences when comparing to other methods (e.g., a β-value of 0.8 might correspond to a level of 30% methylation when the same region is assayed by pyrosequencing). Furthermore, presenting a percent or fold change in DNA methylation values can be misleading when there are small absolute changes in DNA methylation and the mean level of DNA methylation is very low, as is typical for promoter regions.

Degree of DNA methylation change

Small differences in DNA methylation may not be biologically relevant

Establishing a cut-off value for differential DNA methylation, in addition to the inclusion of an appropriate false discovery rate to account for multiple comparisons, is important to limit analyses to biologically relevant changes. Depending on the type of study, the set of criteria for determining "biologically relevant" changes in DNA methylation will vary. These could include: the sample size, the method used, if it is a pilot or follow-up study, what the purpose is (i.e., to identify more subtle changes between cases that may be used to suggest biological pathways involved, or large changes that may themselves be functionally meaningful) and also the heterogeneity of the tissue (e.g., the placenta displays a high level of inter-individual variation). One should also ensure that measured differences are beyond the detection limit of the assay (e.g., 5% for pyrosequencing).[37] Careful consideration of these factors will reduce the false positive rate and focus attention on loci that are most likely to be biologically significant.

Data validation

Data validation will increase support for a true biological effect

Repeating DNA methylation analyses using a second method will strengthen the study conclusions. For example, if the initial analysis is performed using DNA methylation array platforms, subsequent experiments may focus more closely on a specific genomic area using bisulfite pyrosequencing. Using this approach, the study can be extended to include a wider cohort of cases to dissect changes in greater detail and in a cost effective way. While overlapping DNA methylation changes observed in different tissues might in some contexts be used as confirmation of biological relevance, care should be taken that this is not due to: (1) overlap in cellular composition, for example, blood cells may be present in many tissues sampled, including placenta, (2) technical bias toward detecting differences at the loci that are most variable or (3) genetic influences on DNA methylation. Like in any area of biology, reproduction of findings in a fully independent cohort is the best method to validate study results.

Interpretation of DNA Methylation Data

Alternative interpretation of results

Consider other explanations for altered DNA methylation

Epigenetic changes may represent an adaptive rather than a pathological response to adverse exposures or disease. For example in some cases, rather than resulting in fetal macrosomia, the placenta will adapt to some extent, to a maternal diabetic environment to counteract excess maternal glucose, thus reducing fetal

glucose transfer and limiting fetal overgrowth.[38] It is feasible that in utero exposures may leave a stamp on the placenta without causing a major detrimental effect on placental function or leading to epigenetic changes in the fetus. Thus placental DNA methylation may simply represent a "snap-shot" of the epigenetic environment at the time of sampling that does not persist into adulthood.[39] This supports the requirement for longitudinal follow-up studies in the human to determine the biological impact of the placental methylome for long-term health.

Acknowledge the potential for DNA methylation changes to be related to altered cell composition

It is important to recognize that altered DNA methylation levels may be related to changes in the proportions of different cell types,[40] possibly due to the pathology investigated or differences in developmental stage (if not well-matched), rather than true "epigenetic" modifications. The contribution of blood cell composition to epigenetic variation is well documented;[41,42] however, at least in studies of whole blood, this can be corrected for.[23] Correction for cell composition may be less of a concern if the study goal is, for example, to identify a biomarker associated with disease, but would be an important confounder if the working hypothesis is dependent on an epigenetic mechanism.

Negative findings

A negative finding can have positive value

There is a tendency in many studies to expand upon the largest DNA methylation differences as positive findings even when these are non-significant after multiple comparison correction. By limiting over-interpretation of small or non-significant findings we can helpfully exclude DNA methylation as a mechanism in some cases and more responsibly present data to non-experts with an interest in the research. In reality, it is unlikely that DNA methylation per se is the major biological mediator of developmental programming events linked to health and disease all of the time. It is important that we dissect epigenetic mechanisms from non-epigenetic events, which are nowadays often overlooked as potential explanations for phenotypic changes related to DOHAD.

Summary

We hope that the guidelines outlined here, in addition to recommendations recently made elsewhere,[5-7] can provide a basis for the informative reporting of DNA methylation studies in the human placenta, as well as in other tissues. More appropriate and uniform reporting of human EWAS will be a critical step toward the consolidation of the numerous research papers being published in this field. Vigilance in study conception (e.g., prospective, longitudinal studies with appropriate controls), execution (e.g., adequate tissue sampling, avoidance of contamination and reduction of technical variation), and analyses (e.g., accounting for population confounders, increasing statistical power), will be crucial to dissect out biologically relevant changes in the methylome from random events. Improved consistency in the reporting of human DNA methylation data will enable future research into developmental programming mechanisms and, ideally strategies for prevention of adverse outcomes, to move in the right direction.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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