Title: Neurodevelopmental consequences in offspring of mothers with preeclampsia during pregnancy: Underlying biological mechanism via imprinting genes

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Preeclampsia, Genomic Imprinting & Child Neurobehavioral Development: Preeclampsia, a hypertensive disorder unique to human pregnancy, is one of the most common and potentially modifiable metabolic problems, and affects approximately 10-15% of all pregnancies.[1, 2] It is defined by the onset of hypertension (140/90 mm HG) after 20 weeks' gestation in the index pregnancy, accompanied by 300 mg of protein in a 24 hour urine specimen, or persistent > 30mg/dL (1+) protein on a dipstick.[2, 3] While the etiology of preeclampsia is still elusive, it remains a leading cause of mortality and morbidity among mothers and their babies.[3-5]

Recent research has demonstrated that preeclampsia is associated with an increased risk for impaired early language development, lower neurocognitive functioning, and ADHD.[6-9] And there is accumulating evidence to show that pregnancy-induced hypertensive disorders, especially preeclampsia, can have potentially long-term consequences for the offspring's neurobehavioral development [10, 11] via changes in the epigenome. In recent animal studies, genomic imprinting perturbations have been linked to both hypertensive problems and neurodevelopmental syndromes.[13] Similarly, in humans, adverse pregnancy outcomes have been associated with genomic imprinting perturbations, leading to neurodevelopmental syndromes[14, 15] and subsequent disorders.[16, 17] Delineating some of the underlying biological mechanisms by which preeclampsia influences the trajectory of optimal/suboptimal child development and functioning, can help us in our aim to uncover the mechanisms of dysregulated neurobehavioral development and related mental disorders via epigenetic changes.

Severe preeclampsia may cause symptoms such as hypertension, proteinuria, eclampsia, cerebral edema, cerebral hemorrhage, long-term neurocognitive dysfunction, blindness, liver swelling and other liver damage leading to elevated serum transaminase, oliguria,

thrombocytopenia, pulmonary edema necrotizing pancreatitis. All of these symptoms can be fatal to mothers, and, importantly, to their child *in-utero*, resulting in greater mortality in mothers and varying degrees of child morbidity after birth.[2, 18] The main impact of preeclampsia on the fetus is malnourishment, resulting from utero-placental vascular insufficiency hypoxia, which restricts nutrient supplies and oxygen flow from the placenta to the fetus.[19] This leads to various perinatal and neonatal problems, including intra-uterine growth restriction (IUGR, defined as birth weight less than the 10th percentile),[5, 19-25] emergency C-section,[5] preterm delivery,[24, 26, 27] reduced birth weight,[5, 28, 29] lower APGAR scores,[30] more frequent and prolonged neonatal intensive care unit (NICU) stays,[5, 20, 31] and increased acute respiratory distress syndromes after birth.[20, 32] In some cases, fetal damage is so severe that it results in fetal demise, such as stillbirth and neonatal death.[32, 33] Beyond birth, while the long-term health and developmental consequences of exposure to maternal preeclampsia for the surviving child are relatively unexplored, there is some evidence for suboptimal neurocognitive development among infants with IUGR,[34, 35] which is one of the major fetal/child consequences of preeclampsia.[5, 20-25] Recently, with the leadership of the NICHD, growing efforts have been made to find associations between preeclampsia and health consequences in offspring, including IUGR,[35, 36] preterm birth,[37-40] LBW,[41-43] and child neurobehavioral development.[44-46]

The extent of adverse neurobehavioral and other developmental consequences for surviving infants with perinatal problems has been investigated less frequently. Many and colleagues (2003), for example, reported that IQ at age 3 years was significantly lower among IUGR children with maternal preeclampsia, compared to those without (85.5 vs. 96.9, p=.03).[47] Similarly, Cheng and colleagues (2004) documented that preterm infants of preeclamptic mothers, compared to those of non-preeclamptic mothers, had a compromised

neurodevelopmental index,[48] as measured by the Bayley Scales of Infant and Toddler Development- Second Edition (Bayley-II).[49] Children born to mothers with preeclampsia had a lower Mental Developmental Index (MDI) than children born to mothers without at 2 years of age (p=.04), while there was no significant difference between the two groups on the Physical Developmental Index (PDI) (p=.56). Furthermore, they reported that preeclampsia was associated with an over 10-fold increased risk of mildly delayed MDI (p=.007), after controlling for demographic and biomedical confounders. Temperament is thought of as an early biological characteristic similar to a personality trait. Although it is not typically considered to be a neuropsychological construct, there are many overlaps. In particular, the temperamental construct of *effortful control* is very similar to the neuropsychological construct of attention and executive functioning.[50] More recently, *anger* and *negative emotionality* have gained considerable attention as temperamental traits that are closely linked to the limbic system in the brain.[51-53]

Thus, further investigation of the underlying epigenetic mechanisms that show how preeclampsia or pregnancy-induced hypertensive problems are associated with early emerging temperament profiles of the infant as well as child suboptimal neurodevelopment, using validated clinical instruments such as the Early Childhood Behavior Questionnaire (ECBQ),[54] and the Bayley Scales of Infant and Toddler Development – Third Edition (Bayley-III)[49] is warranted.

Placenta Epigenetics and Neurodevelopment:

The medical condition of the mother affects the nature of in-utero condition (i.e., environment) and perturbs gene expression (i.e., genes), which govern the developmental trajectories of offspring. When information from these two areas, environment and genes, are examined

together, they can provide us with important clues as to how the mother's medical complications influence the trajectories of child development.

Over the past decade researchers have begun to consider how the placenta, which is usually discarded at birth, could hold important information about the relationship between the pre- and perinatal environment and cognitive neurobehavioral outcomes in the developing child, over and above the effects of postnatal environment. Many studies have linked poor placentation during pregnancy with a wide range of chronic neurodevelopmental disorders in children,[55-58] including Autism Spectrum Disorder (ASD),[59] Attention Deficit Hyperactivity Disorder (ADHD)[10] and learning disabilities.[60] Moreover, recent data from the CDC showed a 23% increase in identified cases of ASD between 2009 and 2011.[61] It is important to note that the placenta tissue should not be viewed as a surrogate (to the brain) but a target tissue in understanding the genes-environment interplay. The placenta develops from the extra-embryonic cell layer of the blastocyst, as opposed to the embryonic cell mass that will differentiate into the fetus. In an effort to understand the mechanisms of how the environment gets "under the skin," attention has been focused on the potential importance of the link between the placenta and fetal/child brain development.

The placenta has been shown to produce an array of neuropeptide hormones that are analogues to those produced by the hypothalamus and the pituitary gland including GnRH, TRH, CRH, and oxytocin.[62] Rapid advancements in the discovery of integrated regulation of neuropeptide homeostasis within the brain and placenta[63, 64] has led to the concept that the placenta acts as a "third brain" linking the developed (maternal) and developing (fetal) brains.[64,

[65] Maternal perturbations are conveyed to the fetus via the placenta, in the expression of transporters that regulate the flux of glucose, amino acids, and vitamins required for growth and development.[12] Thus, the placenta serves as the "master regulator" *in utero* and plays a highly functional role in shaping fetal development.[65]

Imprinted Genes Expressed in the Placenta and Fetoplacental Development: Fetoplacental development begins with a complex and highly coordinated set of epigenetic events that take place few hours after fertilization and before the implantation of the fertilized egg.[12, 66, 67] During this relatively short but very active window an almost complete reprogramming of the genome methylation takes place accompanied by a reorganization of the histone coding.[67, 68] Other, less characterized, epigenetic events also contribute to preparing the newly fused parental genomes for implantation and embryogenesis.[67] At this stage specific genomic regions carrying a unique set of multilayer epigenetic signals inherited from the germline, known as Imprinting Control Regions (ICRs), are spared this epigenome

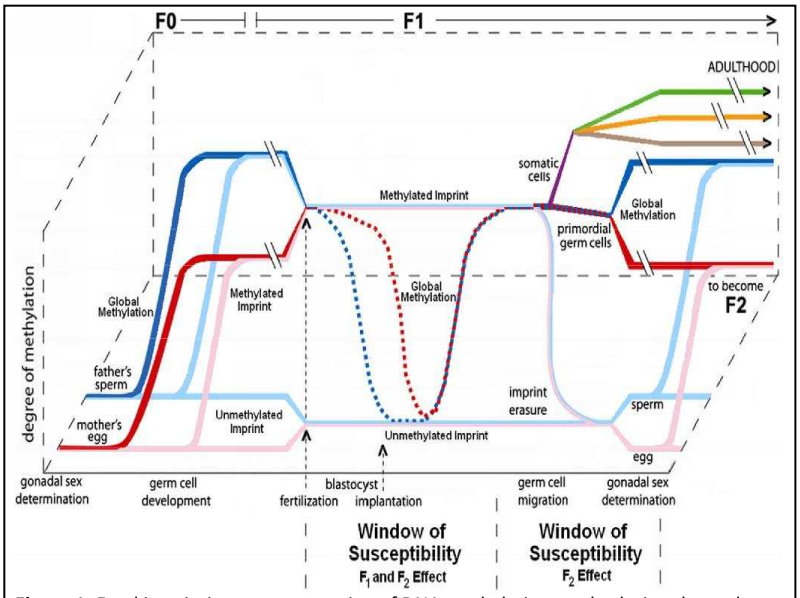

Figure 1. Fetal imprinting reprogramming of DNA methylation marks during the early zygote developmental phases. F_0 sperm and egg carry global (red and blue lines) and imprinting specific methylation signals (pink and light blue). After fertilization global methylation is reprogrammed at the blastocyst stadium. Imprinting signals are maintained unaltered to generate an embryo with distinct parental contributions. Imprinting reprogramming takes place only in the primordial germ cells later in development to generate gametes carrying imprinting marks according to the sex of the developing embryo. Perturbations of the imprinting profiles at the blastocyst stage can directly affect the embryo and also the gametes (F_1 & F_2 windows). Somatic cells separately develop from the embryo carrying the parental imprinting signals and the newly reprogrammed global methylation setting (purple line). They later rearrange their methylation status coherently with the adult tissue they will originate (green, orange, and brown lines) [Adapted from Lambertini *et al.*, 2012[12]]

reprogramming wave (**Figure 1**).[12, 69] ICRs control the allele-specific expression of clusters of about 200 genes (or about 1% of the protein-coding genes) distributed across the human genome. Genes which are monoallelically expressed through the action of these ICRs are known as imprinted genes with their expressed allele determined by the parent of origin. Imprinted genes are thus physically present as two copies but functionally haploid with about 50% of them maternally expressed and 50% paternally expressed.[69-71]

A number of imprinted genes play critical roles in regulating the fetoplacental growth and development and instructing postnatal development. 13, 72 Based on their functional roles, imprinted genes have been classified in four main categories: 1) Genes that directly regulate fetal growth; 2) Genes that indirectly regulate fetal growth by modifying the function of the placenta; 3) Genes that modulate metabolic processes postnatally and 4) Genes that modify behavior postnatally. [13, 72, 73]

Furthermore, recent research suggests that a subset of imprinted genes expressed in the placenta may regulate maternal adaptations to pregnancy i.e. controlling the function of the mother, by regulating the production of placental hormones. [74] The timely expression of imprinted genes has been shown to play an important role in fetoplacental development.[13, 75, 76] These findings are in agreement with the functional importance of imprinted genes which, as shown for other such genes,[77, 78] once altered, can lead to serious phenotypic consequences,[79, 80] some being lethal,[81, 82] which may be further enhanced by the constitutional haplo-insufficiency of imprinted genes.[83]

The endurance of ICRs in the face of extensive epigenetic reprogramming early in development and through the life course raises many interesting points. As the ICR epigenetic setup is not reprogrammed at fertilization, it represents one of the few known instances of epigenetic inheritance.[84] This in turn may explain the influence of the both parental environments prior to conception in determining fetal development. Environmentally-driven alterations of the ICR epigenetic status in the parents' gametes at different stages of life may be preserved. Similarly, environmental exposure may influence the process that protects ICRs from the epigenome reprogramming wave happening at fertilization. As both types of exposure occur very early, the consequences are likely to be wide-ranging impacting the whole embryo and potentially detectable in most tissues.[85, 86] Environmental exposures occurring after implantation that alter the ICR epigenetic setup or function also carry the potential for modifying the fetoplacental development trajectory at different stages.[68, 87-89] Lastly it must be mentioned that the effects of the alteration of the ICR epigenetic setup can extend beyond the F_1 generation. Primordial germ cells (PGCs) are the only embryonic cells that actually undergo ICR reprogramming in order to generate gametes that carry the ICR epigenetic setup specific to the sex of the developing embryo.[12] Alterations that affect the process of erasure, reestablishment or maintenance during maturation into gametes could be passed to the F_2 generation. To summarize, the epigenetic setup of ICRs can be considered as a recording devices of past exposures [4] acting as lasting environmental biosensors of the intrauterine status as conveyed by the mother (e.g., changes in blood pressure). Of note, alterations of both the ICR epigenetic setup and imprinted genes have been linked to different pregnancy and newborn outcomes.[87, 90-97]

Imprinted Genes as a Sensor of Pregnancy-Induced Hypertensive Disorder and Predictor of Child Development: Imprinted genes have been found to respond to common environmental stimuli. For example, peri-conceptional and prenatal exposure to both insufficient and excessive maternal nutrient intake have been found to leave lasting signals on the methylation profile of several imprinted domains, including imprinted genes *INS*, *IGF2*, *GNASAS* and *MEG3*.[65]

Previous studies have examined the expression of imprinted genes in the placenta from preeclamptic pregnancies, often with conflicting results. For example, the maternally expressed imprinted gene *CYCLIN-DEPENDENT KINASE INHIBITOR 1C* (*CDKN1C*) has variously been reported to be significantly decreased,[98] increased [99, 100] or unaltered [101] in preeclamptic placentas. These conflicting results may be explained by a difference in mode of delivery, given recent evidence demonstrating significantly increased *CDKN1C* expression in laboring versus non-laboring placentas. [102] Some mothers carrying babies with loss-of-function of *CDKN1C* have a very severe form of preeclamspia called HELLP (Hemolysis, Elevated Liver enzymes, Low platelet count) syndrome which is a life threatening complication. [103] There is data from an animal model to suggest loss-of-function of *CDKN1C* in the placenta may contribute to preeclampsia-like symptoms in the mother. Genetically unaltered female mice carrying *Cdkn1c* loss-of-function fetuses exhibit increased blood pressure and proteinurea during pregnancy. [104] However, in a separate study, maternal symptoms where less apparent initially suggesting an environmental component. [105] Loss-of-function of *Cdkn1c* in the mouse placenta was associated with increased trophoblast proliferation and a narrowed intervillous space in some studies. [104, 106] A narrowed intervillous space could impede uteroplacental blood flow, which combined with the shallow trophoblast invasion observed, could contribute to the development of

preeclampsia-like symptoms.[104] However, a more recent study revealed a very different placental phenotype with a severely disorganized placenta late in gestation, and with maternal blood hemorrhaging into the blood spaces suggesting that genetic background could influence the phenotypic consequences of loss-of-function of *Cdkn1c.*[107] Taken together, these studies highlight the importance of further research investigating the relationship between preeclampsia and *CDKN1C.*

A second imprinted gene, *PLECKSTRIN HOMOLOGY-LIKE DOMAIN, FAMILY A, MEMBER 2* (*PHLDA2*) has also been linked to preeclampsia. *PHLDA2* was found to be highly overexpressed in placentas from preeclamptic pregnancies.[108] The phenotype of female mice carrying fetuses with loss-of-function of *Phlda2* has not been reported but overexpression of *PHLDA2* in a human placental cell line resulted in impaired cell proliferation, migration and invasion.[108] Placental *PHLDA2* expression may also be important in preeclampsia.

Around 70% of the known imprinted genes are expressed in the placenta[91] and recent work has demonstrated that alterations in placental imprinted gene expression are associated with infant neurodevelopmental outcomes.[109] In two small pilot studies on 50 placenta samples from the Stress in Pregnancy (SIP) Study, we found both an alteration of the methylation profile of the ICR that regulates the parent-of-origin specific expression of two key imprinted genes, *IGF2* and *H19* (imprinted in the opposite direction) in correlation with maternal stress in pregnancy and an alteration of the global DNA methylation in correlation with preeclampsia.[72] We also found that imprinted gene expression in the placenta correlates with fetal growth and development, as measured by head circumference and birth weight.[88, 109] Furthermore, the

imprinting status of each imprinted locus, as defined by the ICR methylation status, has been shown to exert different effects on the reactivation of the silent allele of imprinted genes at that specific locus.[91, 109]

Understanding the molecular mechanisms for fetal programming, through exposure to pregnancy-related medical problems, such as preeclampsia and pregnancy-induced hypertension, is a promising, but neglected, area of research while as a whole, the area of fetal programming represents an important first step towards prevention of *lifelong* negative developmental and mental health consequences for offspring. To achieve this, epigenetically informative longitudinal research that follows a birth cohort from a period in utero through childhood is the key to understanding how maternal preeclampsia *in utero* can influence an infant's developmental trajectory by increasing vulnerability to cognitive and neurobehavioral impairment. If imprinting gene profiles are determined to be early biomarkers for impaired cognitive-neurobehavioral development, they could be used as biomarkers to design more targeted preventive measures for childhood developmental problems by alleviating and reducing the risk for maternal preeclampsia during pregnancy.

In sum, gaining further understanding regarding the ways in which common pregnancy-induced problems such as preeclampsia may lead to suboptimal fetal/infant development, specifically impaired neurobehavioral development via dysregulation in genomic imprinting status in the placenta, may yield effective clues for the prevention of neurodevelopmental disorders in early childhood. Prenatal influence caused by preeclampsia or hypertensive disorders will not fully explain the cause of the neurodevelopmental problems, as there are also

many postnatal assaults that influence the risk for neurodevelopmental disorders in offspring, as well as other possible prenatal mechanism. However, if epigenetic pathways can be used as potential tools for identifying high-risk infants, it is the first step toward developing prevention plans for full-fledged disorders later in childhood, perhaps though educating pregnant mothers. To this end, it is important to encourage collaboration among obstetricians, pediatricians, child psychiatrists, as well as early childhood educators to encourage research around the peri- and prenatal periods to reduce the risk for impaired neurodevelopmental disorders in childhood. Knowledge gained from such studies could contribute to an enhanced capacity for early prevention. At the same time, it will help inform and educate pregnant mothers about the importance of prenatal monitoring of blood pressure, weight gain, and metabolic functioning during pregnancy for the health of their offspring.

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