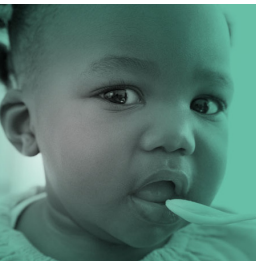
CEHN Research Conference

San Francisco, CA
May 30–June 1, 2012



2012

The Contribution *of* Epigenetics *in* Pediatric Environmental Health

Children's Environmental Health Network

The Contribution *of*

Epigenetics *in* Pediatric Environmental Health

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Keynote Presentations

Developmental Stressors and Epigenetics

Dana Dolinoy

Searle Assistant Professorship in Public Health, University of Michigan School of Public Health

Environmental exposures during early development and other critical life stages may induce changes to the epigenome resulting in potentially deleterious phenotypic effects including metabolic disease, cancer, and neurological disorders. The field of epigenetics is experiencing a rapid advancement in technology, methodology, and data acquisition that now allows for the identification of the constellation of genomic loci with altered epigenetic status following dose-dependent exposures. Utilizing a multi-pronged approach with an *in vivo* mouse model, human clinical samples, and an ongoing 15-year longitudinal epidemiological study, this presentation is intended to elucidate the impact of perinatal bisphenol A (BPA) and lead (Pb) exposure on metabolic homeostasis and DNA methylation, and the interplay between the two. Developmental exposure to environmentally relevant levels of BPA has been shown to affect both global and gene-specific DNA methylation patterns in rodents. We now draw upon data from whole-epigenome platforms to show that multiple dose levels of BPA affect DNA methylation in mice and humans and that these epigenetic effects are non-monotonic in dose response. Preliminary studies also indicate that perinatal Pb exposure exhibits epigenetic effects that may contribute to its known neurotoxic and obesogenic activities. Through these epigenomic profiling approaches, we hope to facilitate the identification of biomarkers of exposure, enabling clinicians to identify at-risk individuals prior to disease onset.

Embryo Vulnerability in An Always Changing World: Epigenetics and Beyond

David Epel

Jane & Marshall Steel Jr. Professor Emeritus in Marine Sciences, Cell and Developmental Biology, Stanford University

Studies on regulation/homeostasis/stress responses that are caused by environmental change have focused on the adult, where such adaptation is reversible. But similar responses during embryonic development can interrupt the developmental program with irreversible effects on the fetus. These effects might be apparent at birth, might first appear at puberty, or might be evident only late in adult life. Epigenetic changes in gene expression have been implicated in these changes, with the focus on modification of DNA or histones. I will review this evidence but also recent work that indicates that such modification is only a part of the story. Epigenetic changes might go far beyond direct effects on gene regulation, encompassing alterations in signaling pathways, cell structure, and protein–protein associations that will later affect developmental outcomes. It appears we are just seeing the tip of the epigenetic iceberg.

Developmental Origins of Health and Disease (DOHaD)—New Insights and Opportunities

Mark A. Hanson

Founding Director of the Institute of Developmental Sciences and Director of the Division of Developmental Origins of Health and Disease in the University of Southampton School of Medicine, British Heart Foundation Professor of Cardiovascular Science

Noncommunicable disease poses a major threat to global health and economies in both developed and developing countries. Risk of such disease is set in part during early life, when environmental influences including a mother's diet, body composition, and exposure to stress affect the development of her fetus and newborn, establishing its responses to later environmental challenges such as an obesogenic lifestyle. If these cues are inaccurate—e.g., unbalanced maternal diet or nutritional transition between generations through migration—offspring's responses are mismatched to environmental challenges, leading to greater risk. Undernutrition remains an enormous problem throughout the developing world, but in both developing and developed societies adverse consequences of over- and undernutrition co-exist. The resulting risk can be transmitted down multiple generations. The consequences extend to reproductive health, behavioral and cognitive problems, and some allergic conditions. Epidemiological, human clinical, and basic science research has now indicated underlying mechanisms, many of which involve epigenetic processes that can serve as early markers of later risk and that are in principle reversible by dietary, endocrine, or pharmacological means. These include DNA methylation, but also changes in histone protein structure and small noncoding RNAs. New evidence is revealing how such processes, which involve more than just imprinted genes, can modify the effects of transcription factors on gene expression and thus responses to later challenges. The specific patterns of CpGs methylated can be important, and the regions of importance are not solely in CpG-rich islands. The bioinformatics needed to analyze such effects is daunting. An urgent priority is to assess such epigenetic markers of risk and to implement the necessary complex interventions, which in many populations will require wider social and educational initiatives as well as public health campaigns.

Acknowledgments: M.A.H. is supported by the British Heart Foundation.

Epigenetics: A Biological Database for "Personalized Medicine"

Robert H. Lane

August L. (Larry) Jung, M.D. Presidential Professor and Chief, Division of Neonatology, & Developmental Origins of Disease Laboratories, Division of Neonatology, University of Utah School of Medicine

The fidelity of environmental epigenetics presents unique opportunities as we pursue the vision of "Personalized Medicine." This concept is particularly important within the practice of pediatrics. Within pediatrics, we know of multiple early-life events such as intrauterine growth restriction, prematurity, mechanical ventilation, that predispose towards—but not necessarily cause—postnatal morbidities such as diabetes and chronic lung disease. To move forward in terms of "personalizing" the care of children, we need to identify which infants suffer the highest risk for postnatal diseases and thereby require more intensive or invasive interventions. Just as important, we need to identify those infants who can afford to bypass intensive or invasive interventions. These identification tasks require a biological "database" that 1) records a personal environmental history; 2) accommodates the heterogeneity of the human condition; and 3) takes into account the complexity of genome–environment interactions. Environmental epigenetics uniquely meets these requirements, which becomes most evident when appreciated at the microscopic level of the individual gene. The key characteristic of the individual gene epigenetic response in meeting these requirements is fidelity, particularly as it relates to adherence to detail and accuracy. The adherence to detail and accuracy reflect the multiple dimensions that determine how an individual gene responds to specific early-life events. Dimensions that determine the response include the timing of the event, the type of event, the sex of the individual, the specific tissue and cell type, and the previous accumulated experiences. Dimensions through which an individual gene responds to early-life events include multiple epigenetic characteristics that can be altered at multiple sites along the whole gene, which can be subsequently modified by maturational developmental processes over time. Often initial responses represent adaptation, while subsequent modifications lead to postnatal morbidities. Despite many sincere efforts to learn more about this multidimensional response, multiple voids in the field obstruct progress towards personalized medical approaches. One void includes a failure to take an integrated approach that accounts for the depth and breadth of information recorded through epigenetics. An important

component of the integrated approach is the recognition that a single epigenetic modification "means" different things depending on the context of other accompanying epigenetic modifications, as well as the location within a gene. Another void also includes a lack of information on how cells record the epigenetic response in terms of specific signals and co-factors that belie environmental epigenetic fidelity. Yet, despite these voids, our growing understanding of the fidelity of the environmental epigenetic response represents a new and exciting opportunity. The complexity that is intrinsic to the fidelity of environmental epigenetics, although frustrating now, provides a depth and breadth of information that will eventually allow us to mine our epigenetic biological "database" to reveal meaningful information about individuals. Further understanding of this database and its construction will form a significant entry point into the world of "personalized medicine."

Effects of Prenatal Environmental Exposures on Child Health and Development

Frederica P. Perera

Professor of Environmental Health Sciences, Director of the Columbia Center for Children's Environmental Health and of the Disease Investigation Through Specialized Clinically-Oriented Ventures in Environmental Research (DISCOVER) Center

There is increasing evidence of the prenatal window of susceptibility to environmental exposures and the role of epigenetic alterations in mediating their effects. The goal of the Columbia Center for Children's Environmental Health (CCCEH) is the prevention of disease and developmental impairment in children. Longitudinal cohort studies of pregnant mothers and children are being conducted by the CCCEH, following the children from *in utero* through adolescence. The interdisciplinary research combines environmental monitoring, biomarkers (such as DNA adducts, DNA methylation and gene expression, eNO and immune markers), imaging techniques, GIS, and clinical assessments within longitudinal designs. Associations have been observed between prenatal environmental exposures such as polycyclic aromatic hydrocarbons (PAHs), pesticides, and other endocrine-disrupting chemicals on child health and development. A striking finding is that multiple outcomes are associated with the same prenatal and/or early postnatal exposure. There is also evidence that epigenetic mechanisms may mediate certain health effects. The research findings have prompted a number of interventions by the CCCEH to reduce the levels of exposure to toxic pollutants. Early identification of risk factors by studies such as ours will have benefits in childhood and possibly over the life course.

Mini-Symposium Abstracts (*Presenting authors in boldface type*)

Biomarkers

Martyn Smith, moderator

Epigenetics and Environment

Robert O. Wright, **Andrea Baccarelli**, **Rosalind Wright**

Harvard Medical School and School of Public Health, Boston, MA

Epigenetic marks include DNA methylation, histone modification, and noncoding RNAs. Previous studies on fetal/child growth and development have identified multiple risk factors including environmental tobacco smoke (ETS), social stress, lead exposure, and air pollution. In parallel, a growing body of literature has demonstrated that all four of these risk factors can alter DNA methylation, suggesting a common pathway by which such environmental factors impair child growth and development. These environmental stressors may affect epigenetic marks at several target genes or may interact with methylation at the level of the retrotransposon, which itself may be a unique, functional epigenetic mark. This talk will highlight issues of environmental interactions with epigenetic marks, with an emphasis on the role of epigenetics as a mediator of environmental risk factors and child development.

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Effects of Maternal Lead Exposure on DNA Methylation in Children

Douglas M. Ruden,[1] **Arko Sen**,[1] **Robert O. Wright**[2]

[1] *Wayne State University, Detroit, MI*

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Early developmental exposure to lead causes long-term deficits in mental abilities (i.e., loss of IQ) and a loss of gray matter in the brains of older children and adults. To explore the possibility that DNA methylation is involved in the long-term effects of developmental lead exposure, we analyzed the global DNA methylation pattern in children from the Early Life Exposure in Mexico to ENvironmental Toxicants (ELEMENT) cohort of Mexico City children who are followed from pregnancy of their mothers to age 10. We chose the 24 children with the highest umbilical cord blood lead levels (between 5 and 10 µg/dL) and 24 children with low blood lead levels and analyzed their CpG DNA methylation status at ~500,000 sites in the genome with the Illumina Human Methylation 450K array (HM450K). We found that 34 mental retardation genes genes on the X chromosome are epigenetically repressed in boys but not in girls. Possible mechanisms and implications will be discussed.

Epigenetic Effects of Air Pollution and Endocrine-Disrupting Chemicals—Methods and Results from Human Research

Andrea Baccarelli

Harvard School of Public Health, Boston, MA

Epigenetics investigates heritable changes in gene expression that occur without changes in DNA sequence. Several epigenetic mechanisms, including DNA methylation and histone modifications, can change genome function under exogenous influence. Results obtained from animal models indicate that *in utero* or early-life environmental exposures produce effects that can be inherited transgenerationally and are accompanied by epigenetic alterations. The search for human equivalents of the epigenetic mechanisms identified in animal models is in progress. I will present evidence from human environmental studies indicating that epigenetic alterations may mediate effects caused by exposure to environmental exposures, including air pollution and its metal components, as well as endocrine active metals. In these investigations, we have shown that environmental exposures are associated with altered methylation of human repetitive elements or genes, as well as with alterations of histone modifications and miRNAs. I will present original data demonstrating that altered epigenetic markings in blood and other tissues can be used to predict environmentally induced disease, such as childhood asthma and cardiovascular disease. I will present novel data providing information on the temporal stability of DNA methylation. On the basis of current evidence, I will propose possible models for the interplay between the environment and the human epigenome, and outline opportunities and challenges in environmental epigenetics.

The Role of Nutrition and Epigenetics in Human Health

Dana Dolinoy, moderator

Epigenetic Effects of Choline During Brain Development

Steven Zeisel

University of North Carolina at Chapel Hill Nutrition Research Institute, Chapel Hill, NC

Choline, an essential nutrient, is an important source of the methyl groups needed for epigenetic marking of DNA and histones. Humans derive choline from the diet or from endogenous biosynthesis (which is induced in pregnant women by estrogen). In rodents, the availability of choline to the fetus is very important for brain hippocampal development; the hippocampus is important for memory function. If pregnant rodents are fed a diet supplemented with choline, their offspring perform as much as 30% better on tests of visuospatial and auditory memory, and this improvement lasts for their entire lifetime. Fetuses from mothers fed a choline-supplemented diet have almost twice the rate of neurogenesis in their hippocampi when compared to fetuses from mothers fed a low-choline diet. In addition, fetuses from mothers fed a choline-supplemented diet have half the rate of neuronal apoptosis (programmed cell death) in their hippocampi when compared to fetuses from mothers fed a low-choline diet. Thus, maternal ingestion of a high-choline diet during pregnancy increases neuronal proliferation and decreases neuronal death in fetal brain. These changes in brain development are, in part, mediated by epigenetic mechanisms. A gene encoding for an inhibitor of cell cycling (CDKN3) is expressed when the gene is undermethylated and is suppressed when the gene is methylated. Fetuses from mothers fed a choline-supplemented diet have more highly methylated CpG islands in CDKN3 in their hippocampi when compared to fetuses from mothers fed a low-choline diet. Highly methylated CDKN3 has reduced expression, and therefore an important brake on cell cycling is removed—hence the increased neurogenesis seen in the choline-supplemented fetal brain. Thus, maternal diet changes epigenetic marks in the fetal brain, and this, in turn, changes brain structure and function.

Acknowledgments: Support for this work was provided by grants from the National Institutes of Health (DK55865, DK56350).

Influence of Prenatal Arsenic Exposure, Folate Nutritional Status, and Newborn Sex on Cord Blood DNA Methylation

Mary V. Gamble

Mailman School of Public Health, Columbia University, New York, NY

Chronic arsenic (As) exposure currently affects over 140 million people worldwide, roughly a third of whom reside in Bangladesh. An emerging body of evidence indicates that As exposure is associated with epigenetic dysregulation. In addition, early-life As exposure may influence the trajectory of health outcomes later in life. However, the mechanisms underlying these observations are unclear. The objectives of this study were to investigate the influence of prenatal As exposure and folate nutritional status on global methylation of cord blood DNA in a study of mother/newborn pairs in Matlab, Bangladesh. Maternal and cord blood DNA were available from a convenience sample of 101 mother/newborn pairs. Measures of As exposure included water As, maternal urinary As (uAs), maternal blood As (mbAs), and cord blood As (cbAs). Measures of DNA methylation included the [^{3}H]-methyl-incorporation assay and 3 pyrosequencing assays: Alu, LINE-1, and LUMA. Nutritional parameters included maternal and cord plasma concentrations of folate, B_{12}, and homocysteine. In the total sample, increasing quartiles of maternal uAs were associated with an increase in covariate-adjusted means of newborn global DNA methylation as measured by the [^{3}H]-methyl-incorporation assay [quartile (Q) 1 and Q2 vs. Q4; $p = 0.06$ and 0.04, respectively]. Sex-specific linear regression analyses, while not reaching significance level of 0.05, indicated that the associations between As exposures and Alu, LINE-1, and LUMA were positive among male newborns ($n = 58$) but negative among female newborns ($n = 43$); tests for sex differences were borderline significant for the association of cbAs and mbAs with Alu ($p = 0.05$ and 0.09, respectively) and for the association between maternal uAs and LINE-1 ($p = 0.07$). Maternal and cord plasma homocysteine concentrations were higher among female than male newborns ($p < 0.01$) and cord homocsteine concentrations were negatively associated with LINE-1 among female newborns ($p < 0.05$). Sex-specific correlations between maternal urinary creatinine and newborn methyl-incorporation, Alu, and LINE-1 were also evident ($p < 0.05$). These results suggests that prenatal As exposure may be associated with global DNA methylation in cord blood DNA, possibly in a sex-specific manner. This is consistent with a growing body of evidence suggesting that epigenetic responses to environmental exposures may differ by sex. Arsenic-induced epigenetic modifications *in utero* could

potentially influence disease outcomes later in life. Several of our previous studies have demonstrated that urinary creatinine is a very strong predictor of As methylation. We believe this may be related to the fact that creatine biosynthesis is a major consumer of methyl groups and is also influenced by dietary creatine intake. The finding of sex-specific correlations between maternal urinary creatinine and newborn DNA methylation was unexpected and may be due to chance; this finding warrants further investigation. Additional studies are needed to confirm these findings and to examine the persistence and potential health impacts of DNA methylation marks over time.

Acknowledgments: This work was a collaboration between the Mailman School of Public Health and The International Center for Diarrheal Disease, Bangladesh (ICDDR,B). We acknowledge with gratitude their support.

Folate Depletion Alters Fetal Hepatic DNA Methylation

Jill McKay

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Growing evidence from animal models suggests that a variety of nutritional insults *in utero* result in altered programming of offspring, ultimately increasing disease risk in later life. Epigenetic markings, including DNA methylation patterns, are one potential mechanism mediating these effects. Since folate is a methyl donor, altered folate supply may influence methyl group availability for DNA methylation. We observed previously that genomic DNA methylation was lower in adult mouse offspring born to folate depleted dams ($p = 0.010$; McKay et al. 2011a). Furthermore, maternal folate depletion altered methylation in a gene-specific manner in the mouse fetal gut (McKay et al. 2011b). In this study we investigated the influence of maternal folate depletion on genome-wide DNA methylation in the male mouse fetal liver. Pairs of female C57BL/J6 mice were assigned randomly to a folate-adequate (2 mg folic acid/kg; control) or folate-deplete (0.4 mg folic acid/kg; test) diet 4 weeks prior to timed mating with a C57BL/J6 male. Dams remained on allocated diets until gestation day 17.5, when dams were killed and fetuses removed. DNA was extracted from fetal livers. To identify regions of the genome that were differentially methylated between test and control groups, methylated DNA was immunoprecipitated and then amplified by PCR, before hybridization to Roche NimbleGen Methylation 385K arrays. Preliminary analysis showed that the promoters of 395 genes were differentially methylated in the fetal liver in response to folate depletion during development. Of these, 123 genes were hypomethylated and 272 genes were hypermethylated in response to the test compared to the control group. Gene ontology analysis using DAVID (http://david.abcc.ncifcrf.gov/) revealed that methylation changes occurred in genes involved in biological processes including chromosome segregation and cell division. If the observed methylation changes alter these biological processes through concordant changes in gene expression, this could potentially lead to chromosomal aberrations and disease.

Mechanisms

Cynthia Bearer, moderator

Epigenetic Regulation after Environmental Intervention

Stephanie Lovinsky-Desir,[1] R. Ridder,[2] D. Torone,[2] H. Jiang,[2] S. Narula,[2] M. Kattan,[1] E. DiMango,[2] R.L. Miller[1,2]

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Indoor allergen remediation may lead to sustained improvement in asthma symptoms, though the mechanism for such improvement is unknown. Epigenetic changes in the T helper (Th) pathways may explain immunomodulation. DNA methylation and expression of interferon (IFN) γ, an epigenetically regulated Th1 gene, may play a role in improved asthma symptoms following a multifaceted environmental intervention. We will compare DNA methylation and gene expression of IFNγ prior to and 6 months after a multifaceted environmental intervention. We hypothesize that decreasing methylation of IFNγ over time is associated with improved asthma outcomes. We also will compare DNA methylation of IFNγ in buccal vs. $CD4^+$ peripheral blood lymphocytes to determine relationships between the two surrogate cell types in allergic asthma. In this randomized control trial of children and adult allergic asthmatics, detailed asthma symptoms, home dust allergen levels, lung function, fractional exhaled nitric oxide, and total and specific immunoglobulin (Ig) E levels are being measured. An intervention counselor visits the home of each subject to identify, educate, and help remove sources of allergen exposure. Blood and buccal samples are obtained for DNA methylation and RNA analysis at randomization and 6 months following intervention. Follow-up measurements are planned through 12 months. Reliable protocols for RNA extraction from buccal swabs have been developed. Following bisulfite conversion, pyrosequencing is being performed. Preliminary data suggest that buccal DNA and RNA are readily extractable from samples collected by children and adults under the observation of a research worker. We are able to demonstrate highly correlated DNA methylation levels of duplicate samples for the target IFNγ

CpG sites, with concordance correlations between sequential pyrosequencing runs of 0.86 ($p < 0.05$) for CpG-186 ($n = 9$) and 0.92 ($p < 0.05$) for CpG-54 ($n = 8$). The CpG-186 and CpG-54 sites in the IFNγ gene promoter demonstrate an intermediate range of percent DNA methylation (54–86% methylation). We also are able to effectively extract RNA from buccal samples with concentrations ranging from 98.8 to 429.4 ng per sample. Comparisons of percent CpG methylation over time as well as with changes in allergen level, IgE, and symptoms following multifaceted intervention will be presented. Buccal DNA and RNA collections are readily feasible when performed in an outpatient setting and yield relatively high-quality amounts of DNA and RNA with good reproducibility. A range of intermediate levels of CpG methylation in buccal cells across samples suggests that these CpG sites may be susceptible to increases or decreases in DNA methylation. This study will be the first to compare changes over time in methylation patterns in association with changes in environmental asthma triggers and asthma symptoms. Novel comparisons in DNA methylation patterns between buccal and blood samples in a pediatric and adult cohort of asthmatics are planned.

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Epigenetic Changes in IFN-gamma and FoxP3 due to SHS

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Secondhand smoke (SHS) has been found to be associated with global methylation as well as allergies and asthma. We hypothesize that SHS exposure is associated with methylation in two key genes, Interferon-gamma (IFN-γ) and Forkhead box protein 3 (FoxP3), in two specific T-cell subsets, T-effector (Teff) and T-regulatory (Treg) cells, respectively. Our main objective was to determine the impact of secondhand smoke on methylation of CpG sites in IFN-γ and FoxP3, since these are important genes associated with allergy and asthma. Moreover, we then tested transcription levels of both IFN-γ and FoxP3 in isolated Teff and Treg cells, respectively. SHS: children whose parent/guardian answered yes to "Do you currently smoke?" and/or "Does anyone who currently spends time with the subject smoke?" ($n = 31$, 11–23 y.o.; mean: 17 y.o.). Age-matched controls (non-SHS): children whose parents answered no to both questions. Teff and Treg cells were isolated, DNA purified, and bisulfate treated. Methylation of 20 CpG sites in the promoter regions of the IFN-γ locus in Teffs and of 16 CpG sites in the promoter and intron regions of the FoxP3 locus in Tregs was determined for each subject by pyrosequencing on Illumina Infinium. Respective transcription profiling for both genes was performed via QT-PCR using beta-glucuronidase for fold expression comparison. SHS-exposed subjects were found to differ significantly from healthy control subjects in all parameters tested to date. Methylation of IFN-γ in Teff cells was found to be significantly greater in SHS subjects than in healthy control subjects (SHS mean: 12.42 ± 0.43; non-SHS mean: 8.26 ± 0.39; $p < 0.0001$), as was methylation of FoxP3 in Treg cells (SHS mean: 11.94 ± 0.3590; non-SHS mean: 8.71 ± 0.45; $p < 0.0001$). Transcription of IFN-γ in Teff cells of SHS subjects was significantly less than in healthy controls (SHS mean: 0.75 ± 0.05; non-SHS mean: 1.52 ± 0.11; $p < 0.0001$); the same was true of FoxP3 transcription in Treg cells of SHS subjects (SHS mean: 0.75 ± 0.05; non-SHS mean: 3.29 ± 0.35; $p < 0.0001$). SHS-exposed subjects had IgE levels significantly greater than non-SHS subjects (SHS mean: 76 kU/L ± 15; non SHS mean: 13 ± 7). This preliminary evidence suggests there are significant epigenetic differences in two key genes known to be associated with immunological changes in allergy and asthma between a group of children exposed to SHS and a group of controls. Our data demonstrate that SHS could exacerbate both allergies and asthma, as evidenced by increased methylation and decreased transcription of IFN-γ and FoxP3 in Teff and Treg cells, respectively. Future studies will examine 1) clinical allergy and asthma profiles in these same subjects, 2) other genes important in allergies and asthma that could be epigenetically modified, and 3) the durability of these changes over time with repeated measures.

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The Role of PBRM1 in Malignant Rhabdoid Tumor Development

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Malignant rhabdoid tumors (MRTs), a pediatric renal cancer, lack SNF5, a subunit of the SWI/SNF chromatin remodeling complex, a regulator of nucleosome positioning and gene expression. Recent studies showed that PBRM1, another subunit of SWI/SNF, was mutated in adult clear cell renal carcinoma (ccRCC). Intriguingly, both SNF5 and PBRM1 regulate the cell cycle through control of expression of $p21^{WAF1/CIP1}$. We hypothesize that SWI/SNF complex mutations provide a link between adult and pediatric renal cancers. To test this notion, we re-expressed SNF5 in MRT cell lines and

followed PBRM1 and p21 expression and cell growth. We also examined SWI/SNF complex composition and recruitment to the p21 promoter. We also investigated PBRM1's role in SNF5-induced G1 cell cycle arrest using PBRM1-deficient MRT cell lines. We utilized adenoviral vectors co-expressing hSNF5 and GFP (Ad-SNF5-GFP), HA tagged SNF5 (Ad-SNF5-HA), GFP (Ad-GFP), and empty vector (Ad-CMV) to express human SNF5 in four MRT cell lines (A204.1, G401 TTC549, TTC642). The expression of hSNF5 and other complex members was quantified through immunoblotting and QT-PCR. Immunoprecipitation (IP) was utilized to determine the composition of the SWI/SNF complex. Cell cycle analysis was conducted by using flow cytometry with BrdU and PI staining. PBRM1 knockdown cell lines were generated using lentiviral-RNAis (Open Biosystems). Re-expression of SNF5 in MRT cell lines led to increased PBRM1 protein levels without concomitant increases in mRNA levels. Immunoprecipitation experiments showed that SNF5 re-expression caused PBRM1 levels in the complex to increase, as compared to the BRG1 ATPase levels that remained constant after SNF5 re-expression. These results were consistent across the four MRT cell lines examined. Interestingly, levels of other complex proteins, such as BAF155 and BAF250, increased in a similar fashion to PBRM1. After re-expression of SNF5, we also observed an increase of both SNF5 and PBRM1 at the p21 promoter, with a peak at the transcription start site. In contrast, other complex members showed only a modest increase across the entire promoter region with no apparent peak of binding. We are currently testing three PBRM1 knockdown cell lines for their ability to undergo growth arrest and p21 induction after SNF5 re-expression. Our data show that SNF5 expression increases PBRM1 levels globally, presumably through stabilization within the SWI/SNF complex. This finding suggests a high degree of interplay between PBRM1 and SNF5 during chromatin remodeling activity. Therefore, identifying genes regulated by both SNF5 and PBRM1 will prove critical in further understanding their mechanism of interaction and their contribution to MRT development. We will use high-throughput approaches including ChIP-seq, nucleosome positioning and gene expression analyses to accomplish these goals. Ultimately, our studies will identify key signaling pathways activated by environmental agents that contribute to the initiation and/or progression of renal cancers including MRTs and ccRCCs.

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Choline Prevents Ethanol Inhibition of Neurite Outgrowth

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Ethanol is a known developmental neurotoxicant, with 1% of all newborns manifesting signs of fetal alcohol spectrum disorder. Exposure can occur from alcohol in beverages, foodstuffs, medications, personal products, gasoline, and hand sanitizers. L1 cell adhesion molecule (L1), critical for brain development, mediates neurite outgrowth via trafficking through lipid rafts. Ethanol inhibits this process. Choline has been shown to prevent ethanol developmental neurotoxicity. Our objective is to determine if choline prevents ethanol inhibition of L1-mediated neurite outgrowth and signal transduction. Cerebellar granule neurons from 6-day-old rat pups were prepared and plated on poly L-lysine (PLL) or L1. For neurite length measurements, different additives were added to the media: ethanol (EtOH) 25 mM and/or 40 mM choline (Cho). Cells were fixed 24 hr after plating. Neurite length was determined by a blinded investigator. For signal transduction, cells were plated on PLL, and 40 mM Cho was added. Following incubation overnight, L1 signaling was triggered by addition of a crosslinking antibody. Signal transduction was measured by immunoblots of tyrosine dephosphorylation of L1. Ethanol significantly reduced L1-mediated neurite length but not neurite length of neurons grown on PLL alone. Cho significantly reduced ethanol inhibition of L1-mediated neurite outgrowth. Ethanol significantly reduced tyrosine dephosphorylation of L1, which was significantly reduced by prior incubation with Cho. Choline reduces ethanol inhibition of L1 function. There are at least two different mechanisms that could mediate the neuroprotective effect of choline: 1) via stabilization of lipid raft signaling or 2) via DNA methylation. Choline is metabolized to sphingomyelin, a crucial component of lipid rafts. We will be determining if the mechanism of choline neuroprotection is incorporation into sphingomyelin or methylation of DNA.

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Environmental Epigenetics: From Mice to Humans

Nina Holland, moderator

miRNA Profiles Following Air Pollutant Human Exposures

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Inhalation of air pollutants is associated with increased mortality and morbidity in cardiac, inflammatory, and airway diseases. Changes in immune responses, inflammation, and other adverse impacts from acute exposure are associated with altered gene expression. We have previously shown that *in vitro* exposure of airway epithelial cells by ozone and diesel exhaust (DE) can alter expression of microRNAs. *In vitro* studies have shown that miRNAs altered by ozone or DE are associated with regulation of disease-related pathways. We hypothesize that *in vivo* inhalation of these pollutants will result in dysregulation of these pathways and are associated with immune and physiological responses. We therefore aimed to determine changes in miRNA profiles from airway cells following human *in vivo* DE or ozone exposure. We performed a double-blind placebo-controlled random exposure study. Fifteen healthy individuals were exposed in a crossover fashion to clean air (CA), DE (300 µg/m^3) or ozone (0.3 ppm) for 2 hours in a controlled chamber with exposures separated by a minimum of 28 days. Bronchoscopy was performed the next day to recover bronchial epithelial cells (BEC). Airway inflammation was assessed by cytokine analysis and differential cell counts in bronchoalveolar lavage fluid. RNA was isolated from recovered cells and integrity assessed. RNA was used for hybridization to Agilent miRNA arrays and for confirmation of candidate miRNAs by RT-PCR. Compared to clean air, exposure to both ozone and DE resulted in airway inflammation (5–15% neutrophilia). miRNA profiles identified in the arrays were analyzed by PartekGS for those altered [≥ 1.5 fold-change (fc) and $p < 0.05$] compared to clean air. Despite considerable interindividual heterogeneity, hierarchical clustering demonstrated that both ozone and DE exposure resulted in significantly altered profiles. Over 30 miRNAs were identified as altered uniquely by each exposure. In BECs from ozone-exposed individuals altered miRNAs were down-regulated while in DE exposed subjects 21% were up-regulated. The most prominent alteration of miRNAs by ozone included those in the miR-449 family (fc = –2.0, $p = 0.0024$); for DE miR-1246 (DE, fc = –1.7, $p = 0.022$). Using a candidate approach miRNA-181a was shown to be decreased by more than 50% in 10/16 subjects exposed to DE. IL-25, a cytokine involved in TH2 promotion, is predicted to be regulated by this miRNA. RT-PCR confirmed that it was increased 3.5-fold in those subjects where miRNA-181a was decreased, and unchanged in those where miRNA-181a was unchanged. These results demonstrate that acute exposure to air pollutants can cause changes in miRNA expression in airway cells that correlate with changes in gene regulation of their predicted targets. Confirmation of miRNAs identified by arrays to be altered by exposure will be confirmed along with their putative targets. Changes in miRNA expression may represent a novel mechanism by which air pollutants may perturb disease-related pathways and induce adverse health effects.

This abstract does not necessarily represent EPA policy.

Prenatal BPA: Epigenetic Disruption, Brain, and Behavior

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Bisphenol A (BPA) is an estrogenic endocrine disruptor widely used in the production of plastics. There is increasing concern about effects of early-life exposure to BPA on child health. Animal studies suggest that *in utero* exposure to BPA may produce a variety of prenatal and postnatal adverse effects, including impairments in brain development, sexual differentiation, learning, and behavior. The main aim of this study was to explore the molecular mechanisms that underlie long-lasting effects of developmental BPA exposure on brain development, learning, and behavior. We first examined whether low-dose maternal exposure to BPA during gestation affects postnatal brain gene expression and behavior in the offspring. We further explored possible epigenetic mechanisms that may mediate these effects. The pregnant Balb/c dams were orally exposed to BPA dissolved in corn oil (2, 20, or 200 µg/kg/day) or only corn oil (vehicle control) during the entire gestational period (GD0–19). Gene expression analysis was performed on the RNA extracted from the placental tissue (GD18) and brain tissue (cortex, hypothalamus, and hippocampus) of the 4-week-old offspring using real-time qRT-PCR. Methylation at specific CpG sites in the ERα gene region was determined using bisulfite-pyrosequencing method and the DNA isolated from

the cortical tissue of the 4-week-old offspring. The behavioral analysis included the open-field test, which was performed on 2-month-old mice using standard procedures. At all three doses, prenatal BPA treatment resulted in a significant up-regulation of the genes relevant for neuroendocrine function and behavior, such as the genes encoding estrogen receptors (ERs), in the cortex (both ERα and ERβ), hypothalamus (ERα), and hippocampus (ERβ) of 4-week-old female offspring. These animals also showed increased expression of the reelin (hippocampus) and the BDNF (cortex) genes, consistent with BPA's effect on neurodevelopment and synaptic plasticity. Moreover, maternal BPA exposure led to a down-regulation of the mRNAs encoding DNA methyltransferases DNMT1 and DNMT3A in both the placenta (GD18) and the cortex of 4-week-old offspring. In the cortex, decrease in the DNMT1/3A expression was further associated with reduced DNA methylation in the ERα gene region. Last, the open-field behavior analysis suggested a hyperactive phenotype that emerges as a function of elevated *in utero* BPA exposure. In summary, our study shows that low-dose maternal exposure to BPA results in significant postnatal changes in the expression of the genes relevant for neurodevelopment, neuroendocrine function, learning, and behavior in the offspring, and this is associated with altered behavioral phenotype. Moreover, prenatal and persisting postnatal changes in DNMT1 and DNMT3A expression provide possible mechanism for the developmental epigenetic disruption and long-lasting effects on brain function induced by *in utero* BPA exposure. Further work will examine whether developmental and early life interventions may alleviate BPA's effects on brain and behavior. We will also explore whether BPA's epigenetic and behavioral effects may extend to future generations.

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PAH Linked Aberrant DNA Methylation of Asthma Genes in Sensitized Mice

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Polycyclic aromatic hydrocarbons (PAH) are common urban air pollutants emitted during combustion. Previous studies indicated that young children exposed to higher levels of PAH are at greater risk of respiratory problems. Previously, our group also linked prenatal exposure to PAHs to aberrant DNA methylation of asthma candidate genes in young children. However, the mechanisms of prenatal PAH exposure on asthma in older age groups has not yet been well demonstrated in an animal model. The objective of this study was to explore the mechanisms of pre- and postnatal exposure to PAH in relation to asthma closer to adulthood. We investigated if early-life PAH exposure altered the methylation status of gene promoters in lung versus spleen in an asthma mouse model. Mice were prenatally (gestation day 3–19, dam) and postnatally (postnatal day 2–19, dam and offspring,) exposed to aerosol PAHs 5 hours a day or vehicle control (six mice each group). Offspring were sensitized to ovalbumin (OVA), and challenged to either OVA or phosphate buffered saline (PBS) at age 5 weeks. Serum IgE level in each animal was measured to verify OVA sensitization. Using lung and spleen tissues, the promoter methylation status of candidate asthma genes Adrb2, Arg1, Acsl3, Hmgn5/Nsbp1, Ifng, IL4, Il4r, and Pde4d were analyzed by bisulfite PCR sequencing. Of the eight genes analyzed, remarkable differential methylation was identified in two promoters in lung but not spleen. From lung samples, Arg1, a type I arginase associated with the production of fractional exhaled nitric oxide in children with airway inflammation, was hypermethylated in PAH-exposed OVA sensitized and challenged group, compared to the vehicle control group with OVA sensitized and challenged; Hmgn5, high-mobility group nucleosome binding domain 5 or nucleosomal binding protein 1 that is involved in nucleosome remodeling, was first identified hypomethylated in PAH group compared with the vehicle control group (both OVA sensitized and challenged). Further, the absence of differential methylation in spleen according to PAH exposure or OVA sensitization suggested that the lung is more susceptible to PAH-induced epigenetic modification. Our study revealed novel associations between prenatal exposures to ambient PAH and asthma gene promoter methylation in mouse lung, but not spleen, tissue. Further study of the gene expression in relation to promoter methylation is required to address the biological function of the promoter differentiation and the gene function.

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Effects of Age, Sex, and Prenatal Exposure on Methylation

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Epigenetic mechanisms, particularly DNA methylation, have attracted increasing interest in the etiology of disease as a possible link between the genetic and environmental determinants of health. Prenatal exposures to organochlorines and polybrominated diphenyl ethers (PBDEs) have been associated with decreased fertility, abnormal mental development, and altered thyroid function in children. We aim to determine whether DNA methylation, measured at birth and 9 years of age, is associated with prenatal environmental exposures to persistent pollutants and may be a potential mechanism of adverse health effects. We are also investigating how site-specific epigenetic markers differ by age and sex in children, and whether these biological factors are effect modifiers for environmental exposures. The CHAMACOS birth cohort study is investigating environmental exposures and children's health and development in low-income Mexican-American immigrant families in California with high exposures to DDT and PBDEs. DNA methylation was assessed in cord blood from 254 CHAMACOS newborns and blood from the same children at 9 years of age by Illumina Infinium HumanMethylation450K BeadChips that interrogate 485,577 CpG sites. Extensive quality control procedures confirmed the high quality of the data. We employed bootstrapping, unsupervised clustering, and pathway analyses to assess gene- and site-specific differences, and minimize the number of tests. About 15.5% of all CpG sites assessed by 450K BeadChip, representing >15,000 genes, were differentially methylated between children at birth and 9 years of age, adjusting for multiple testing by controlling for the false discovery rate. More than 2% of CpG sites investigated in > 1,900 genes showed significant differences by sex. As expected, most were located in sex chromosomes; however, some 731 CpG sites with significant differences between girls and boys were found in autosomes. Markers of DNA methylation were also associated with prenatal exposure to persistent organic pollutants. Different patterns of epigenetic changes were observed for shores, shelves, gene bodies, and other regions that were additionally modified by age and sex. DNA methylation undergoes significant changes from birth to early adolescence, and differs in boys and girls. Prenatal exposure to persistent organic pollutants is associated with genome-wide and site-specific differences in DNA methylation that are also modified by age and sex. We plan to explore how these changes are clustered by region, gene, and pathway, and whether they are associated with health effects such as obesity and puberty. Numerous candidate genes, CpG site hits, and their overlaps by age and sex remain to be explored, and confirmed by alternative methodologies such as pyrosequencing. Finally, we plan to evaluate the effects of prenatal and postnatal exposure to other toxicants on the full cohort of CHAMACOS children at different ages.

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Critical Windows of Early Exposure & Sensitivity I

Robert O. Wright, moderator

DNA Methylation Signatures and IL10 Levels in Childhood ALL

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We have previously found a significant deficit of the anti-inflammatory cytokine IL10 in neonatal blood spots of children with acute lymphoblastic leukemia (ALL) compared to healthy controls, which supports the hypothesis that ALL may develop as a result of an abnormal immune response to early infection. We aimed to confirm our finding of low IL10 levels in children with ALL in a second sample cohort and using a different technology, in addition to testing for associations between genetic markers at the *IL10* gene locus and ALL disease status as well as IL10 expression. We also wished to assess whether IL10 levels at birth are related to DNA methylation status at birth in a subset of samples. An IL10 Human Ultrasensitive ELISA kit (Invitrogen) was used to quantify levels of IL10 in neonatal blood spots of 111 ALL cases and 130 age-matched controls. IL10 levels were normalized against total protein content using a Bradford protein assay. Genotyping of three SNPs at the *IL10* gene locus on chromosome 1q32.1 was carried out in 525 ALL cases and 775 controls using Sequenom MALDI-TOF mass spectrometry. Genome-wide DNA methylation analysis was carried out using the Illumina Infinium HumanMethylation450 Beadchip on both neonatal bloodspots and leukemia cells taken from a subset of ALL cases and controls with IL10 measurements. Mean IL10 levels were significantly lower in ALL cases versus controls ($p = 0.007$), with an odds ratio of 0.362 (95% CI: 0.142, 0.909) comparing the highest quartile to the lowest quartile. Two SNPs lying within 1 kb upstream of the *IL10* gene promoter were found to be associated with case–control status ($p < 0.05$), and a trend towards significance was shown between the genotype of these two SNPs and levels of IL10 in the neonatal bloodspots. In addition, DNA methylation levels of several CpG loci across the genome at birth (neonatal blood spots) were significantly associated with IL10 levels at birth. We have replicated our previous discovery that neonatal levels of IL10 are significantly lower in children who develop ALL compared to healthy controls. In addition, we have identified two novel SNP associations with ALL at the *IL10* locus, which appear to correlate with IL10 expression. These data provide further evidence for the role of this anti-inflammatory cytokine, and abnormal immune function, in the etiology of childhood ALL. Elucidation of the interaction between neonatal IL10 levels and DNA methylation both at the *IL10* gene locus and genome-wide may shed further light on the relationship between immune responses and risk of developing ALL.

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Placental Epigenetics and Infant Neurobehavior

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The intrauterine environment can impact the developing fetus through alterations of the epigenetic control of critical genes in the placenta. These alterations lead to changes in placental function that can impact infant health, including behavioral and mental health, at birth and throughout life. In this study, we specifically examined variation in placental DNA methylation of the promoters of the *HSD11B2* (encoding 11-beta-hydroxysteroid dehydrogenase-2) and *NR3C1* (encoding the glucocorticoid receptor) genes and its association with infant neurobehavior assessed using the NICU Network Neurobehavioral Scales. Forming the population for our examinations, the Rhode Island Child Health Study (RICHS) is focused on examining the impact of the environment on epigenetic regulation in the placenta, and how variation in epigenetic features, such as DNA methylation, is associated with infant neurobehavioral profiles. DNA methylation was assessed using quantitative bisulfite pyrosequencing of 187 human term placenta samples from infants enrolled in RICHS, and the genotype of a common SNP in the promoter region of the *NR3C1* gene was also assessed using Taqman allelic discrimination assays. After controlling for genotype and confounders, we identified significant associations of *NR3C1* methylation with infant quality of movement ($p = 0.05$) and with infant attention ($p = 0.05$), and a potential interaction between methylation and genotype on infant attention score. We also identified, controlled for confounders, that *HSD11B2* methylation extent is greatest in infants with the lowest birth weights ($p = 0.04$), and that this increasing methylation

was also associated with reduced scores of infant quality of movement ($p = 0.04$). These results suggest that genetic variation in addition to factors in the intrauterine environment which can impact lifelong health outcomes are associated with placental methylation of the *HSD11B2* and *NR3C1* genes and that these epigenetic alterations are, in turn, associated with prospectively predictive early neurobehavioral outcomes, suggesting in some part a mechanism for the developmental origins of child mental health. Ongoing studies are validating these findings in additional subjects, as well as utilizing discovery-based techniques to identify new genes and pathways for more in-depth examination.

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Methylation Levels at Imprinting Control Regions Are Not Altered with Fertility Treatments

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Assisted reproductive technology (ART) has been associated with an increase in the risk of rare childhood disorders caused by loss of imprinting. Hormone stimulation and the culturing and manipulation of the gametes may disrupt the establishment and maintenance of important developmental marks such as DNA methylation and genomic imprinting. With the rising use of ART and fertility treatments, it has become increasingly important to examine what effect (if any) these technologies may have on epigenetic marks that are determined during gametogenesis and embryogenesis. Our main question in this study is, Do fertility treatments, including ovulation induction, alter epigenetic mechanisms such as DNA methylation at imprinted loci? We examined the effects of different methods of conception [ovulation induction ($n = 27$), *in vitro* fertilization ($n = 59$), and spontaneous ($n = 61$)] on DNA methylation patterns at six imprinted differentially methylated regions (DMR) (*MEST*, *KCNQ1*, *SNRPN*, *GRB10*, *H19*, and *IGF2*DMR0) in a birth cohort. Bisulfite pyrosequencing was performed on embryonic (cord blood) and extra-embryonic (placenta) biospecimens. Quantitative PCR was used to assess whether differences in methylation levels of imprinted DMRs affects the overall transcription levels of the genes corresponding to these elements. Data and biospecimens in this study were from participants of the Epigenetic Birth Cohort, comprised of 1,941 mother–child dyads recruited at Brigham and Women's Hospital. Overall, we found that the methylation levels across the ICRs of *GRB10*, *MEST*, *H19*, *SNRPN*, and *KCNQ1* as well as the somatic *IGF2*DMR0 were not disrupted by either ovulation induction or *in vitro* fertilization fertility treatments in cord blood and placenta in our birth cohort. We observed small but statistically significant differences in certain imprinting control regions based on the method of conception; however, these small changes in methylation did not correlate to the overall transcriptional levels of the genes adjacent to the ICRs (e.g., *H19, KCNQ1,* and *SNRPN*). This study provides novel epigenetic analyses on infants conceived by ovulation induction and examines how methylation levels correlate with gene expression. Our observations suggest that large methylation changes at imprinted genes following IVF are infrequent events, and are unlikely due to ovulation stimulation. As growing research using birth cohorts reveals limited adverse effects of reproductive technology on human development, the research focus should shift to understand how ART may influence long-term health outcomes as these children age. Moreover, future studies are needed to properly address any effect of the underlying fertility problem itself on these childhood imprinting disorders.

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A Genomic & Epigenomic Integration Approach on Obesity

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Recent efforts in large-scale genome-wide association studies (GWAS) have identified numerous loci associated with obesity or related traits. Emerging evidence also shows that epigenetic dysregulation plays an important role in the pathogenesis of obesity. We predicted that integration of genomic and epigenomic alterations would increase power in prioritizing top signals and provide extra information on causality. We applied this integrative approach to our previous genome-wide methylation profiling (Illumina HumanMethylation27 BeadChip) on 7 obese adolescents and 7 age-matched lean controls. First, we did a literature search to identify genes related to monogenic obesity and genes discovered by GWAS. Second, we merged these genes with our methylation profile to examine if the methylation levels of the CpG sites in these genes were also significantly different ($p < 0.01$) between the two groups. We further replicated the significant CpG sites in 46 obese subjects and 46 lean controls by using pyrosequencing

technology. Of total 108 genes identified from literatures, 81 were mapped on Illumina 27k Chip. Four CpG sites showed significant differential methylation between the obese and lean controls. They locate in 4 genes: LEPR (p = 0.002), SNRPN (p = 0.009), KREMEN1 (p = 0.009), and LY86 (p = 0.009). These 4 CpG sites were then validated in the replication cohort. The assays for two genes (SNRPN and LY86) have been finished and the other two are ongoing. For the target CpG site in LY86, the methylation level in obese subjects was significantly higher than that in lean controls (0.57 vs. 0.52, p = 0.009), consistent with the initial finding. In addition, 5 more CpG sites around were measured, and all showed significant hypermethylation in obese subjects (p ranges from 0.0003 to 0.03). After adjusted for age, 5 of 6 CpG sites still showed significant association with obesity. The target CpG site and a surrounding one in SNRPN did not show significant result. By merging recent GWAS findings for obesity and related traits, we re-analyzed our previous epigenomic data for obesity. LY86 gene was "double hit" and successfully replicated in a larger cohort. Our finding suggests that LY86 is an important gene in the pathophysiology of obesity. Moreover, the confirmation by GWAS provides the clue to the causal direction of epigenetic changes: The methylation changes in LY86 might be a cause rather than a consequence of obesity. Our study emphasizes the importance of integrating genomic and epigenomic alterations to improve our understanding of complex diseases.

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Critical Windows of Early Exposure & Sensitivity II

Richard Finnell, moderator

Epigenetic Marks of Impaired Glucose Tolerance in Pregnancy

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Recent studies have shown that development of diabetes is associated with changes in gene expression. DNA methylation is involved in regulation of gene expression. We hypothesized that DNA methylation patterns would be different among women with and without impaired glucose tolerance (IGT) in pregnancy. Since diabetes in pregnancy is associated with adverse effects in both mothers and their infants, including future risk of both diabetes and obesity, we sought to understand if hyperglycemia in pregnancy is associated with epigenetic alterations that may explain the risk of adverse events in mothers and offspring. We used PCR-pyrosequencing to quantify global methylation in *Alu* and *LINE-1* repetitive elements in bisulfite-treated DNA derived from peripheral blood leukocytes of 184 pregnant women. Gene-specific methylation was also assessed in the imprinted loci *H19* (non-protein coding RNA transcript promoter and *H19* ICR, differentially methylated domain) and *KCNQ1* (potassium voltage-gated channel, KQT-like subfamily, member 1) and the non-imprinted glucocorticoid receptor gene (*GCR*). The degree of methylation was expressed as the percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines (%5mC). IGT was assessed at 24–28 weeks gestation by blood glucose measured 1 hour after a 50 g challenge and defined as glucose ≥ 140 mg/dL. Global DNA methylation was correlated with blood glucose (*Alu* rho = 0.26, p = 0.0004; *LINE-1* rho = 0.13, p = 0.09). Women with IGT had global hypermethylation in *Alu* when compared to women without IGT (p = 0.002) but showed no differences in *LINE-1* methylation. Gene-specific methylation in GCR was also correlated with blood glucose (rho= 0.29, p = 0.0001; 52.25% vs. 49.96% comparing women with and without IGT, p = 0.0009). The distal region of the *H19*-associated imprinting center (ICR1) was also hypermethylated (p = 0.006), while the *H19* promoter was hypomethylated (p = 0.002), in women with IGT compared to those without IGT. In this population, IGT during pregnancy was associated with epigenetic alterations in both imprinted and nonimprinted loci related to growth, which provides a novel mechanism that may explain the adverse maternal and fetal effects associated with hyperglycemia in pregnancy and long-term risk of developing diabetes and obesity. It will be important next to measure the effects among infants born to these women and to validate these results in larger and other populations.

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DNA Methylation of 11βHSD2 Correlates with Infant Growth

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The placenta plays an important role in maintaining fetal endocrine autonomy. The enzyme 11beta-hydroxysteroid dehydrogenase type 2 (11βHSD2) converts maternal cortisol to cortisone, preventing passage into the fetal–placental compartment. Placental 11βHSD2 is highly regulated. Prior studies have demonstrated an inverse relationship of 11βHSD2 activity and birth weight. We sought to evaluate the role of epigenetics in regulation of 11βHSD2. We evaluated the DNA methylation in the promoter region of 11βHSD2. We compared the extent of methylation to the infant's birth weight and birth weight percentile. Placentas from 150 term births were collected within 2 hours of delivery and transferred to RNAlater™. Placental genomic DNA was extracted and bisulfite treated. Modified DNA was pyrosequenced examining 4 CpG sites in the promoter region of 11βHSD2. Birth weight percentiles were determined using the Fenton growth chart algorithm by the gestational age and birth weight into small for gestational age (SGA), ≤ 10%, appropriate for gestational age (AGA), 11–89%, or large for gestational age (LGA), ≥ 90%. *p*-Values are calculated using Kruskal–Wallis one-way analysis of variance test and Dunn's multiple comparison test. A consistent increase in 11βHSD2 methylation was found in all four CpG loci with the extent of methylation ranging from 2% to 27%. The mean of all 4 CpG sites varied from 6.5% to 19%. The data were normally distributed with all four CpG loci examined. There was an increase in methylation with an increase in birth weight ($p = 0.0031$). Dunn's multiple comparison test revealed significant differences in mean methylation between both SGA and AGA, and SGA and LGA infants, with $p < 0.05$. The expression of 11βHSD2 is under tight regulation in the placenta. Previous studies have demonstrated a decrease in 11βHSD2 activity with increasing birth weight. Epigenetic mechanisms such as DNA methylation may be involved in this regulation. Multivariable regression will be used to explore the association between methylation and birth weight, controlling for infant sex, maternal age, maternal body mass index, and maternal smoking during pregnancy. Associated expression analyses will also be performed to solidify the relationship between 11βHSD2 DNA methylation and expression.

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Birth Defects as Clues to the Etiology of Cancer in Children

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The etiology of childhood cancer is largely unknown. Although a small proportion is thought to be directly attributable to a genetic syndrome, we do not know how most pediatric malignancies may relate to genetic alterations not readily linked to a syndrome or chromosomal anomaly, nor the degree to which environmental exposures, gene–environment interactions, or epigenetic factors may play a role. For clues to these associations, we examined whether the incidence of childhood cancer is elevated among children with birth defects, including those with and without chromosomal anomalies. We examined cancer risk in a population-based cohort of over 3 million children with and without major birth defects, born 1988–2004, by linking data from the California Birth Defects Monitoring Program, the California Cancer Registry, and birth certificates. Cox proportional hazards models generated hazard ratios (HRs) and 95% confidence intervals (CIs) based on person-years at risk to compare risk of childhood cancer among infants born with and without specific types of birth defects. Among 4,869 cancer cases in the birth cohort, 222 had a major birth defect. While expected elevations in cancer risk were observed among children with chromosomal birth defects (HR = 12.44; 95% CI: 10.10, 15.32), especially for the leukemias (HR = 28.99; 95% CI: 23.07, 36.42), children with nonchromosomal birth defects also had elevated overall cancer risk (HR = 1.58; 95% CI: 1.33, 1.87). The risk was not elevated for leukemia in this group, but instead for brain tumors, lymphomas, neuroblastoma, and germ cell tumors. In this study, one of the largest such studies to date, we saw that children with nonchromosomal birth defects exhibited increased risk for solid tumors, but not leukemia. Dysregulation of early human development likely plays an important role in the etiology of childhood cancer, and these findings underscore the importance of the *in utero* period as a critical window of susceptibility. Next steps include continued inquiry of the predisposition to cancer among young children with structural birth defects in order to help elucidate etiologic commonalities between these two outcomes, and exploration of the environmental exposure and genomic profiles of children with cancer, with or without birth defects.

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The Effects of Prenatal Alcohol Exposure on Choline Metabolism: How Epigenetic Mechanisms May Mediate Alcohol's Teratogenic Effects

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Prenatal alcohol exposure leads to a diversity of physical and behavioral abnormalities. The severity of these consequences varies widely, and prenatal nutrition is an important factor contributing to variation in fetal alcohol spectrum disorders (FASD). Epigenetic mechanisms may have an underlying role in the adverse effects of alcohol as well as the protective effects of nutritional supplementation in FASD. Choline, an essential nutrient, is an important methyl group donor and influences brain development. Evidence shows that pre- and postnatal supplementation can reduce some fetal alcohol effects. Clinical and animal studies were combined to examine if alcohol influences choline metabolism during pregnancy in order to understand whether choline supplementation compensates for an alcohol-related nutritional deficiency. Two groups of pregnant women participated in the clinical component of this project: women who reported moderate to heavy drinking during pregnancy ($n = 85$) and those who reported little or no alcohol use ($n = 83$). These women were recruited as a part of a longitudinal prospective study in the Ukraine examining maternal nutritional status and its contribution to FASD risk. Baseline maternal blood samples were analyzed for choline, betaine, and dimethylglycine (DMG). In a parallel rodent study, Sprague-Dawley dams were intubated with 6.0 g/kg/day ethanol on gestational days (GD) 5–20; yoked pair-fed and ad libitum chow controls were included. Levels of plasma choline, betaine, and DMG were measured on GD10, 15, and 21 in dams and fetuses. In the clinical study, the two groups of pregnant women showed no difference in plasma concentrations of any nutrient. In the animal study, prenatal alcohol exposure did not decrease choline levels in either dams or fetuses. However, there were age-dependent effects of alcohol on plasma betaine levels and a transient decrease in plasma DMG in alcohol-exposed dams during the period of intoxication. These data suggest that alcohol does not induce a choline deficiency, but may transiently interfere with choline-related pathways, which may contribute to alcohol's damaging effects on the fetus. Given the importance of betaine and DMG in the methionine-homocysteine cycle, interference in this pathway due to prenatal alcohol exposure may lead to epigenetic changes in the fetus. These findings have important implications for the elucidation of mechanisms by which alcohol disrupts development and may lead to a better understanding of how alcohol–nutrition interactions may influence alcohol's teratogenic effects as well as the mechanism by which choline supplementation may ameliorate the severity of brain and neurobehavioral impairments in FASD.

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Session I: The Association between Epigenetics and Pediatric Disease

Rachel Miller, moderator

Epigenetics in Epidemiologic Research

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As the prevalence of childhood obesity continues to escalate almost unabated in developed countries, etiologic studies of obesity have focused on the interaction between genetic variants and modifiable risk factors that encompass energy balance, estimated as caloric intake less caloric expenditure. However, an alternative explanation is that the environment directly induces dysregulation of epigenetic mechanisms that guide expression of genes involved in energy balance, culminating in gene expression profiles that predispose to an obese phenotype. Because imprinted genes are regulated by epigenetic mechanisms, and because they are normally expressed from only one parental chromosome, they are particularly vulnerable to genetic and/or epigenetic deregulation. Aberrant DNA methylation at imprint regulatory elements of the paternally expressed growth factor, *IGF2,* have been associated with increased risk of obesity and overgrowth disorders. *DLK1* is elevated in the circulation of obese humans, and both *DLK1* and *NNAT* are involved in adipogenesis. Mechanisms associated with imprint deregulation are only now being unraveled. Feeding female mice a methyl group donor-rich diet during pregnancy triggers altered methylation of the *Agouti* locus in pups, which subsequently modulates risk

of obesity and diabetes. There is a higher propensity for persistent childhood obesity in children born to mothers who smoke during pregnancy, and our data support that *IGF2* is epigenetically altered and up-regulated in neonates born to smoking mothers. However, outside cigarette smoking, other exposures have not being examined. The goal of our research program is to identify early exposures that increase the risk of epigenetic dyregulation, resulting in alterations in gene expression imprinted genes. Our central hypothesis is that such early dysregulation of epigenetic profiles is stable throughout the individual's life course, and that it orchestrates risk of common chronic diseases. We have previously proposed that these stable epigenetic marks can be developed to serve as biosensors or archives of past exposure. This could be useful in retrospective studies where recall concerns, especially if differential among groups being compared, could lead to spurious findings. Also, because there necessarily has to be some degree of malleability in the epigenetic marks, we posit that some could be useful biosensors to monitor long-term interventions. We present a summary of results for some of the work aimed at developing these potential markers of early exposure.

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Neonatal Epigenetic Signatures of Asthma and Asthma-Protective Exposures

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Asthma can currently be managed but not really cured. Therefore, prevention would be an ideal approach to this disease. Obviously, availability of asthma predictors detectable in early life, or even better at birth, would greatly improve the effectiveness of prevention by identifying those individuals within the population for whom more drastic, and therefore less easy to implement, preventive measures might be warranted. Because asthma typically begins in early life, and the asthma status of the child is strongly related to that of the mother, we have hypothesized that signatures detectable in the epigenome, and more specifically in the methylome, at birth can serve as predictors of asthma status later in life. To test this hypothesis, we have assessed genome-wide patterns of DNA methylation in cord blood samples isolated from Tucson children enrolled in the Infant Immune Study (IIS). The longitudinal nature of the IIS study offers a unique opportunity to address and answer questions about early epigenetic predictors of asthma. Indeed, the cord blood donors have now reached an age (8–9 years) at which a firm diagnosis of asthma can be established. Moreover, in order to assess whether a farming environment, a prototypic asthma-protective exposure, is associated with specific epigenetic signatures, we have also examined genome-wide methylation patterns in DNA isolated from the cord blood of European children born to mothers exposed or not exposed to farming during pregnancy. The results and implications of both these analyses will be discussed.

DNA Methylation Patterns in Siblings with and without Asthma

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Our group previously demonstrated that *in utero* dietary supplementation with methyl donors regulates locus-specific DNA methylation and predisposes mice to allergic airway disease by skewing towards a Th2 phenotype. To further investigate the role of epigenetics in asthma, we used genome-wide DNA methylation profiling to identify differentially methylated regions (DMRs) associated with asthma development in sibling pairs from the MRCA cohort of British families that were ascertained through an affected proband with at least stage 3 asthma. Using the comprehensive high-throughput arrays for relative methylation (CHARM) method, we collected DNA methylation profiles in peripheral blood of 48 discordant (one asthmatic and one nonasthmatic sibling) and 46 concordant (both asthmatic siblings) sibling pairs. Using percent methylation estimates from the normalized and scaled data set, we applied a linear regression model that fits percent methylation to asthma status, age of the two siblings, sex of both siblings, and family clustering. Asthma-associated DMRs appeared throughout the genome, mostly in CpG island shores (87%) and in gene bodies (61%). Nineteen percent of DMRs are in promoters while 61% are in gene bodies. The average length of the DMR encompasses 16 probes (560 bases) and average percent methylation change in asthmatics compared to controls is 14%. A number of DMRs are near genes that have previously been associated with pathophysiology of asthma, namely cyclooxigenase 2 (Cox-2 or Ptgs22), inducible nitric oxide synthase (iNOS or NOS2), and one of the subunits of the IL-17 receptor (IL17RD). In a recent study of 940 children, hypomethylation of iNOS was also associated with short-term exposure to particulate matter ($PM_{2.5}$), consistent with our results demonstrating hypomethylation of iNOS (*q*-value = 0.00988; 10% hypomethylation; 540 bases length) in

asthmatic compared to nonasthmatic children. The majority of DMRs we identified, however, are associated with genes that have potential to be involved in immune regulation of asthma but have not been explored. One such DMR (q-value = 1.2E–5; 17% hypomethylation; 610 bases length) is in the intron of one of the isoforms of Lmo1, a transcription factor that binds GATA3 and maps to an area of consistent chromosomal translocation on chr 11 that disrupts the gene in T-cell leukemia. We have confirmed direction of methylation change for three asthma-associated DMRs by pyrosequencing and are in the process of pyrosequencing additional DMRs for confirmation. Our analysis identified novel differentially methylated sites associated with asthma. Understanding epigenetic regulation of biological processes in the lung may lead to the development of novel diagnostic and therapeutic approaches for asthma.

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Session II: The Association between Epigenetics and Pediatric Exposures

Cynthia Bearer, moderator

Maternal Tobacco Exposure Is Associated with Alterations in Placental DNA Methylation and Gene Expression

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While many fetuses are exposed to tobacco *in utero*, not all experience adverse outcomes as a result of this exposure. Specific genetic polymorphisms alter the metabolism of polycyclic aromatic hydrocarbons (PAHs) found in tobacco. We have reported that these polymorphisms are associated with a reduced birth weight. However, the potential for epigenetic changes in the fetus to contribute to altered PAH metabolism remains largely unexplored. We have recently reported that the promoter of CYP1A1, a gene involved in PAH metabolism, is hypomethylated with maternal tobacco exposure. This hypomethylation correlates with an increase in gene expression. We wanted to further this study on a genome-wide scale. We aimed to study the correlation between DNA methylation and gene expression in placenta from smokers and nonsmokers. Placentas were collected within a half hour of delivery and frozen at –80°C for later use. A total of 36 subjects, 18 smokers and 18 nonsmokers, were matched for maternal age and ethnicity. RNA was extracted and hybridized to the Illumina WG6 expression bead chip which contains > 48,000 probes. DNA was extracted, bisulfite treated, and hybridized to the Illumina Infinium HumanMethylation 27 bead chip, which probes over 27,000 individual CpGs. Data was analyzed using Partek and Ingenuity Pathway Analysis (IPA) software. Pearson coefficients were determined using R. We found that 1,024 specific CpG dinucleotides are differentially methylated and 623 genes are differentially expressed between smokers and nonsmokers in the placenta. Significant correlation of methylation and expression data (identified as having a Pearson correlation ≥ 0.7 or ≤ –0.7) reveals that within the cohort exposed to maternal tobacco smoke, expression of 438 genes correlates with DNA methylation. Only 25 genes show such a correlation in the nonsmoking group. The genes that showed a significant correlation in the smoking cohort were analyzed using IPA. We found that members of the oxidative phosphorylation, mitochondrial dysfunction, and the hypoxia inducible factor (HIF) signaling pathways are enriched in this list. Maternal tobacco smoke exposure is associated with a modest alteration in genome-wide DNA methylation in the placenta. We show that these alterations correlate with meaningful changes in gene expression. Our studies indicate that the complex interplay of genomic and epigenomic factors may contribute to specific phenotypic outcomes and can help begin to elucidate the differential susceptibilities to tobacco smoke *in utero*. We propose that genome-wide site-specific changes in DNA methylation contribute to the mechanism of altered susceptibility to *in utero* tobacco smoke.

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DNA Methylation during Development and Alteration by Alcohol Exposure

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Fetal Alcohol Spectrum Disorder (FASD), resulting from drinking during pregnancy, is the leading cause of mental retardation. How alcohol as an environmental factor leads to brain retardation in FASD is unclear. Epigenetics is key to development and tissue specification. Our recent understanding of alcohol on epigenetics leads to a potential mechanism for FASD. DNA methylation is known to regulate embryonic stem cell and zygotic development. Here, we demonstrate how DNA methylation is a program that mediates neural tube

maturation and brain development. And we demonstrate how alcohol, which is known to affect the methyl donor, alters the program to affect the progression of neural differentiation and brain development. Embryos as well as neural stem cells during development were exposed to alcohol in culture or through maternal drinking to allow specific cellular and genomic analyses. The cellular distribution of DNA methylation marks including 5-methylcytosine (5mC) and 5-hydroxylmethylcytosine (5hmC), the 5hmC synthesis enzyme (Tet1/2), and binding proteins (MBD1 and MeCP2) were analyzed with immunocytochemistry to elucidate the temporal and spatial distribution. The genomic DNA methylation was also analyzed using DNA methylation immunoprecipitation followed by microarray chip assay (MeDIP-Chip). We demonstrated that *de novo* 5mC marked the neural stem cell differentiation temporally in the neural tube. Further, 5mC, 5hmC, and Tet1/2 were brain-region and neuronal-type specific, and temporally progressed with regional brain maturation. The MBD1 and MeCP2 arrived in distinctly different patterns during neuronal migration and both increased progressively over neuronal maturation. At the genomic level, a featured diversification of moderately methylated genes into hypo- and hypermethylation occurred during differentiation. These orderly cellular and genomic DNA methylation changes dovetailed with the progression of maturation of the nervous system—we referred to this as the DNA methylation program (DMP). Alcohol was found to deter the progression of the DMP during neural tube and brain development. We demonstrated that the intricate and long DNA methylation program leaves wide entries for environmental inputs. Alcohol exposure altered the methylation program described above. The alcohol's alteration of the intricate DNA methylation program is at cellular as well as genomic levels. These findings add further support that alcohol through epigenetics is a key mechanism underpinning neurodevelopment deficit in FASD.

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Behavioral Experience Determines Pb and Stress Effects

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Behavioral experience is a critical determinant of later behavior and brain function. Yet animal models typically evaluate the effects of developmental insults in the absence of any context, even though the human environment is highly dynamic: All organisms undergo a wide variety of behavioral experiences that have the potential to enhance or ameliorate the consequences of these developmental insults. This study examined the hypothesis that contrasting behavioral experiences would modify the trajectory of CNS consequences of low-level lead (Pb) exposure, prenatal stress (PS), or combined Pb and PS in a mouse model. Mice exposed to 0 or 100 ppm Pb from 2 months prior to dam breeding (blood Pb ~7 µg/dL), to prenatal restraint stress (30 min 3x/day to dams from approximate gestational days 11–19), or to Pb+PS, were subjected, over the same time period, to behavioral experience that was either positive [food rewarded responding on a fixed interval (FI) schedule of reinforcement] or negative [4 forced swim (FS) tests at 3-week intervals]. Levels of monoamines and glutamatergic neurotransmitters in brain regions associated with executive function (hippocampus, frontal cortex, ventral striatum) were measured at the termination of the imposed behavioral experience. Different behavioral experience significantly altered levels of many monoamine and glutamatergic neurotransmitters in multiple brain regions (hippocampus, frontal cortex, striatum, and midbrain) even in controls. Different behavioral experiences also altered the direction and/or magnitude of Pb, PS, and Pb+PS-induced changes in multiple neurotransmitters. These neurotransmitter changes differed by sex and were related to subsequent Pb±PS-induced behavioral effects. For example, Pb- and PS-induced alterations in FS behavior in test 1 in males correlated significantly with the Pb, PS, and Pb+PS-induced increases in striatal 5-HIAA and frontal cortex GABA levels produced by FS but not FI experience. The Pb- and PS-induced alterations in FS test 1 in females correlated significantly with Pb- and PS-induced reductions in frontal cortex dopamine turnover and increases in hippocampal GABA produced by FS but not FI experience. Behavioral experience can modulate the ultimate CNS consequences of developmental Pb, PS, or Pb+PS, producing neurotransmitter differences related to corresponding behavioral effects. It seems likely that other CNS developmental insults would also be influenced by early behavioral experience. We are currently beginning to examine differences in epigenetic profiles associated with Pb, PS, and Pb+PS, focusing initially on methylation (hippocampus) and acetylation (frontal cortex) changes in the glucocorticoid receptor (GR) gene Nr3c1 given the impacts of both Pb and PS on GR function. We also seek to determine the extent to which specific behavioral experiences might restore 'normal' epigenetic profiles and attenuate Pb, PS, and Pb+PS CNS effects.

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Poster Presentation Abstracts

(*Presenting authors in boldface type*)

Role of Mercury in the Alteration of Global DNA Methylation

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According to the National Health and Nutrition Examination Survey, the blood mercury concentrations of 15.7% of childbearing-age women were above the level that would cause developmental effects in their children. Methylmercury (MeHg) has been shown to be associated with epigenetic modifications. This study was conducted to identify the changes in global DNA methylation due to MeHg exposure. DNA methylation has been identified as one of the molecular mechanisms behind the physiological deficits due to exposure to environmental toxicants. The hypothesis of the study was that MeHg exposure during the embryonic stage would be associated with alteration in DNA methylation. The objective of the study was to identify the change in DNA methylation in zebrafish due to direct exposure to MeHg. Zebrafish embryos were treated with 0.0, 0.001, 0.003, 0.01, 0.03, and 0.1 µM MeHg solutions for 24 hr. The adult fish were raised from the treated embryos and maintained according to standard procedures. The brain tissues of adult fish were removed and immediately placed in RNAlater and flash frozen. Genomic DNA from the brain tissue was isolated using DNeasy kit. The overall methylation status of the genomic DNA isolated from the brain tissues was identified using LUminometric Methylation Assay by Qiagen PyroMark Q96D pyrosequencer. Global methylation data was analyzed using SigmaPlot 11.0 software. The relationship between the DNA methylation and MeHg exposure was analyzed with one-way ANOVA. The global methylation pattern of the MeHg exposed zebrafish brain tissues were identified by LuMA. The percentage of methylation was identified by the ability of restriction enzymes to differentially digest methylated DNA from unmethylated DNA. The method of collection of brain samples and the amount of DNA used for digestion were significant in the sensitivity of the assay. DNA isolated from brain samples collected in RNAlater and immediately flash frozen showed lesser degradation compared to the tissues that were only flash frozen. The assay was also sensitive to the amount of DNA used for enzyme digestion. Global DNA methylation was identified as percentage by the assay. The range of values of percentage of DNA methylation was between 78 and 86. One-way ANOVA analysis showed that the percentage of global methylation in the brain tissues of treated animals was not significantly different than the control animals. Global hypomethylation and site-specific hypermethylation are the hallmarks of various diseases including cancer. This study investigated the role of MeHg exposure in the alteration of DNA methylation in the brain tissues of zebrafish. Embryonic exposure to MeHg did not significantly alter the global DNA methylation pattern in zebrafish. It has become clear that epigenetic alterations, particularly gene silencing via DNA hypermethylation, plays a critical role in the initiation and progression of diseases. The next step of this study is to identify gene specific alteration of methylation pattern due to MeHg exposure by Methylated DNA immunoprecipitation (MeDIP)-Chip assay.

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Ventilation Affects Histone Marks in Lung and Brain of Preterm Lambs

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Mechanical ventilation (MV) injures the lung and brain of chronically ventilated preterm neonates. Our studies using chronically ventilated preterm lambs show that both organs have genome-wide *hypo*acetylation of histones. Lung and brain injury is reduced and genome-wide *hyper*acetylation of histones occurs with a gentler ventilation mode: nasal high-frequency ventilation (HFV; similar to nasal CPAP). Dichotomy of genome-wide acetylation state of histones between MV and nasal HFV suggests that epigenetic mechanisms may participate in the pathogenesis of multiple-organ injury that typifies neonatal chronic lung disease (CLD). We hypothesized that treating preterm lambs with histone deacetylase (HDAC) inhibitors during MV would lead to histone *hyper*acetylation and reduce injury to the lung and brain. Preterm lambs (~132 days gestation; term ~ 150 days), treated with antenatal steroids and postnatal surfactant, were managed by MV, MV+valproic acid (VPA; nonspecific HDAC inhibitor), MV+trichostatin A (TSA; specific HDAC inhibitor), or nasal HFV (n = 4/group). Each inhibitor was given once/day, intravenously. At the end of 3 days, lung parenchyma and temporal lobe white matter were analyzed by immunoblot for acetylated (ac) H3K14, H3K27ac, H3K18ac, trimethylated (me3) H3K36, and histone deacetylase (HDAC). Immunoblot results were normalized for total H3. Structural

measurements were made of alveolar formation in the lung and apoptosis/proliferation of neurons and glia in the brain. Histone marks H3K14ac, H3K27ac, H3K18ac, H3K36me3 were significantly lower ($p < 0.05$) in the lung and temporal lobe of preterm lambs that were supported by MV than nasal HFV. HDAC protein abundance was significantly higher in the MV group. During MV, inhibition of histone deacetylation, with either VPA or TSA, significantly increased H3K14ac, H3K27ac, H3K18ac, H3K36me3 in the lung and white and gray matter of the brain. HDAC protein abundance was significantly decreased in both organs. Structurally, the lung of both groups of treated preterm lambs had significantly greater morphometric indices of alveolar formation in the lung. Markers of apoptosis (cleaved caspase 3) and proliferation (proliferating nuclear cell antigen) were significantly lower in white matter of the temporal lobe of the brain. Treating preterm lambs with HDAC inhibitors during MV leads to histone *hyper*acetylation and reduces injury to the lung and brain. These molecular and structural outcomes are similar to the outcomes following support of preterm lamb with nasal HFV. These results suggest an epigenetic mechanism for the pathogenesis of neonatal CLD. We speculate that the lung epigenetic and structural effects are triggered by ventilator-induced stretch. We further speculate that the brain epigenetic and structural effects result from release of pro-inflammatory cytokines and/or chemokines from the injured lung that triggers local inflammatory responses in the brain.

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Global Methylation as a Biomarker of Diesel Exhaust

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In adults, various components of diesel exhaust particles (DEP) have been shown to induce global DNA hypomethylation in blood after recent exposure; however, most studies are limited by having used surrogate measurements to estimate the change in global methylation and not having controlled for secondhand smoke exposure over time. Further, the effect of DEP on global methylation in children remains unclear. The objective of this analysis was to evaluate the difference in global methylation among children with high and low DEP exposure using saliva DNA. We hypothesized that global demethylation would be significantly associated with early childhood exposure to DEP compared with children exposed to low levels of DEP. The Cincinnati Childhood Allergy and Air Pollution Study completed exposure assessments from birth through age 7 years. Time-weighted average daily DEP exposure was estimated from a land-use regression model. Children who had never moved and had no reported exposure to secondhand tobacco smoke were randomly selected ($n = 92$). High and low exposure to DEP was defined as having an exposure above ($n = 23$) or below ($n = 69$) the 75th percentile at age 6 months. Global methylation was assessed as methylated cytosine using the MethylFlash™ Methylated DNA Quantification Kit with 50 ng of genomic DNA from saliva. The association between global methylation and dichotomized OEP was analyzed using linear regression. Of the children selected, 80% were Caucasian and 58% male. The time-weighted average daily exposure to DEP of the high and low exposed groups was 0.57 µg/m^3 and 0.30 µg/m^3, respectively. The average percent methylated cytosine (SD) in the high group was 1.39 (0.81) and 2.23 (1.32) in the low group. In the high versus low exposure group, the percent methylated cytosine was significantly decreased ($p < 0.01$). This finding was further validated by a regression model, which controlled for race and sex, showing an inverse relationship between DEP exposure and global methylation ($p < 0.01$). Children exposed to high levels of OEP exhibited decreased methylated cytosine suggesting global methylation may serve as a biomarker of DEP exposure. Global methylation levels may potentially be used to identify children at greater risk for the development of asthma and allergic disease; however, this requires additional investigation. Further, this study utilized a method which analyzes global methylation in the total genome rather than using surrogate markers, such as LINE1 and Alu, to estimate changes, and is the first study to utilize saliva as a DNA source to experimentally determine the level of global methylation with respect to DEP exposure.

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Endocrine Disrupters and Obstetric Complications in a Cohort of Massachusetts Women

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Phthalates and phenols are found in many personal care products and household items, thereby exposing both women and their developing fetuses. Preconception exposure is thought to increase obstetrical complications such as infertility while *in utero* exposure is hypothesized to alter the course of development. We investigated the hypotheses that first-trimester urinary phthalate and phenol concentrations were positively associated with obstetric outcomes, including use of assisted reproductive technology (ART), gestational diabetes, and preterm birth, while decreasing maternal weight gain, birth weight, and birth length. We prospectively followed women concurrently enrolled in two large birth cohorts at Brigham and Women's Hospital in Boston, Massachusetts: the Predictors of Preeclampsia Study (POPS) and the Epigenetic Birth Cohort (EBC). We examined associations between urinary phenols and phthalates and binary obstetric outcomes (ART, gestational diabetes, and preterm birth) using multivariate logistic regression and associations between urinary phthalates and phenols with continuous outcomes (pregnancy weight gain, birth weight, and birth length) using multivariate linear regression. The study population included 196 women who ranged in age from 19 to 43 years. A total of 11.7% used ART, 4.6% were diagnosed with gestational diabetes, and the mean weight gain was 34.0 lbs with a range of –2 to 76 lbs. An estimated 6.1% of the infants were born preterm; mean birth weight and birth length were 3,434 g and 19.4 inches, respectively. Compared to women with mEHHP concentrations in the lowest quartile, women in the highest quartile had 2.07 times the odds (95% CI: 0.64, 6.73) of having ART ($p = 0.02$). Relative to infants born to mothers in the lowest quartile of mCOP concentration, infants born to mothers in the highest quartile had 15.62 times the odds (95% CI: 0.95, 257.52) of being born preterm ($p = 0.01$). All other associations were null for both individual and summed urinary phthalate and phenols concentrations. We observed little association between urinary phthalate and phenol concentrations with six maternal and fetal complications. Future studies should assess this association throughout the preconception and gestational periods since a critical period, in which exposure is most relevant, may be present and multiple samples can capture any within-person variation. Meanwhile, improved labeling of products containing phenols and phthalates could help pregnant women, among others, avoid exposure.

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Indian Children's Exposure to Poisonous Substances: Prospective Assessment of Patterns, Severity, and Clinical Outcome

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Children's exposure to poisonous substances (e.g., pesticides, household products, medicines) in rural and urban settings is a growing concern worldwide. Children have multiple sources and routes of poisonous substances exposure that differ from adults. Prospective assessment of patterns and severity using clinical indices helps to provide intense monitoring, treatment, and public assistance to prevent future exposure. This study assessed the patterns, severity, and clinical outcome of poisonous substances exposure in the pediatric population by a prospective method in Indian tertiary-care hospitals. A prospective assessment was conducted over a period of 1 year. Poisonous substances exposure cases were identified from the medico-legal case register and relevant data were collected from the patients' case notes, treatment chart, nursing notes, laboratory reports, discharge summaries, patient's relatives or caretakers, and health care professionals as appropriate. Glasgow coma scale (GCS) and poisoning severity score (PSS) were used to predict the severity of illness caused by exposure. The sensitivity/correlation of severity grade/score with the clinical outcome was evaluated. About 23% and 43% of children were exposed to pesticides and household products, respectively. Accidental exposure (55%) was found to be the major cause of poisoning. Children 0–5 years of age had higher accidental exposure. Male children had higher exposure than female children. Exposure cases were higher in urban area (42.7%), followed by rural (31.7%) and semi-urban (25.6%) areas. Exposure was higher in middle (60%) or poor (30%) socioeconomic class children when compared to rich (1%) socioeconomic class children. A majority (95%) of children with mild to moderate predicted severity level were recovered from the illness. Notably, children with severe predicted illness were either discharged with severe morbidity or expired. There was a significant ($p < 0.001$) correlation between predicted severity level/score and clinical outcome, thereby indicating excellent sensitivity of GCS and PSS to predict the severity of exposure. Notably, we observed moderate correlation ($r = 0.51$, $p < 0.001$) between GCS and PSS systems, thereby similar efficacy of both the

clinical indices. The current study demonstrated the exposure patterns, causes of incidences, and simple, easy, and sensitive clinical indices to predict the severity of exposure in the hospitals. Next, we will implement this prospective assessment as a part of treatment protocol in the hospitals. This approach focuses equally on treating and preventing the probable causes and recurrence, and changing children's knowledge about poisonous substances.

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Type 2 Diabetes in Belize: Education & Analysis

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In May, 14 undergraduate students from Boston University traveled to Punta Gorda, Belize, to teach elementary school students about general health, positive lifestyle choices, and risk factors and prevention of type 2 diabetes. The Boston University students organized and staffed health clinics in Punta Gorda and surrounding communities. The students screened for general precursors and risk factors for type 2 diabetes. This paper will show the impact of health education on general health and awareness of type 2 diabetes risk factors in a lower socioeconomic status cohort in Belize. The interesting findings of this study include a lack of relationship between Belizeans' body mass index and blood glucose levels, irrespective of the most significant risk factor for type 2 diabetes being obesity. Two types of health clinics were run where 234 willing adults participated from communities surrounding Punta Gorda. The first type of health clinic was mobile where the students traveled from residence to residence in six villages surveying those that were willing to participate in diabetes screening. The second type of clinic was a walk-in clinic that was held at the local schools and in the town center of Punta Gorda. Signs were posted describing the purpose of the clinics and participants willingly volunteered. There were stations set up for height and weight, as well as tables with three Boston University students recording blood glucose levels (mh/dL), blood pressure, age, sex, and time from last meal. The prevalence of type 2 diabetes is higher in the more rural communities where their diet is more limited than to the community in Punta Gorda. There were 234 surveyed participants; however, 181 of them had complete data. The average age was 38 years old, the average blood glucose level was 143 mg/dL, the average weight was 151 lbs, and the average blood pressure was 129/77. Blood glucose levels of 126 mg/dL or above are classified as high levels. 52.6% of the participants had high levels of glucose in their blood. The highest blood glucose level was over 600 mg/dL and the highest BMI was 40. Of those that were above normal blood glucose levels, less than 5% said they felt symptomatic. The prevalence of type 2 diabetes is rising in Belize due to an increase in obesity and physical inactivity, aging, population growth, and urbanization. In a holistic approach to combat these factors, the present cross-sectional study took part in increasing the general public's knowledge regarding overall health. Based on this data and methodology, Boston University is in the planning stages to transform this pilot service trip into a sustainable yearly project due to its community-focused approach and benefits of type 2 diabetes risk reduction education.

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EDCs and Childhood Obesity—Exposure Assessment

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Prevalence of obesity continues to grow worldwide, as does production of chemicals. Several chemicals may disturb hormonal function (EDCs). In multiple cohorts the effects of perinatal exposure to EDCs on the risk of childhood obesity have been studied, but conclusive evidence of the obesogenic effect of EDCs is not observed. However, matrix type and level of exposure vary across cohorts. Our goal was to review effects of EDCs on obesity in the literature according to type of matrix and level of exposure. A systemic review of the literature (PubMed) was performed. Primary search terms were specific endocrine disruptors combined with obesity related terms, all with the limiters "English" and "human." Studies were included only if they measured perinatal exposure and specifically reported matrix type and exposure levels. To increase comparability, studies were excluded if exposure was not reported as or could not be converted to lipid corrected values. Dose–response information was available predominantly for PCB and DDE, which were assessed in maternal serum collected during pregnancy, cord blood, or breast milk. PCB exposure ranged from 1.8 pg/g lipid, measured in breast milk, to 1.52 µg/g lipid, also measured in breast milk. DDE exposure ranged from 113 ng/g lipid, measured in breast milk, to 6.85 µg/g lipid, measured in maternal blood. Few effects of exposure are seen when assessment was done in breast milk. For PCB, only between the range of 117 ng/g lipid to 696 ng/g lipid relations are observed with growth outcomes. Both in the lowest range of exposure as well as in the highest range no significant long-term changes in growth are observed. Regarding DDE a dose–response relation is less clear. The obesogenic effect of perinatal EDC exposure appears to depend on the type of matrix in which exposure was assessed and the level of exposure. Therefore studies relating EDC exposure to growth related outcomes should be interpreted with great caution. Further studies to

systematically elucidate the effect of EDC exposure levels on childhood obesity are warranted.

Prenatal Folic Acid Supplements and Infant Bronchiolitis

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Bronchiolitis, a viral lower respiratory tract infection, affects 20% of infants and is a risk factor for asthma. Maternal folic acid supplementation is recommended during the periconceptional period to prevent neural tube defects; however, there is a question of whether supplementation, through potential epigenetic effects on the fetal immune system, may increase the risk of infant bronchiolitis. This study examines the association of maternal prenatal vitamin supplementation, the primary source of supplemental folic acid during pregnancy, with increased risk of bronchiolitis during infancy. We tested the hypothesis that there is a timing-dependent association of folic acid supplementation during pregnancy with an increased risk of bronchiolitis during infancy. We conducted a retrospective cohort study of term infants in TN Medicaid, 1995–2007. Maternal prenatal vitamin prescription filling was ascertained using linked administrative and pharmacy data. Maternal prenatal vitamin use was categorized as prescription filled in first trimester only, only after first trimester, during and after first trimester, or none filled. The outcome was an infant health care visit with a bronchiolitis ICD-9 diagnosis. We assessed the association of maternal prenatal vitamin category with bronchiolitis using multivariable logistic regression after adjusting for birth weight, sex, and maternal race, asthma history, age, prenatal care adequacy, smoking and education. A total of 167,333 mother–infant dyads were included. Overall 10% of mothers filled prescriptions in the first trimester only, 26% only after the first trimester, 45% both during and after the first trimester, and 19% did not fill a prescription. The prevalence of infant bronchiolitis was 25%, 19%, 22%, and 20% for the aforementioned categories, respectively. Infants whose mother filled a prescription only during the first trimester or during and after the first trimester had increased relative odds of bronchiolitis compared to infants whose mother did not fill a prenatal vitamin prescription during pregnancy, with adjusted odds ratios and 95% confidence intervals (CI) of 1.17 (95% CI: 1.11, 1.22) and 1.06 (95% CI: 1.02, 1.09), respectively. Bronchiolitis is a common cause of infant morbidity. Infants whose mothers filled a prenatal vitamin prescription during the first trimester of pregnancy had a 17% increased relative odds of having a health care visit for bronchiolitis compared to infants whose mother did not fill a prescription during pregnancy. Taken into consideration with the work that will investigate maternal folate levels during pregnancy and child respiratory and atopic disease risk, these findings provide insight into the potential timing of epigenetic effects that may influence child respiratory and allergic diseases. Future work in this cohort will investigate the association of folic acid dose and child bronchiolitis, asthma, and atopic disease outcomes.

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Perinatal Phthalate Exposure and the Developing Brain

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In utero phthalate exposure impairs reproductive development; however, few studies have examined impacts on other hormone-sensitive systems. White matter tract formation is highly sensitive to hormonal disruption. Both aberrant myelination and phthalate exposure are negatively correlated with attentional, cognitive, and social dysfunction in children. The aim of these studies is to explore the mechanisms by which phthalates alter neurodevelopment. We hypothesize that phthalate exposure during pregnancy and lactation leads to behavioral deficits via epigenetic changes that perturb myelination in brain areas associated with developmental disorders such as ADHD and autism. We are utilizing rodent and cell culture models to examine the effects of low-dose chronic phthalate exposure on nervous system development. To assess the effects of phthalates on formation of myelin, oligodendrocyte precursor cells (OPCs) will be treated with metabolites of DEHP, and effects on differentiation, myelin gene expression, and DNA methylation will be quantified. Brains of offspring of animals administered DEHP during pregnancy and lactation will be examined for alterations in expression of myelin genes and DNA methyltransferases as well as DNA methylation. Behavioral assays that measure social interaction and attention will also be conducted on the offspring. Offspring of animals administered 0.05, 0.5, or 5 mg/kg body weight•day DEHP during pregnancy did not display overt abnormalities. Preliminary data suggest an increase in myelin basic protein (MBP) and DNMT3a expression at PND15 in the cerebellum of offspring of 5mg/kg•day treated dams relative to control animals. As previously reported, DNMT1 and DNMT3a expression was elevated in testes of DEHP exposed offspring relative to controls. Exposure to phthalates during early brain development may cause changes in myelin gene expression as well as modifications to the epigenome that contribute to behavioral changes such as those observed

in human epidemiological studies. Studies are ongoing to assess epigenetic changes, myelin formation, and behavioral outcomes in rodents exposed to DEHP throughout gestation and lactation.

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Possible Epigenetic Mechanisms of Developmental Toxicity from Organohalogen Flame Retardants

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Organohalogen flame retardants have been used to comply with California's furniture flammability standard since 1975. Pre- and postnatal exposures are associated with adverse developmental effects in children such as lower IQ, impaired cognitive and motor development, and low birth weight. Epigenetic mechanisms may be involved in these outcomes. Epigenetic changes caused by maternal flame retardant exposure may affect gene expression and subsequently modify a child's development. This possible long-term consequence of adding organohalogen flame retardants lacking adequate toxicological information to consumer products should be better understood. The literature was reviewed on the developmental toxicity mechanisms and possible epigenetic pathways of organohalogen flame retardants. The evidence around epigenetic mechanisms of similar chemicals that are also associated with developmental toxicity, such as organohalogen pesticides, was also reviewed. Neurotoxicity following developmental exposure to PBDEs is expected because of structural similarity to known neurotoxins such as PCBs. Prenatal exposure to BDE-47 has been linked to global hypomethylation of the DNA in the brains of adult mice and reduced sociability in a genotype-independent manner suggesting epigenetic changes in mice brains that resulted in reduced sociability. Also, after pentaBDE exposure in rats, clusters of genes involved in neurodevelopment were altered, including several down-regulated genes of cation channels suggesting possible epigenetic alteration. PCBs have transgenerational neuroendocrine effects. Significant inverse relationships were found for several organohalogen concentrations and blood global DNA methylation, estimated in Alu repeated elements. Due to California's furniture flammability standards, virtually every North American tested has the flame retardant pentaBDE in their body. Although pentaBDE is no longer used, its replacements are also organohalogens and may exert similar toxicity. The possibility of epigenetic mechanisms of organohalogen flame retardants raise concerns about developmental health of new generations of children whose mothers have been exposed to pentaBDE and/or its replacements. This should be explored further to assess the multi-generational persistence of health risks posed by organohalogen flame retardants.

Ambient Air Toxics and Preterm Birth

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Studies have linked prenatal traffic-related air pollution exposures to several adverse birth outcomes. Some studies have suggested that air toxics such as polycyclic aromatic hydrocarbons (PAHs) or benzene found in traffic exhaust can cross the placenta, form DNA adducts, and affect fetal development via oxidative stress and other pathways. The principal objective was to examine associations between prenatal exposures to air toxics and the risk of delivering a baby preterm (< 37 weeks completed gestation) or very preterm (< 32 weeks completed gestation). We also sought to demonstrate the utility of a land use–based regression (LUR) model in a population-based pregnancy outcome study. We used birth certificates to identify 36,428 preterm births, including 3,463 very preterm births from 1/1/1995 through 12/31/2006 to women living ≤ 5 miles of a California Air Resources Board (CARB) air toxics station in Los Angeles County. For each case, we selected 10 controls matched by gestational age. We averaged monitoring station data to create air toxics exposure estimates for the first and second trimesters, last pregnancy month, and entire pregnancy. We used conditional logistic regression to analyze the data, adjusting for maternal age, race/ethnicity, education, and parity. In adjusted models, the odds of preterm birth increased 1–4% per interquartile range (IQR) increase in seasonalized and unseasonalized LUR quantified pollution. First-trimester IQR increases in PAHs increased very preterm birth odds by 2–3%. IQR increases in entire pregnancy and first-trimester benzene, toluene, ethyl benzene, and xylenes (BTEX) increased very preterm birth risk by 6–13%. Last pregnancy month carbon monoxide (CO), NO, NO_2, NO_x, and PM_{10} exposures increased preterm birth odds by 3–5% per IQR increase in pollutant concentrations. Very preterm birth odds also increased by 3–15% in each pregnancy period for per IQR increase in exposures to CO, NO, NO_2, NO_x, and $PM_{2.5}$. Our study provides evidence that PAHs and BTEX exposures from traffic and industrial sources may increase a mother's risk of delivering a (very) preterm baby. Both early and late pregnancy appear vulnerable to the effects of prenatal

exposure to air pollution. Furthermore, we demonstrated the utility of LUR models to estimate traffic-related air pollution exposures in a population spanning a large geographical region. Future studies should investigate air toxics as possible causative agents, and explore whether such prenatal exposures to environmental toxicants can induce epigenetic changes in the fetus.

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Ethical Implications of Epigenetics

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Much of our basic ethical construct has been based around "do no harm." But is this simple approach good enough? We have tremendous knowledge about the adverse effects of chemicals gained from research and experience. Recent advances in our understanding of how DNA expression can be modified indicates that subtle changes are possible beyond changing the DNA sequence. Examine the possible ethical implications of epigenetics. The power and subtlety of epigenetic changes can be passed to the next generation. Epigenetic changes can occur as the result of exposure to environmental contaminates such as cigarette smoke, arsenic, alcohol, phthalates, and BPA, as well other hazards. More important and with profound ethical implications are studies indicating that nutrition, methyl content of diet, intake of folic acid and vitamins, or even social and maternal behavior toward the offspring have epigenetic consequences. In rodents, maternal grooming or lack of grooming results in significant epigenetic changes. This means that it is not enough just to have a developmental environment free of chemical contaminants, but there must be a loving and supportive environment during development. We must move beyond just "doing no harm" to "doing good." The concept of epigenetics provides the scientific and biological foundation for the necessity of "doing good." This concept is called "epiprecaution" to signify the need to move above and beyond preventing exposures to harmful material but to one that is nurturing and supportive. Our expanding appreciation of the influence of development on epigenetics will have profound effect on our ethical thinking. We have an ethical responsibility to ensure that our children have an environment in which they can reach and maintain their full potential, not just free of exposure to chemicals but an environment that is supportive and nurturing. Epiprecaution moves beyond just doing no harm to one of creating a positive and supportive environment for our children.

Folate Protection of Embryogenesis and Gene Expression

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We demonstrated that folate (FA) prevents cardiac birth defects induced acutely during gastrulation on embryonic day (ED) 6.75 by a binge level of alcohol, by the drug lithium (Li) or by homocysteine (HCy). All three factors inhibit Wnt-mediated gene expression in the heart fields. Despite FA fortification and its use in periconceptional vitamins, the mechanism by which FA protects embryogenesis is unknown. This study is to identify genes in the cardiac outflow (OFT) that are altered by embryonic exposure on ED6.75 to ethanol, Li, or HCy, with and without FA supplementation 1) in the Wnt pathway that is important in cell fate decisions, 2) in its second messenger phosphatidylinositol (PtIn) signaling pathway, 3) in the folic acid cycle, and 4) in genes associated with epigenetic mechanisms. Pregnant mice and their embryos were exposed acutely on ED6.75 to Li or HCy, with and without dietary FA supplementation. We used Affymetrix microarray analyses of gene expression changes in the embryonic cardiac outflow tract (OFT) tissues a week later at mid-gestation on ED15.5. The cardiac OFT region and neural development are primarily affected after exposure at this specific developmental window. We extended these studies to alcohol exposure. Validation of specific gene expression was done a half-day after exposure and again at ED15.5 in embryos displaying abnormal cardiac function as monitored noninvasively *in utero* by echocardiography. In the Li- affected ED15.5 OFT, 51 genes associated with Wnt and phosphatidylinositol signaling were down-regulated; 21 genes were up-regulated. Sixteen Wnt related genes were down-regulated by both Li and HCy; 23 were up-regulated. A number of the genes overlapped. Thirty-eight genes that were misexpressed by Li but reversed by FA in the OFT related to genes associated with chromatin modification reactions and with methylation. Key gene expression changes in the above pathways have been validated by RT-PCR and *in situ* hybridization in the heart regions a half a day after the acute exposure to Li or to alcohol on ED7.5, and at mid-gestation. Taken together, these results confirm that Li, HCy, and alcohol exposure misexpresses key genes in Wnt/PtIn signaling and in FA metabolism that leads to methylation and chromosome modifications. Changes in these pathways we suggest contribute to heart defects seen after embryonic exposure. FA appears to protect one carbon metabolism, and hence methylation reactions to lead to normal cardiogenesis and embryogenesis. Optimal protection of the embryo occurs with early supplementation initiated right after conception. In the future epigenetic modulation of specific genes within the Wnt and phosphatidylinositol pathways during early cardiogenesis and dysmorphogenesis will be necessary to define.

Quality Assessment Tools in Published Animal Studies

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Although bias in clinical drug studies is well documented, little is known about bias in animal research. Animal data provide an important basis for pre-clinical and environmental health evaluation. Although researchers have developed guidelines to inform the design and execution of animal research, little has been done to systematically gather and evaluate the assessment instruments. We performed a systematic review to evaluate the available instruments and develop an evidence base for evaluating the methodological quality of animal studies that can be used in pre-clinical research or for evaluating potential health effects of environmental chemicals. We searched Medline from January 1965 to November 2011 to identify all articles meeting our inclusion criteria, namely that the article be 1) a published report related to the design of a quality assessment instrument for animal research studies and 2) written in English. Articles reporting applications of an instrument were excluded. We extracted data on the study design criteria (e.g., randomization, blinding, allocation concealment, statistical model used) assessed by each instrument. We recorded the number of criteria assessed by each instrument, whether the instrument calculated an overall quality score, and the method used to develop the criteria (empirically based or consensus). Of the articles matching our inclusion criteria, 12 reported modifications or updates of previously developed instruments, and 12 articles described new instruments, for a total of 24 distinct instruments. Core methodological criteria identified include randomization (20 of 24 instruments), blinding (20 of 24 instruments), and sample size calculation (17 of 24 instruments). In general, authors failed to empirically justify why these items were included while others were not. Twenty-three of 24 instruments were not tested for validity or reliability but were developed through consensus. The number of items assessed by these instruments ranged from 2 to 26. Eleven of 24 instruments were developed as general animal research guidelines and were not designed for a specific disease model. The most commonly modeled disease used for developing the instruments was stroke (8 out of 24 instruments). By describing the components, benefits, and limitations from known animal research quality assessment instruments, we hope to not only provide researchers with a framework for conducting and evaluating animal research but also provide the rationale for empirically testing present and future quality assessment instruments. Based on the results from this study, we will develop a quality assessment instrument to assess the relationship between methodological quality and research outcomes.

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Mother's Age at DDT Exposure Modifies Infant Birth Weight

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Previously we reported that age at exposure modified *p,p′*-DDT associations with maternal risk of breast cancer. Women exposed before age 14 years, but not after, showed increased risk of breast cancer with increasing DDT, but not DDE, during pregnancy. We wondered whether early-life exposure to DDT compounds results in epigenetic changes that impact other health outcomes. Here we test the hypothesis that mother's age at exposure modifies DDT associations with the birth weight of her infant and whether *p,p′*-DDT and *p,p′*-DDE have the same effect. The study subjects included 2,209 mother–infant pairs from the Child Health and Development Studies (CHDS) pregnancy cohort with existing organochlorine assays from pregnancy serum. Mothers entered the study between 1959 and 1967 before DDT was banned. High levels of DDT compounds (*p,p′*-DDT and *p,p′*-DDE) have already been documented in this cohort. There were 89 (4%) low birthweight infants (< 2,500 g). We used logistic regression to test the hypothesis that *p,p′*-DDT and *p,p′*-DDE in maternal prenatal serum predict incidence of low birth weight, and if mother's age in 1945, the year when DDT use became widespread, modified these associations. We found that maternal prenatal DDT and DDE exposures had opposing effects on risk of low birth weight in her infant. DDT was associated with lower risk while DDE was associated with higher risk of low birth weight. Moreover, these effects depended on age at mother's exposure (*p* for interaction = 0.04 for DDT and 0.05 for DDE). DDT and DDE associations with low birth weight were substantial for mothers exposed before birth or in infancy [DDT odds ratio (OR) = 0.3; 95% confidence interval (CI): 0.1, 0.9; DDE OR = 3.1; 95% CI: 1.4, 6.7], but approached the null for mothers initially exposed to DDT after age 14. Findings were not explained by mother's age at pregnancy, body mass, height, parity, serum lipids, or race. Findings suggest an opportunity for early-life prevention of low birth weight, but illustrate complexity. For DDT, exposure in early life may have contradictory effects at different life stages. Slower DDT metabolism (higher DDT/lower DDE) in mothers may enhance fetal growth, reducing the incidence of clinically significant low birth weight. But because higher birth weight is associated with increased breast cancer risk, efforts to reduce low birth weight may increase risk of breast

cancer in the next generation. Follow-up through the fourth generation of the CHDS will result in timely tests of the hypothesis that exposures in early life, during windows of susceptibility, may have lasting transgenerational effects on reproduction and cancer.

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DNA Methylation in Childhood B-Lymphoblastic Leukemias

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Childhood B-cell acute lymphoblastic leukemias (B-ALL) arise from diverse genetic and epigenetic events. Gene dysregulation through abnormal DNA methylation has been suggested to play an important role in the tumorigenesis of B-ALL, and this role is believed to be modified by many environmental factors. The current study was performed to identify methylation signatures that characterize B-ALL, and furthermore, to elucidate relationships between methylation profiles and various environmental factors that were previously known to be associated with B-ALL. We performed a genome-wide methylation analysis for 238 childhood B-ALL patients, including 77 hyperdiploid and 64 t(12;21)(p13;q22) [ETV6/RUNX1–fusion] patients. Additionally, we obtained methylation profiles for 24 purified normal precursor B-cell samples at four differentiation stages, including pro-B, pre-B-I, pre-B-II, and immature B cells. The Infinium HD450 Methylation array (Illumina) was used for this purpose, which assays a comprehensive set of 450K CpG loci throughout the genome. For 81 of the 238 B-ALL patients and all of the normal pre-B cell samples, we also obtained genomewide expression data. To identify CpG loci involved in oncogenic transformation from normal pre-B cells, we compared methylation profiles between B-ALL samples with normal pre-B cells. Differential methylation analysis nominated a set of 37,328 loci (7,945 genes) that are significantly different between these two groups (Bonferroni-corrected $p < 0.05$ and beta difference > 0.3). Integration with gene expression data yielded 872 genes whose expression changes were associated with methylation alterations. Unsupervised clustering analysis identified a demethylation cluster and a hypermethylation cluster that were enriched for hyperdiploid, and a demethylation cluster that was specific for ETV6/RUNX1. In addition, the loci residing in clusters that were highly variable amongst ALL patients were found to be associated with epidemiological factors including paternal smoking and exposure to pesticide, insecticide, solvent, or paints. These data indicate that numerous genes were aberrantly hypermethylated or demethylated in B-ALL, many of which were associated with transcriptional regulation with potential functional consequence that promotes leukemogenesis. Furthermore, heterogeneity in methylation profile was also present even within a well-established cytogenetics group and some loci in the heterogeneous clusters showed significant correlation with environmental factors, suggesting a possible role of such environmental factors in modifying leukemia susceptibility.

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Exposure & Fetal Growth Dysregulates Placental miRNA

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Exposure of the developing fetus to harmful chemicals, such as xenoestrogens, heavy metals, alcohol, and cigarette smoke, is associated with poor fetal and developmental outcomes. Research has suggested that altered placental miRNA expression is associated with harmful xenobiotic exposures and adverse pregnancy outcomes. Aims included identifying placental miRNA expression associated with maternal cigarette smoking during pregnancy and aberrant fetal growth and assessing how such expression profiles in the placenta may elucidate mechanisms by which adverse prenatal conditions may alter the growth and development of the fetus. Real-time PCR was used to measure the expression of candidate miRNA in two independent sets of human placentas. Differential miRNA expression associated with maternal cigarette smoking during pregnancy, controlling for confounders, was analyzed in a set of 25 placentas. Further, a placental cell culture model was used to investigate which components of cigarette smoke may alter miRNA expression in placental cells. In a separate set of 107 placentas, differential miRNA expression associated with fetal growth, controlling for confounders, was investigated. In 25 human placentas, miR-16, miR-21, and miR-146a were significantly down-regulated in cigarette smoke-exposed placentas compared to controls, and TCL-1 cells exposed to nicotine and benzo[*a*]pyrene exhibited significant,

down-regulation of miR-146a. In an independent set of 107 placentas, miR-16 and miR-21 expression was significantly reduced in infants with the lowest birth-weights. Logistic regression models suggested that low expression of miR-16 in the placenta predicts an over 4-fold increased odds of small for gestational age (SGA) status ($p = 0.009$). Having both low miR-16 and low miR-21 expression in the placenta predicted a greater increase in odds for SGA than having just low miR-16 or miR-21 expression ($p < 0.02$), suggesting an additive effect of both of these miRNA. Taken collectively, this work further elucidates the association of placental miRNA expression with maternal cigarette smoking during pregnancy and fetal growth and seeks to better understand how an adverse intrauterine environment acts through epigenetic mechanisms to alter infant and child health. Current and future directions include investigating how epigenetic mechanisms may be an important mode by which *in utero* exposures and adverse prenatal conditions may influence child neurobehavioral outcomes.

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Placental 11β-HSD2and Birth Weight in Hamilton County, TN

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Hamilton Country has one of highest rates of low birth weight births in the state of Tennessee. An excess *in utero* exposure to cortisol has been linked to restricted fetal growth and reduced birth weight. Placental production of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) inactivates cortisol before passage into the fetus, and thus is considered to be a protective mechanism. The current study investigates whether the expression of 11β-HSD2 in placenta tissue correlates with birth weight in infants born in Hamilton County, TN. Additional comparison were made based on maternal age, marital status, BMI, maternal drug, race, and maternal education to see if these groupings had any influence on infant birth weight, 11β-HSD2 expression or a calculated birth weight centile. The expression of 11β-HSD2 was measured in 242 samples. Five-gram placental samples were preserved RNAlater within an hour of delivery. The relative abundance of 11β-HSD2 mRNA in preserved placenta samples was assessed by a two-step qRT-PCR. Gene transcript levels of ACTB, GAPDH, and 11β-HSD2 were quantified with the Ssofast Eva Green assay using the relative standard curve method and assay specificity was verified by melting curve analyses. Reference genes were analyzed for consistency of expression using geNorm software. Statistical tests were nonparametric. A birth weight centile was computed, adjusting for several maternal characteristics, infant sex, ethnicity, and gestational age. Using a Spearman rank order correlation, 11β-HSD2 expression and birth weight centile in the uncomplicated pregnancies ($n = 191$, $p = 0.024$), but not with raw birth weight. Complications included IUGR, oligohydramnios, PIH/PE, NRFHR, and preterm labor. Birth weight was significantly different between married and single women ($p < 0.001$), Caucasian and African-American mothers ($p = 0.023$), and obese and underweight women ($p = 0.029$), with no accompanying difference in 11β-HSD2 expression. Significant differences in infant birth weight centile were found between married and single women ($p = 0.011$), with no accompanying difference in 11β-HSD2 expression. To our knowledge, no research has used such a large sampling to assess the relationships between the expression of this enzyme using qRT-PCR over a range of birth weights and various maternal parameters and fetal parameters. The disparity between Caucasian and African-American infant birth weight, as well as between single and married women, is well supported in the literature. The use of raw birth weight alone may not be a sensitive enough endpoint for subtle correlations, as with the current study. Confounding variables that have been shown to influence birth weight need to be accounted for in order to see these extraneous variables. Additional studies will be done correlating metal concentrations in the placental tissue with the expression of 11β-HSD2.

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Epigenetics of Wood Smoke Exposure and Diet in Child Asthma

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Asthma is an increasingly common chronic disease among children, and data point toward a complex mechanism involving genetic, environmental, and epigenetic factors. Epigenetic modifications such as DNA hypo- or hypermethylation have been shown to occur in response to factors such as particulate matter (PM) or diet. Current research suggests that children may be especially susceptible to these factors. Within the context of a study of asthmatic children we collected buccal cells for epigenetic analysis. The central hypothesis is that exposure to biomass smoke $PM_{2.5}$ is associated with DNA hypo- or hypermethylation at specific sites, that diet may also influence methylation events, and that such alterations are associated with respiratory health. Prior to the study, buccal cell storage and collection strategies were evaluated by assessing DNA yield and quality. Children in the wood stove intervention study were recruited to participate in this epigenetics study. Concurrent with indoor air sampling and asthma outcome assessment,

buccal cells were collected using cytology brushes. At each collection visit, one buccal cell sample was stored in Cell Lysis Solution (Qiagen) for future DNA methylation analysis, and three samples were stored in RNAlater (Qiagen) for future RNA expression analysis. Dietary intake was assessed using the 2004 NutritionQuest Block Kids Food Frequency Questionnaire (FFQ). Height and weight were also measured. Using an optimized method, average (SD) DNA yield was 2.5 (2.0) µg. DNA quality was high with an average (SD) 260/280 wavelength output of 1.9 (0.2), which is sufficient for the planned studies. Of the 48 children in the main intervention study during the recent winter (2011–2012), 83% ($n = 40$) agreed to participate in the epigenetics/diet study. Of the 40 participants, 45% ($n = 18$) were female, 55% ($n = 22$) were male, and ages ranged from 7 to 17 years. Two buccal cell sample collection events were completed for most subjects (78 events total). Average (SD) transportation time on ice from field collection to archiving samples at –80°C was 159 (82) minutes. Each subject completed one FFQ during the winter with completion times averaging approximately 25 minutes. This epigenetics/diet study is in the initial stages of data collection. We will be evaluating DNA samples for methylation status at CpG promoter sites of asthma-relevant Th1/Th2 genes. Epigenetic results will be evaluated with respect to exposure assessment (i.e., indoor $PM_{2.5}$ and endotoxin) and outcomes measures (i.e., PEF and FEV_1 monitoring and biomarkers of inflammation and oxidative stress). Corresponding RNA expression will also be evaluated from archived samples. As this study is conducted within the context of a randomized intervention trial, we will be able to assess epigenetic changes that correspond to exposure reduction strategies. Children's dietary intake of methyl-rich nutrients will be assessed as potential effect modifiers.

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In Utero Smoke Exposure and Pediatric Asthma

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Among people with asthma, the clinical impact and relative contribution of maternal smoking during pregnancy (*in utero* smoking) and current secondhand smoke exposure on asthma control is poorly documented, and there is a paucity of research involving minority populations. We examined the association between poor asthma control and *in utero* smoking and current secondhand smoke exposure among Latino and African-American children with asthma. Case-only analysis of two multi-center case–control studies conducted in parallel using similar protocols from 2008 through 2010. We recruited 2,346 Latinos and African Americans with asthma (ages 8–21 years) from the mainland United States and Puerto Rico. Ordinal logistic regression was used to estimate the effect of *in utero* smoking and current secondhand smoke exposures on National Heart, Lung, and Blood Institute–defined asthma control. Regression analyses were adjusted for eczema, exposure to home indoor allergens, IgE, socioeconomic status, race/ethnicity, and study center. Poor asthma control was associated with *in utero* smoking [odds ratio (OR) = 1.5; 95% CI: 1.1, 2.0], independent of its association with current secondhand smoke exposure (OR = 1.3; 95% CI: 1.0, 1.5). After pooling our samples with two additional populations of Latino and African-American children with asthma (pooled sample size = 2,839), poor asthma control remained associated with *in utero* smoking (OR = 1.5; 95% CI: 1.1, 1.9). Current smoking was also associated with poor asthma control (OR = 1.3; 95% CI: 1.1, 1.6), with the odds of poor asthma control increasing 20% for each additional household smoker (OR = 1.2; 95% CI: 1.0, 1.4). *In utero* tobacco smoke exposure is associated with

poor asthma control more than 8 years postexposure. This observation suggests that prenatal exposures have persistent effects long after the initial exposure. Potential mechanisms may include epigenetic modifications while *in utero*. There is mounting evidence that prenatal cigarette smoke exposure results in DNA methylation that has been measured at birth, childhood, and adulthood, suggesting that *in utero* exposure can lead to DNA modifications which persist long after the exposure has occurred. Our next steps are to examine if our observed associations are mediated by DNA methylation.

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Role of Epigenetics in Child and Adolescent Mental Health

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Until recently, the etiology of behavioral disorders in children and adolescents was poorly understood. Recent studies suggest that sustained genetic and environmental prenatal insults could predispose the human brain to manifest symptoms of disordered behavior, even without altering DNA sequence. Changes during periods of increased brain plasticity such as DNA methylation and histone modification (methylation, acetylation) can alter signaling pathways and leave epigenetic footprints. We summarize results of recent meta-analysis and critical reviews of recent studies that implicate adverse environmental events in the development of pediatric psychopathology. The goal was to outline the role of epigenetic regulatory mechanisms and the manner in which an alteration or deregulation can predispose a child to development of mental disorders. We used systematic review of recent literature in past 24 months using Pubmed and Ovid. It appears that epigenetics plays a role in mental retardation, suicidal behavior, addiction, depression, anxiety, schizophrenia, and autism spectrum disorders including Rett and Asperger's syndromes. Epigenetic interpretations can provide clues to discordance of monozygotic twins, as regards mental disorders. Environmental insults to the hippocampal neurons can affect the learning process of children with resultant consequences in cognition and emotions. Recent publications suggest that some imprints of epigenetic deregulations are potentially reversible. There could be a potential role for the use of "epigenetic biomarkers" in mental disorders. At the end of this presentation, participants should be able to understand the link between genes and disease and hence the potential for using epigenetic interventions in management of neuropsychiatric disorders. Therapeutic interventions that target the epigenome could provide a new frontier for tackling mental disorders in children and adolescents.

The Epigenetic Effects of Human Milk on Obesity

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Metabolic programming is the epigenetic response to early nutrition during a critical prenatal and postnatal window. Research has shown that obesity starts between 9 months and 2 years of age and that overweight infants become obese adults. Without the influence of high fat foods and sedentary lifestyle, what is contributing to the start of obesity at 9 months age? The goal was to evaluate the evidence using a literature review to describe possible long-term clinical effects of diet and nutrition resulting from metabolic programming. The objective of the literature review is to examine the clinical evidence of the epigenetic effects of human milk on future body mass index (BMI) and to identify a possible biological epigenetic mechanism responsible for the long lasting effects. A systematic literature review was conducted utilizing the keywords epigenetics, body mass index, obesity, weight gain, breastfeeding, diet, and nutrition. References addressing an epigenetic or biological mechanism contributing to metabolic programming were included. Multiple disciplines were examined in the literature review including biology, neurology, nutrition, genetics, medicine, and biochemistry, utilizing the Web of Knowledge and the Web of Science. There is strong support across a variety of disciplines that suggests that diet and nutrition during the first year of life influence metabolic programming, BMI, and obesity. In a meta-analysis of 17 studies, the risk of being overweight was reduced by 4% for each month of breastfeeding and the effect lasted until adulthood. A clear dose–response effect of breastfeeding on the prevalence of obesity was documented in a study with 13,000 5-year-old children. A neurobiological study in mice suggests that the action of leptin is necessary during the critical prenatal window for establishing neural pathways to regulate and program metabolism and may contribute to the "leptin-dependent childhood obesity." Genetic research suggests that the gene involved in the production of the hormone, leptin, is highly regulated epigenetically through methylation and that this methylation is affected by diet. There is strong clinical support across multiple disciplines suggesting that diet and nutrition during the first year of life may influence metabolic programming and obesity. The evidence suggests there is a critical newborn window in which the diet affects adult BMI in a specific measurable dose–responsive outcome that lasts until adulthood, consistent with the definition of metabolic programming. The next step is to determine the epigenetic mechanisms that lead to the 4% reduction in the incidence of obesity for each month the infant receives human milk. Understanding the interactions of diet and nutrition with our epigenome allow health care providers to tailor education focusing on early life nutritional habits and help prevent obesity later in life.

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Family-Based Association Study of ADHD and Genes Increasing the Risk for Smoking Behaviors

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ADHD and smoking behavior are highly comorbid, and this comorbidity could be due to shared genetic factors. Several genes increasing smoking behavior risk were reliably identified by genome-wide association studies (GWAS). We investigated five top single nucleotide polymorphisms (SNPs) located in different genes and loci (*CHRNA3*, *BDNF*, *DBH*, and *LOC100188947*) that were highly associated with different dimensions of smoking behavior, in relation to ADHD. Family-based association tests were used to study transmission of risk alleles within these five genetic markers in families that totaled 454 children with ADHD between 6 and 12 years old. Clinical diagnosis of ADHD and a number of behavioral and neurocognitive phenotypes relevant to the disorder were investigated. One SNP (rs1329650) from a noncoding RNA (*LOC100188947*) was significantly associated with overall ADHD diagnosis with the C* risk allele being overtransmitted from parents to children with ADHD ($p = 0.02$). It was also over transmitted to children with higher scores on Conners' Parents ($p = 0.01$) and Conners' Teacher ($p = 0.002$) index scores, CBCL withdrawn ($p = 0.001$) and aggressive ($p = 0.007$) behaviors. Children with poorer performances on executive and attention tasks were more likely to inherit the risk allele. The C* allele of rs1329650 may be increasing the risk for ADHD and smoking behavior through a common mechanism, possibly externalizing behaviors and specific cognitive deficits that manifest as ADHD in childhood and are the gateway to smoking behavior later in life. This exploratory study illustrates the use of comorbid disorders to investigate ADHD genetics. In spite of its relatively large sample size, replication in future studies is warranted.

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Low Birth Weight and The Metabolic Syndrome in Young Adults: Evidence from The Butantã Cohort

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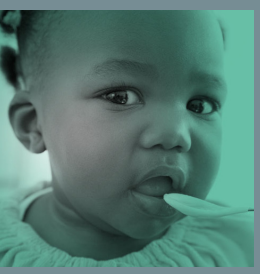
Chronic diseases such as hypertension and cardiovascular diseases have become a major challenge worldwide. According to Barker's Hypothesis, many diseases manifestations that occur in adulthood have their origins in early life. Thence, investigating the mechanisms of those diseases and the contribution of low birth weight as a risk factor for later diseases onset became a priority. The present study aims at understanding if there is a correlation between birth weight and the metabolic syndrome onset in young adults. Patients born between 1977 and 1989 and followed in the Butantã Cohort were invited for a follow-up and medical check-up. Eighty-nine patients were included. From patient's medical records, we collected birth weight data. Two groups were formed: the low birth weight group (≤ 3,000 g); and the normal birth weight group (> 3,000 g). We collected social-demographic and clinical data as well as lab tests results. Cardiovascular disease risk was assessed according to the Framingham Risk Score. Data were then correlated and statistically calculated. The cumulative frequency of the weight was analyzed with Pearson's chi-square test for independent groups. Two-tailed *p*-values ≤ 0.05 were considered significant. The low birth weight group (group 1) comprised 37 patients and the normal birth weigh group (group 2) comprised 51 patients. There was no significant difference between the two groups in terms of social demographic characteristics (age, educational level, per capita income, substance abuse, and existing chronic diseases conditions). Of these, 29.7% ($n = 11$) in group 1 and 43.1% ($n = 22$) in group 2 had a BMIP 25.0 kg/m^2; 13.9% ($n = 5$) in group 1 and 11.5% ($n = 6$) had arterial hypertension. There was also no significant difference between the two groups in terms of metabolic profile, intima media thickness of carotid artery, and visceral adipose tissue accumulation. All 89 patients we classified as low risk (< 10%) according to the Framingham risk score. Recent epidemiological literature suggests that birth weight is associated with metabolic syndrome in adulthood. In this study, we did not find a significant correlation between birth weight and arterial hypertension, metabolic profile, intima media thickness of carotid artery, and visceral adiposity, as well as cardiovascular disease risk according to Framingham score. These findings could be related to the sample size or to the group age (younger than 30 years), suggesting that maybe metabolic syndrome starts later in life. As next steps, the two study groups follow up shall be continued and more patients will be included.

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