Published in final edited form as:

Reprod Toxicol. 2015 November ; 57: 43–49. doi:10.1016/j.reprotox.2015.05.002.

DNA Methylation Changes in the Placenta Are Associated With Fetal Manganese Exposure

Jennifer Z.J. Maccani, PhD¹, Devin C. Koestler, PhD², E. Andrés Houseman, ScD³, David A. Armstrong, MS⁵, Carmen J. Marsit, PhD^{4,5}, and Karl T. Kelsey, MD, MOH^{6,7}

¹Penn State Tobacco Center of Regulatory Science (TCORS), College of Medicine, Department of Public Health Sciences, Hershey, PA, USA

²Department of Biostatistics, University of Kansas Medical Center, Kansas City, Kansas, USA

³College of Public Health and Human Sciences, Oregon State University, Corvallis, Oregon, USA

⁴Section of Biostatistics & Epidemiology, Department of Community and Family Medicine, Geisel School of Medicine at Dartmouth, Hanover, New Hampshire, USA

⁵Department of Pharmacology and Toxicology, Geisel School of Medicine at Dartmouth, Hanover, New Hampshire, USA

⁶Department of Pathology and Laboratory Medicine, Brown University, Providence, Rhode Island, USA

⁷Department of Epidemiology, Brown University, Providence, Rhode Island, USA

Abstract

Adequate micronutrient intake, including manganese (Mn), is important for fetal development. Both Mn deficiencies and excess exposures are associated with later-life disease, and Mn accumulates in the placenta. Placental functional alterations may alter fetal programming and lifelong health, and we hypothesized that prenatal exposures to Mn may alter placental function through epigenetic mechanisms. Using Illumina's HumanMethylation450 BeadArray, DNA methylation of >485,000 CpG loci genome-wide was interrogated in 61 placental samples and Mn associations assessed genome-wide via omnibus test ($p=0.045$). 713 loci were associated with Mn exposure ($p<0.0001$). 5 significantly differentially-methylated ($p<1.3\times 10^{-7}$) loci reside in neurodevelopmental, fetal growth and cancer-related genes. cg22284422, within the uncharacterized *LOC284276* gene, was associated with birth weight; for every 10% increase in methylation, lower birth weights were observed, with an average decrease of 293.44 grams. Our

Corresponding Author: Carmen J. Marsit, Associate Professor, Departments of Pharmacology & Toxicology and Community and Family Medicine, Geisel School of Medicine at Dartmouth, HB 7650, Pharmacology & Toxicology, Hanover, NH, 03755, USA; phone: 603-650-1825; fax: 603-650-1129; Carmen.J.Marsit@Dartmouth.edu.

CONFLICT OF INTEREST AND FINANCIAL DISCLOSURES: The authors declare that there are no conflicts of interest or competing financial interests.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

observations suggests a link between prenatal micronutrient levels, placental epigenetic status and birth weight, although these preliminary results require validation.

Keywords

epigenetics; in utero; methylation; manganese; placenta; pregnancy; LOC284276

1.1 INTRODUCTION

Proper placental growth and function are important for normal fetal growth and development. This is particularly true early in gestation, when inadequate nutrition or exposure to environmental toxicants may disturb placental development, an important process with the potential to affect fetal growth^{1–3}. The growth and function of the placenta is controlled by the complex interplay of various cellular pathways, which themselves can be altered at the molecular level through epigenetic mechanisms. These epigenetic mechanisms may be susceptible to perturbation via adverse environmental exposures. In fact, several distinct exposures have been reported to alter DNA methylation patterns in the placenta, suggesting that its function is malleable by the environment^{4–8}. It is logical to hypothesize that nutrition, particularly intake of micronutrients, may have the potential to influence placental DNA methylation patterns; however, to date, no epigenome-wide studies have investigated placental DNA methylation patterns in association with intake of the crucial micronutrient manganese (Mn).

Mn is naturally found in water, soil, air, and most foods, and is generally found in the body in microgram (µg) levels⁹. Adequate amounts of Mn in the diet, predominantly from grain sources¹⁰, are important for normal growth and development; however, too much Mn can inhibit growth¹¹. Excess Mn exposure can occur from industrial or occupational exposures as well as from air pollution as Mn can be a byproduct of combustion from motor vehicles and coal burning power plants. Mn levels have commonly been assessed in keratin rich tissues such as hair and toenails. Levels of Mn in these media have observed to be associated with various cancers^{12–13}, demonstrating that nail Mn can be utilized as a biomarker of internal dose of Mn in the body.

During pregnancy, decreased levels of Mn have different mechanisms of effect from increased levels of Mn¹⁴, although both low¹⁵ and high^{16–17} Mn levels in the *in utero* environment can have negative results. Previous research has suggested that Mn crosses into¹⁶ and can concentrate¹⁸ in the placenta, suggesting that levels of Mn in fetal tissues may be more relevant to fetal health than maternal Mn levels. There is a growing body of literature implicating epigenetic mechanisms as contributing to the development of chronic adult diseases arising in association with prenatal exposures. Further, there are several reports associating epigenetic alterations with exposure to manganese and other heavy metals^{19–21}, and a study by Wang and colleagues²² has raised the possibility that DNA methylation may mediate adverse effects associated with prenatal Mn exposure. In addition, many of these metals may co-occur due to their environmental and anthropogenic sources²³. For example, lead and manganese levels have been correlated amongst children living in urban environments^{24–26}. Prenatal Mn exposure has been associated with childhood

neurodevelopmental problems^{27–29} and later life diseases such as cancer^{13,30}. Hence, we hypothesized that alterations to normal placental DNA methylation patterns are associated with infant toenail Mn and, further, that these changes were associated with changes in measures of fetal growth.

2.1 MATERIALS AND METHODS

2.1.1 Study Design

In this study, the methylation status of >485,000 CpG loci in 61 placental samples was interrogated using an Illumina Infinium HumanMethylation450 BeadArray³¹. These placental samples were collected from infants enrolled in an ongoing birth cohort of healthy, term infants from non-pathologic pregnancies delivered at Women and Infants' Hospital in Providence, RI, USA, according to protocols approved by both Women and Infants' Hospital and Dartmouth College Institutional Review Boards³². Full thickness sections of fetal placental tissue were collected 2 cm from the umbilical cord insertion site, free of maternal decidua, within 2 hours of birth. Four samples were taken from each placenta, one from each quadrant of the placenta. These sections were immediately placed in RNAlater™ (Applied Biosystems, Inc., AM7020). Samples were blotted dry of RNAlater after being stored at 4°C for at least 72 hours, snap-frozen in liquid nitrogen and homogenized into a powder using a liquid nitrogen cooled mortar and pestle. The homogenized sample was stored at –80°C prior to processing. Homogenizing 4 full thickness samples per placenta, one from each quadrant, is performed to minimize potential variations in methylation within placental tissue.

2.1.2 Exposure assessment

Following hospital discharge, toenail clippings from mothers and infants were requested, and were available for 61 of these infants. Infant toenail clippings were taken from all toes and were analyzed for µg Mn per g of toenail material following methods previously described³³ in the Dartmouth trace element analysis laboratory. Samples were read in four batches. Within each batch, samples with Mn content below the limit of detection were assigned a value equal to half of the lowest observed Mn value in that batch.

2.1.3 DNA extraction and bisulfite modification

The QIAmp DNA Mini Kit (Qiagen, Inc., 51304) was used for placental DNA extraction according to manufacturer's protocol; once extracted, quantification of placental DNA was achieved with a NanoDrop ND-1000 spectrophotometer (NanoDrop). 1 µg of placental DNA was bisulfite modified using an EZ DNA Methylation Kit (Zymo Research, D5008) for subsequent genome-wide DNA methylation analysis.

2.1.4 DNA methylation profiling

The Illumina Infinium HumanMethylation450 BeadChip array platform³¹ was utilized to interrogate >485,000 CpG loci in the placental DNA samples. This assessment was performed at the University of Minnesota Genomics Center following standardized protocols of the manufacturer. β values representing the methylation status at each CpG locus were calculated from methylated (M) and unmethylated (U) allele intensity; the ratio

of fluorescent signals $\beta = \text{Max}(M,0)/[\text{Max}(M,0) + \text{Max}(U,0) + 100]$ and $0 < \beta < 1$. Complete methylation is indicated by β values near 1 and absence of methylation is indicated by β values near 0. Following array quality assurance as previously described³⁴, 384,474 autosomal, non-single nucleotide polymorphism (SNP)-associated CpG loci were subsequently analyzed. The reliability of these arrays has been validated through repeated samples³⁴ and through orthogonal techniques by our lab and others^{35–37}.

2.1.5 Gene Expression

Total RNA was extracted from an aliquot of homogenized placenta using the RNeasy Mini Kit (Qiagen, Valencia, CA) following manufacturer's protocols and quantified using a Nanodrop 2000 (ThermoFisher Scientific, Waltham, MA). RNA samples were aliquoted and stored at -80°C and samples were thawed only once for expression analysis using the BioRad CFX Connect Real-Time PCR Detection System (BioRad, Hercules, CA). First strand reaction was performed with BioRad iScript cDNA Synthesis Kit and qPCR reaction with BioRad iQ SYBER Green Supermix following manufacturer's protocols. All reactions were run in triplicate; results were normalized to the placental expression of housekeeping gene succinate dehydrogenase subunit A (*SDHA*) and low placental methylation status, which served as a control for the experiment. Significance was determined by two-tailed Student's t-tests were used to assess potential associations between low or high methylation status of *LOC284276* and gene expression, as sample for gene expression were chosen to be of the highest and lowest extents of methylation. Expression of *LOC284276* and *SDHA* were measured using the following primers: Transcript 1 (GenBank hypothetical *Homo sapiens* mRNA transcript BC121150.1): F-5'-ACGCCTTCTCTTGGGTCTG, R-5'-CAGGCTTCTGCTCCTCTCAC. Transcript 2 (GenBank hypothetical *Homo sapiens* mRNA transcript BC035336.1): F-5'-CACCGGTTTCATCGGTCTGA, R-5'-CACCCACCGAATTCTCACA. *SDHA*: F-5'-TGCTCAGTATCCAGTAGTGGA, R-5'-TTCTCTTACCTGTGCTGCAA.

2.1.6 Statistical analysis

Prior to analysis, methylation β values were logit-transformed (i.e., $\log_2(\beta/1-\beta)$) as in previous studies to obtain the DNA methylation M-value for each CpG locus³⁸. To adjust for the presence of plate-effects that might confound subsequent analyses, the ComBat normalization method³⁹ was applied to the methylation M-values using the R-package 'sva'. Combat is an empirical Bayes batch adjustment methodology that uses a location and scale adjustment for standardizing the mean and variability in methylation levels across batches and has been shown to perform effectively and efficiently compared to competing batch/plate-adjustment methodologies. Successful attenuation of plate-effects was assessed using a Principal Components Analysis (PCA) of the plate-adjusted methylation data following ComBat. To avoid assuming a linear response in the association between infant toenail Mn and placental methylation, tertiles were generated for infant toenail Mn data and used for subsequent analysis³⁸. Referent tertile for Mn analyses was medium Mn, due to the fact that both high and low levels of Mn have been shown to have detrimental health effects^{12,27}. This ensured that our analyses would capture placental DNA methylation patterns associated with either high or low infant toenail Mn tertile. A permutation test⁴⁰ was used to test the omnibus association between infant toenail Mn and methylation status over the 384,474

CpG loci, controlled for maternal age, birth weight percentile, delivery method, and infant gender⁴. Specifically, minimum p-value (over individual regression models for each of 384,474 CpG loci) was used as a test statistic, and its null distribution was obtained by 1000 draws from the permutation distribution obtained by permuting infant toenail Mn values with respect to methylation and putative confounders. CpG loci passing a Bonferroni p-value threshold of $p < 1.3 \times 10^{-7}$ were selected to minimize type I error. The Bonferroni threshold is calculated by dividing the desired p-value threshold ($p < 0.05$) by the number of loci investigated (384,474)⁴¹.

3.1 RESULTS

Table 1 describes the demographics of the population of mother-infant pairs included in this study. The mothers who participated in this study were predominantly Caucasian and the infants were term births (>37 weeks, mean 39 weeks) with a mean birth weight of 3426.9 grams (standard deviation 678.3 grams). None of the mothers reported smoking during their pregnancies. Mn levels ranged from 0.131 to 5.666 μg Mn per gram of toenail, and these levels were categorized into tertiles for subsequent analyses. The low infant toenail Mn tertile ranged from 0.131 μg Mn per gram of toenail to 0.374; the medium, or referent, tertile from 0.394 to 0.771; and the high tertile from 0.858 to 5.666. It is important to note that although individuals may be exposed to multiple heavy metals in the environment, for most heavy metals, these concentrations will fall largely within a reference range of values; in this cohort, toenail Mn concentrations largely fell within a proposed reference range for toenail Mn derived from concentrations found in healthy individuals' toenail samples⁴².

Genome-wide DNA methylation status of placenta-derived DNA from 61 placental samples was assessed and the overall association between methylation status and infant toenail Mn tertiles was examined. An omnibus test suggested significant variation in DNA methylation associated with infant toenail Mn tertile ($p = 0.045$). 57,377 CpG loci were found to have p-values < 0.05 ; 713 CpG loci were found to have p-values < 0.0001 . Of the CpG loci with p-values < 0.0001 , 46.4% were found to reside within CpG islands, 20.3% were located in CpG island shore regions (regions immediately upstream (5') or downstream (3') of a CpG island), and 5.9% were located in CpG island shelf regions (regions slightly further upstream (5') or downstream (3') of a CpG island, beyond shore regions). Differential methylation patterns at any of these sites are theoretically capable of influencing gene expression, particularly in promoter regions, although gene body methylation can also influence gene expression, alternative splicing, and spurious transcription. 158 of these CpG loci were promoter-associated and 241 were located within the gene body. 83 CpG loci were found to reside in genes with multiple CpG loci with p-values < 0.0001 ; these CpG loci, their p-values for the association with infant toenail Mn tertile, their gene symbols and annotations can be found in the Supplemental Material, Table S1.

We next asked if there were individual loci that were associated with the Mn tertiles. This analysis found that 5 CpG loci, presented in Table 2, passed the Bonferroni threshold of $p < 1.3 \times 10^{-7}$. These CpG loci were in the *EMX2OS*, *ATAD2B*, *FTO/RPGRIP1L*, *EN1*, and *LOC284276* genes' regions. Box plots of the methylation of these 5 CpG loci by infant toenail Mn tertile can be found in Figure 1.

To examine if variability in methylation at these loci was associated with newborn outcomes, such as growth and gestational time, we modeled these outcomes as a function of DNA methylation of each of the loci controlled for confounders. The placental methylation status of one of these loci, cg22284422, in the uncharacterized *LOC284276* region on chromosome 18 was significantly associated with birth weight in a multivariable linear regression model controlled for maternal age, infant gender, and gestational age⁴ (see Table 3). Based on this model, a 10% increase in placental cg22284422 methylation corresponds to an expected decrease in birth weight of 293.44 grams (see Figure 2 and Table 3). In addition, the expression in placental tissue of two known mRNA transcripts of the *LOC284276* gene was analyzed in a subset of these samples. We observed no significant differential gene expression to be associated with placental cg22284422 methylation.

4.1 DISCUSSION

In this study, we have conducted an epigenome-wide study to discover potential associations between placental methylation patterns and prenatal Mn exposure. We found 713 CpG loci with placental DNA methylation patterns associated with infant toenail Mn tertile at a significance threshold of $p < 0.0001$, of which 5 CpG loci demonstrated Mn-associated placental DNA methylation levels at a Bonferroni-adjusted significance threshold of $p < 1.3 \times 10^{-7}$.

Four of the five CpG loci associated with placental Mn are of crucial importance in central nervous system differentiation and development (*EMX2OS*⁴³, *ATAD2B*⁴⁴ and *ENI*⁴⁵) and, when expressed in the placenta, fetal growth (*FTO*⁴⁶). Moreover, a well-characterized link exists between *FTO* and obesity⁴⁶. *ENI* is a homeobox gene⁴⁵, underscoring its importance in development. Interestingly, the *EMX2OS* gene encodes a long non-coding RNA transcript regulating the *EMX2* gene⁴³. This might suggest that metal exposures influence non-coding RNA expression, although this awaits experimental proof. While the function of the fifth gene region, *LOC284276*, is not currently known, we found that the placental methylation status of cg22284422 within this region was significantly associated with birth weight. The mechanism responsible for the association between methylation of this loci and birth weight is unknown, but it has been suggested that Mn levels in maternal and umbilical cord blood are linked to impaired fetal growth^{47–49}. The absence of data characterizing this putative gene limit our ability to understand the nature of this association, but GEO datasets report *LOC284276* expression changes to occur in colorectal cancer, prostate cancer, glial brain tumors, and adult acute myeloid leukemia⁵⁰ suggesting that it has an important role in growth and development. Our real-time PCR experiments, did not indicate a strong relationship between methylation at this region of *LOC284276* and gene expression. The function and gene product of this locus are unclear, particularly in placenta, and so methylation of this region associated with this gene may be performing other functions. Further characterization of methylation of this region and any potential functional significance in the placenta are needed to elucidate the role it may play in infant growth.

The 713 CpG loci we have found to be associated ($p < 0.0001$) with infant toenail Mn reside in genes that also play roles in fetal and placental development, particularly neurodevelopment. In addition, they have been associated with susceptibility to a myriad of

diseases and disorders. After inputting these 713 genes into the DAVID functional annotation tool⁵¹, we found that these genes are involved in multiple biological processes and pathways of potential interest. Many of these genes are involved in neurodevelopment and neurogenesis. These include *CNP*⁵², *EN1*⁴⁵, *ROBO3*⁵³, *ZNRF2*⁵⁴, *NEUROD1*⁵⁵, *BMP2*⁵⁶, *FGF18*⁵⁷, *SALL1*⁵⁸, *TULP3*⁵⁹, *PTEN*⁶⁰, *ILK*⁶¹, *TSC2*⁶², *CALD1*⁶³, *HOXA3*⁶⁴, *UTRN*⁶⁵, *NTF3*⁶⁶ and *EPHB2*⁶⁷, among others. Further, additional genes were associated with the development of or risk for various neurological disorders, including autism (*ROBO3*⁵³, *SYN3* and *GRIK4*⁶⁸), attention deficit hyperactivity disorder (*ISL1*⁶⁹), Tourette's syndrome (*SLITRK1*⁷⁰), schizophrenia (*NEUROD1*⁵⁵ and *CYFIP1*⁷¹), and Alzheimer's disease (*NQO1*⁷² and *FKBP1B*⁷³). Several genes on this list, including *G2E3*⁷⁴, *GNAS*⁷⁵, and *TSC2*⁷⁶, are crucial for normal embryonic development. *VEGF*⁷⁷ is important for angiogenesis, a key placental function; *CYR61*⁷⁸ has been implicated in preeclampsia; and two others of the identified genes are involved in trophoblast differentiation and placental development (*PARP1*⁷⁹ and *NEUROD1*⁸⁰). These results suggest that *in utero* Mn exposure may result in potentially harmful disruption to normal placental and fetal growth and development, which is important considering existing links between placental methylation patterns and fetal growth^{6,34} and neurobehavior^{81–82}.

We cannot say with certainty what mechanism Mn or other environmental toxicants may be using to associate with differential methylation, as there remain questions even on how *de novo* methylation occurs⁸³. We believe it is unlikely that these exposures are having effects on the expression pattern of the methyltransferase enzymes themselves, as this would potentially lead to broader, more global effects, while we observe only targeted effects. Likely, the variability in methylation is a reflection of the cellular response to stressors, including toxicants. This is supported by recent work describing distinct fetal and maternal leukocyte methylation patterns, or “environmental footprints,” associated with cadmium exposure *in utero*⁸⁴. An additional possibility is that DNA-binding factors may play a role in mediating the turnover of methylation marks in placental tissue, an idea known as the transcription factor occupancy hypothesis⁸⁵. Future targeted mechanistic studies are warranted to address this issue.

There are a number of limitations to this study which should be considered. First, we are limited, at this point, to a relatively small sample size (n=61). It is possible that, due to this small sample size, our model is over-adjusted; however, this would bias our results toward the null, strengthening our findings. We also do not have access to an independent cohort in which to validate these findings, and so it will be beneficial to confirm the methylation and expression patterns we have observed in independent samples as well. Finally, we considered infant Mn exposure as a single exposure in isolation, but recognize that this exposure is one of many that will be occurring coincidentally during pregnancy, and so future studies need to examine the additive and interactive effects of chemical and environmental mixtures, and their impacts on molecular features as well as health outcomes.

5.1 CONCLUSIONS

This study is an important but preliminary first step in defining placental DNA methylation patterns associated with *in utero* exposure to Mn; however, further investigation of altered

placental methylation patterns in a larger sample set with complete data on Mn in corresponding infant hair samples or toenail clipping samples is needed to validate our findings, and this represents a limitation of this study. Future research aimed at further elucidating the mechanism of the association between Mn and other micronutrients and placental epigenetic effects, including array-based studies, and studies considering the potential mechanism relating these alterations to outcome, will add to the understanding of how perturbations in prenatal Mn levels may contribute to the developmental origins of health and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FUNDING: This work is supported by NIH-NIMH R01MH094609, NIH-NIEHS R01ES022223 and NIH-NIEHS P01 ES022832 and by US EPA grant RD83544201. Its contents are solely the responsibility of the grantee and do not necessarily represent the official views of the US EPA. Further, the US EPA does not endorse the purchase of any commercial products or services mentioned in the presentation. Also by T32ES007272 from the NIH-NIEHS, and T32HL076134-04 from the NHLBI (to J.Z.J. Maccani while a postdoctoral fellow at Brown University). Dr. Maccani is now at the Penn State College of Medicine Tobacco Center of Regulatory Science (TCORS) and is funded by grant P50-DA-036107-01 from the National Institutes of Health.

ABBREVIATIONS

SNP	single nucleotide polymorphism
Mn	manganese
PCA	Principal Components Analysis

References

1. Dandrea J, Wilson V, Gopalakrishnan G, Heasman L, Budge H, Stephenson T, et al. Maternal nutritional manipulation of placental growth and glucose transporter 1 (GLUT-1) abundance in sheep. *Reproduction*. 2001; 122(5):793–800. [PubMed: 11690540]
2. Desai M, Hales CN. Role of fetal and infant growth in programming metabolism in later life. *Biological reviews of the Cambridge Philosophical Society*. 1997; 72(2):329–348. [PubMed: 9155245]
3. Mattison DR. Environmental exposures and development. *Current opinion in pediatrics*. 2010; 22(2):208–218. [PubMed: 20216314]
4. Maccani JZJ, Koestler DC, Houseman EA, Marsit CJ, Kelsey KT. Smoking-related changes to placental DNA methylation at the RUNX3 gene are associated with gestational age. *Epigenomics*. 2013; 5(6):619–30. [PubMed: 24283877]
5. Novakovic B, Ryan J, Pereira N, Boughton B, Craig JM, Saffery R. Postnatal stability, tissue, and time specific effects of AHRR methylation change in response to maternal smoking in pregnancy. *Epigenetics*. 2014; 9(3):377–86. [PubMed: 24270552]
6. Wilhelm-Benartzi CS, Houseman EA, Maccani MA, Poage GM, Koestler DC, Langevin SM, et al. In utero exposures, infant growth, and DNA methylation of repetitive elements and developmentally related genes in human placenta. *Environmental health perspectives*. 2012; 120(2):296–302. [PubMed: 22005006]

7. Suter M, Abramovici A, Showalter L, Hu M, Shope CD, Varner M, et al. In utero tobacco exposure epigenetically modifies placental CYP1A1 expression. *Metabolism: clinical and experimental*. 2010; 59(10):1481–1490. [PubMed: 20462615]
8. Suter M, Ma J, Harris A, Patterson L, Brown KA, Shope C, et al. Maternal tobacco use modestly alters correlated epigenome-wide placental DNA methylation and gene expression. *Epigenetics : official journal of the DNA Methylation Society*. 2011; 6(11):1284–1294.
9. Nielsen FH. How should dietary guidance be given for mineral elements with beneficial actions or suspected of being essential? *The Journal of nutrition*. 1996; 126(9 Suppl):2377S–2385S. [PubMed: 8811801]
10. Pennington JA, Young BE. Total diet study nutritional elements, 1982–1989. *Journal of the American Dietetic Association*. 1991; 91(2):179–183. [PubMed: 1991931]
11. Antonova MV. Effect of different amounts of manganese in the daily food ration on the growth and development of the progeny of white rats. *Voprosy pitaniia*. 1978; (1):65–68. [PubMed: 629035]
12. Wozniak A, Kujawa A, Senczuk-Przybylowska M, Kulza M, Gaweck W, Szybiak B, et al. Physiological metals in the serum, hair and nails of patients with head and neck cancer. *Przegląd lekarski*. 2012; 69(10):785–797. [PubMed: 23421034]
13. Karimi G, Shahar S, Homayouni N, Rajikan R, Abu Bakar NF, Othman MS. Association between trace element and heavy metal levels in hair and nail with prostate cancer. *Asian Pacific journal of cancer prevention : APJCP*. 2012; 13(9):4249–4253. [PubMed: 23167323]
14. Zota AR, Ettinger AS, Bouchard M, Amarasiwardena CJ, Schwartz J, Hu H, et al. Maternal blood manganese levels and infant birth weight. *Epidemiology*. 2009; 20(3):367–373. [PubMed: 19289966]
15. Mistry HD, Williams PJ. The importance of antioxidant micronutrients in pregnancy. *Oxidative medicine and cellular longevity*. 2011; 2011:841749. [PubMed: 21918714]
16. Misselwitz B, Muhler A, Weinmann HJ. A toxicologic risk for using manganese complexes? A literature survey of existing data through several medical specialties. *Investigative radiology*. 1995; 30(10):611–620. [PubMed: 8557501]
17. Takser L, Mergler D, Hellier G, Sahuquillo J, Huel G. Manganese, monoamine metabolite levels at birth, and child psychomotor development. *Neurotoxicology*. 2003; 24(4–5):667–674. [PubMed: 1290080]
18. Miller RK, Mattison DR, Panigel M, Ceckler T, Bryant R, Thomford P. Kinetic assessment of manganese using magnetic resonance imaging in the dually perfused human placenta in vitro. *Environmental health perspectives*. 1987; 74:81–91. [PubMed: 3691434]
19. Cantone L, Nordio F, Hou L, Apostoli P, Bonzini M, Tarantini L, et al. Inhalable metal-rich air particles and histone H3K4 dimethylation and H3K9 acetylation in a cross-sectional study of steel workers. *Environmental health perspectives*. 2011; 119(7):964–969. [PubMed: 21385672]
20. Goodrich JM, Basu N, Franzblau A, Dolinoy DC. Mercury biomarkers and DNA methylation among Michigan dental professionals. *Environmental and molecular mutagenesis*. 2013; 54(3):195–203. [PubMed: 23444121]
21. Baccarelli A, Bollati V. Epigenetics and environmental chemicals. *Current opinion in pediatrics*. 2009; 21(2):243–251. [PubMed: 19663042]
22. Wang L, Shiraki A, Itahashi M, Akane H, Abe H, Mitsumori K, et al. Aberration in epigenetic gene regulation in hippocampal neurogenesis by developmental exposure to manganese chloride in mice. *Toxicol Sci*. 2013; 136(1):154–65. [PubMed: 23976782]
23. Hu H, Shine J, Wright RO. The challenge posed to children's health by mixtures of toxic waste: the Tar Creek superfund site as a case-study. *Pediatr Clin North Am*. 2007; 54(1):155–75. [PubMed: 17306689]
24. Delves HT, Clayton BE, Bicknell J. Concentration of trace metals in the blood of children. *Br J Prev Soc Med*. 1973; 27(2):100–7. [PubMed: 4724642]
25. Joselow MM, Tobias E, Koehler R, Coleman S, Bogden J, Gause D. Manganese pollution in the city environment and its relationship to traffic density. *Am J Public Health*. 1978; 68(6):557–60. [PubMed: 655314]

26. Zielhuis RL, del Castillo P, Herber RF, Wibowo AA. Levels of lead and other metals in human blood: suggestive relationships, determining factors. *Environ Health Perspect.* 1978; 25:103–9. [PubMed: 720295]
27. Claus Henn B, Ettinger AS, Schwartz J, Tellez-Rojo MM, Lamadrid-Figueroa H, Hernandez-Avila M, et al. Early postnatal blood manganese levels and children's neurodevelopment. *Epidemiology.* 2010; 21(4):433–439. [PubMed: 20549838]
28. Roels HA, Bowler RM, Kim Y, Claus Henn B, Mergler D, Hoet P, et al. Manganese exposure and cognitive deficits: a growing concern for manganese neurotoxicity. *Neurotoxicology.* 2012; 33(4): 872–880. [PubMed: 22498092]
29. Ericson JE, Crinella FM, Clarke-Stewart KA, Allhusen VD, Chan T, Robertson RT. Prenatal manganese levels linked to childhood behavioral disinhibition. *Neurotoxicology and teratology.* 2007; 29(2):181–187. [PubMed: 17079114]
30. Bunin GR, Li Y, Ganguly A, Meadows AT, Tseng M. Parental nutrient intake and risk of retinoblastoma resulting from new germline RB1 mutation. *Cancer causes & control : CCC.* 2013; 24(2):343–355. [PubMed: 23224327]
31. Illumina. [accessed 2013 Mar 7 2013] Illumina HumanMethylation450 BeadChip Kit. 2013. Available: http://www.illumina.com/products/methylation_450_beadchip_kits.ilmn
32. Lesueur C, Gilbert-Diamond D, Andrew AS, Ekstrom RM, Li Z, Kelsey KT, et al. A case-control study of polymorphisms in xenobiotic and arsenic metabolism genes and arsenic-related bladder cancer in New Hampshire. *Toxicology letters.* 2012; 210(1):100–106. [PubMed: 22306368]
33. Karagas MR, Andrew AS, Nelson HH, Li Z, Punshon T, Schned A, et al. SLC39A2 and FSIP1 polymorphisms as potential modifiers of arsenic-related bladder cancer. *Human genetics.* 2012; 131(3):453–461. [PubMed: 21947419]
34. Banister CE, Koestler DC, Maccani MA, Padbury JF, Houseman EA, Marsit CJ. Infant growth restriction is associated with distinct patterns of DNA methylation in human placentas. *Epigenetics : official journal of the DNA Methylation Society.* 2011; 6(7):920–927.
35. Koestler DC, Avissar-Whiting M, Houseman EA, Karagas MR, Marsit CJ. Differential DNA methylation in umbilical cord blood of infants exposed to low levels of arsenic in utero. *Environ Health Perspect.* 2013; 121(8):971–7. [PubMed: 23757598]
36. Marsit CJ, Houseman EA, Christensen BC, Gagne L, Wrensch MR, Nelson HH, Wiemels J, Zheng S, Wiencke JK, Andrew AS, Schned AR, Karagas MR, Kelsey KT. Identification of methylated genes associated with aggressive bladder cancer. *PLoS One.* 2010; 5(8):e12334. [PubMed: 20808801]
37. Blair JD, Langlois S, McFadden DE, Robinson WP. Overlapping DNA methylation profile between placentas with trisomy 16 and early-onset preeclampsia. *Placenta.* 2014; 35(3):216–22. [PubMed: 24462402]
38. Kuan PF, Wang S, Zhou X, Chu H. A statistical framework for Illumina DNA methylation arrays. *Bioinformatics.* 2010; 26(22):2849–2855. [PubMed: 20880956]
39. Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics.* 2007; 8(1):118–127. [PubMed: 16632515]
40. Westfall, PH.; Young, SS. Resampling-based multiple testing : examples and methods for P-value adjustment. New York: Wiley; 1993.
41. Dunn OJ. Multiple comparisons among means. *JASA.* 1961; 56(293):52–64.
42. Goullé JP, Sausseureau E, Mahieu L, Bouige D, Groenwont S, Guerbet M, Lacroix C. Application of inductively coupled plasma mass spectrometry multielement analysis in fingernail and toenail as a biomarker of metal exposure. *J Anal Toxicol.* 2009; 33:92–8. [PubMed: 19239734]
43. Spigoni G, Gedressi C, Mallamaci A. Regulation of Emx2 expression by antisense transcripts in murine cortico-cerebral precursors. *PLoS One.* 2010; 5(1):e8658. [PubMed: 20066053]
44. Leachman NT, Brellier F, Ferralli J, Chiquet-Ehrismann R, Tucker RP. ATAD2B is a phylogenetically conserved nuclear protein expressed during neuronal differentiation and tumorigenesis. *Development, growth & differentiation.* 2010; 52(9):747–755.
45. Alves dos Santos MT, Smidt MP. En1 and Wnt signaling in midbrain dopaminergic neuronal development. *Neural development.* 2011; 6:23. [PubMed: 21569278]

46. Bassols J, Prats-Puig A, Vazquez-Ruiz M, Garcia-Gonzalez MM, Martinez-Pascual M, Avelli P, et al. Placental FTO expression relates to fetal growth. *Int J Obes (Lond)*. 2010; 34(9):1365–1370. [PubMed: 20351740]
47. Zota AR, Ettinger AS, Bouchard M, Amarasiwardena CJ, Schwartz J, Hu H, Wright RO. Maternal blood manganese levels and infant birth weight. *Epidemiology*. 2009; 20(3):367–73. [PubMed: 19289966]
48. Vigeh M, Yokoyama K, Ramezanzadeh F, Dahaghin M, Fakhriazad E, Seyedaghamiri Z, Araki S. Blood manganese concentrations and intrauterine growth restriction. *Reprod Toxicol*. 2008; 25(2): 219–23. [PubMed: 18242051]
49. Guan H, Wang M, Li X, Piao F, Li Q, Xu L, Kitamura F, Yokoyama K. Manganese concentrations in maternal and umbilical cord blood: related to birth size and environmental factors. *Eur J Public Health*. 2014; 24(1):150–7. [PubMed: 23543679]
50. Barrett T, Wilhite SE, Ledoux P, Evangelista C, Kim IF, Tomashevsky M, et al. NCBI GEO: archive for functional genomics data sets—update. *Nucleic Acids Res*. 2013 Jan.41:D991–5. Database issue. [PubMed: 23193258]
51. Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, et al. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome biology*. 2003; 4(5):3.
52. Aston C, Jiang L, Sokolov BP. Transcriptional profiling reveals evidence for signaling and oligodendroglial abnormalities in the temporal cortex from patients with major depressive disorder. *Molecular psychiatry*. 2005; 10(3):309–322. [PubMed: 15303102]
53. Anitha A, Nakamura K, Yamada K, Suda S, Thanseem I, Tsujii M, et al. Genetic analyses of roundabout (ROBO) axon guidance receptors in autism. *American journal of medical genetics Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics*. 2008; 147B(7):1019–1027.
54. Araki T, Milbrandt J. ZNRF proteins constitute a family of presynaptic E3 ubiquitin ligases. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2003; 23(28):9385–9394. [PubMed: 14561866]
55. Aston C, Jiang L, Sokolov BP. Microarray analysis of postmortem temporal cortex from patients with schizophrenia. *Journal of neuroscience research*. 2004; 77(6):858–866. [PubMed: 15334603]
56. Bani-Yaghoob M, Felker JM, Sans C, Naus CC. The effects of bone morphogenetic protein 2 and 4 (BMP2 and BMP4) on gap junctions during neurodevelopment. *Experimental neurology*. 2000; 162(1):13–26. [PubMed: 10716885]
57. Bao X, Nishimura S, Mikami T, Yamada S, Itoh N, Sugahara K. Chondroitin sulfate/dermatan sulfate hybrid chains from embryonic pig brain, which contain a higher proportion of L-iduronic acid than those from adult pig brain, exhibit neuritogenic and growth factor binding activities. *The Journal of biological chemistry*. 2004; 279(11):9765–9776. [PubMed: 14699094]
58. Bohm J, Buck A, Borozdin W, Mannan AU, Matysiak-Scholze U, Adham I, et al. Sall1, sall2, and sall4 are required for neural tube closure in mice. *The American journal of pathology*. 2008; 173(5):1455–1463. [PubMed: 18818376]
59. Carroll K, Gomez C, Shapiro L. Tubby proteins: the plot thickens. *Nature reviews Molecular cell biology*. 2004; 5(1):55–63. [PubMed: 14708010]
60. Christensen R, de la Torre-Ubieta L, Bonni A, Colon-Ramos DA. A conserved PTEN/FOXO pathway regulates neuronal morphology during *C. elegans* development. *Development*. 2011; 138(23):5257–5267. [PubMed: 22069193]
61. Dai X, Jiang W, Zhang Q, Xu L, Geng P, Zhuang S, et al. Requirement for integrin-linked kinase in neural crest migration and differentiation and outflow tract morphogenesis. *BMC biology*. 2013; 11:107. [PubMed: 24131868]
62. de Vries PJ, Howe CJ. The tuberous sclerosis complex proteins--a GRIP on cognition and neurodevelopment. *Trends in molecular medicine*. 2007; 13(8):319–326. [PubMed: 17632034]
63. Fukumoto K, Morita T, Mayanagi T, Tanokashira D, Yoshida T, Sakai A, et al. Detrimental effects of glucocorticoids on neuronal migration during brain development. *Molecular psychiatry*. 2009; 14(12):1119–1131. [PubMed: 19564873]

64. Gaufo GO, Thomas KR, Capecchi MR. Hox3 genes coordinate mechanisms of genetic suppression and activation in the generation of branchial and somatic motoneurons. *Development*. 2003; 130(21):5191–5201. [PubMed: 12954718]
65. Grady RM, Merlie JP, Sanes JR. Subtle neuromuscular defects in utrophin-deficient mice. *The Journal of cell biology*. 1997; 136(4):871–882. [PubMed: 9049252]
66. Yang T, Kersigo J, Jahan I, Pan N, Fritzsche B. The molecular basis of making spiral ganglion neurons and connecting them to hair cells of the organ of Corti. *Hearing research*. 2011; 278(1–2): 21–33. [PubMed: 21414397]
67. St John JA, Key B. EphB2 and two of its ligands have dynamic protein expression patterns in the developing olfactory system. *Brain research Developmental brain research*. 2001; 126(1):43–56. [PubMed: 11172885]
68. Griswold AJ, Ma D, Cukier HN, Nations LD, Schmidt MA, Chung RH, et al. Evaluation of copy number variations reveals novel candidate genes in autism spectrum disorder-associated pathways. *Human molecular genetics*. 2012; 21(15):3513–3523. [PubMed: 22543975]
69. Laurin N, Lee J, Ickowicz A, Pathare T, Malone M, Tannock R, et al. Association study for genes at chromosome 5p13-q11 in attention deficit hyperactivity disorder. *American journal of medical genetics Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics*. 2008; 147B(5):600–605.
70. Kowalska A, Midro AT, Janik P, Gogol A, Sluzewski W, Rajewski A. Searching for Tourette's syndrome gene. Part 2. Patient's genome variability. *Postepy Hig Med Dosw (Online)*. 2012; 66:89–95. [PubMed: 22371411]
71. Hosak L. New findings in the genetics of schizophrenia. *World journal of psychiatry*. 2013; 3(3): 57–61. [PubMed: 24255876]
72. Bian JT, Zhao HL, Zhang ZX, Bi XH, Zhang JW. Association of NAD(P)H:quinone oxidoreductase 1 polymorphism and Alzheimer's disease in Chinese. *Journal of molecular neuroscience : MN*. 2008; 34(3):235–240. [PubMed: 18253865]
73. Gant JC, Blalock EM, Chen KC, Kadish I, Porter NM, Norris CM, et al. FK506-binding protein 1b/12.6: A key to aging-related hippocampal Ca dysregulation? *European journal of pharmacology*. 2013
74. Brooks WS, Helton ES, Banerjee S, Venable M, Johnson L, Schoeb TR, et al. G2E3 is a dual function ubiquitin ligase required for early embryonic development. *The Journal of biological chemistry*. 2008; 283(32):22304–22315. [PubMed: 18511420]
75. Schwindinger WF, Reese KJ, Lawler AM, Gearhart JD, Levine MA. Targeted disruption of Gnass in embryonic stem cells. *Endocrinology*. 1997; 138(10):4058–4063. [PubMed: 9322912]
76. Campioni M, Severino A, Manente L, Tudu IL, Toldo S, Caraglia M, et al. The serine protease HtrA1 specifically interacts and degrades the tuberous sclerosis complex 2 protein. *Molecular cancer research : MCR*. 2010; 8(9):1248–1260. [PubMed: 20671064]
77. Clark DE, Smith SK, Licence D, Evans AL, Charnock-Jones DS. Comparison of expression patterns for placenta growth factor, vascular endothelial growth factor (VEGF), VEGF-B and VEGF-C in the human placenta throughout gestation. *The Journal of endocrinology*. 1998; 159(3): 459–467. [PubMed: 9834463]
78. Gellhaus A, Schmidt M, Dunk C, Lye SJ, Kimmig R, Winterhager E. Decreased expression of the angiogenic regulators CYR61 (CCN1) and NOV (CCN3) in human placenta is associated with pre-eclampsia. *Molecular human reproduction*. 2006; 12(6):389–399. [PubMed: 16675545]
79. Hemberger M, Nozaki T, Winterhager E, Yamamoto H, Nakagama H, Kamada N, et al. Parp1-deficiency induces differentiation of ES cells into trophoblast derivatives. *Developmental biology*. 2003; 257(2):371–381. [PubMed: 12729565]
80. Westerman BA, Chhatta A, Poutsma A, van Vegchel T, Oudejans CB. NEUROD1 acts in vitro as an upstream regulator of NEUROD2 in trophoblast cells. *Biochimica et biophysica acta*. 2004; 1676(1):96–103. [PubMed: 14732494]
81. Bromer C, Marsit CJ, Armstrong DA, Padbury JF, Lester B. Genetic and epigenetic variation of the glucocorticoid receptor (NR3C1) in placenta and infant neurobehavior. *Dev Psychobiol*. 2013; 55(7):673–83. [PubMed: 22714792]

82. Lesseur C, Paquette AG, Marsit CJ. Epigenetic regulation of infant neurobehavioral outcomes. *Med Epigenet.* 2014; 2(2):71–79. [PubMed: 25089125]
83. Marsit CJ. Influence of environmental exposure on human epigenetic regulation. *J Exp Biol.* 2015; 218(Pt 1):71–9. [PubMed: 25568453]
84. Sanders AP, Smeester L, Rojas D, DeBusscher T, Wu MC, Wright FA, et al. Cadmium exposure and the epigenome: Exposure-associated patterns of DNA methylation in leukocytes from mother-baby pairs. *Epigenetics.* 2014; 9(2):212–21. [PubMed: 24169490]
85. Feldmann A, Ivanek R, Murr R, Gaidatzis D, Burger L, Schübeler D. Transcription factor occupancy can mediate active turnover of DNA methylation at regulatory regions. *PLoS Genet.* 2013; 9(12):e1003994. [PubMed: 24367273]

Highlights

- 713 CpG loci in the placenta were associated with *in utero* Mn exposure ($p < 0.0001$).
- 5 loci ($p < 1.3 \times 10^{-7}$) reside in developmental, fetal growth and cancer-related genes.
- cg22284422, in the uncharacterized *LOC284276* gene, was associated with birthweight.
- A 10% methylation increase at this CpG corresponds to a 259.04 g lower birthweight.
- This suggests a link between prenatal Mn, placental methylation and birthweight.

Figure 1.

Box plots of the placental DNA methylation status of the five Mn-associated CpG loci passing a Bonferroni significance threshold of $p < 1.3 \times 10^{-7}$. A) cg07419575; B) cg08192560; C) cg16063747; D) cg22284422; E) cg26692097.

Figure 2.
Plot of cg22284422 placental methylation vs birth weight, with line of best fit. p-value = 0.014; Pearson's product-moment correlation coefficient=-0.31.

Table 1

Study population demographics.

Total, n (%)	61 (100%)
Infant gender	
Male, <i>n</i> (%)	27 (44.3%)
Female, <i>n</i> (%)	34 (55.7%)
Maternal age (years)	
Mean ($\pm$ SD)	32.2 $\pm$ 3.7
Median (Range)	32 (23 – 39)
Tobacco use during pregnancy[†]	
Yes, <i>n</i> (%)	0 (0%)
No, <i>n</i> (%)	60 (98.4%)
Birth weight (grams)	
Mean ($\pm$ SD)	3426.9 $\pm$ 678.3
Median (Range)	3460 (2160 – 4530)
Gestational Age (weeks)	
Mean ($\pm$ SD)	39.0 $\pm$ 1.2
Median (Range)	39 (37 – 41)
Maternal race	
Caucasian, <i>n</i> (%)	53 (86.9%)
Non-Caucasian, <i>n</i> (%)	8 (13.1%)
C-section^{††}	
Yes, <i>n</i> (%)	19 (31.1%)
No, <i>n</i> (%)	41 (67.2%)
Recreational drug use during pregnancy	
Yes, <i>n</i> (%)	1 (1.6%)
No, <i>n</i> (%)	60 (98.4%)

[†] 1 sample missing tobacco smoking data;^{††} 1 sample missing delivery method data.

Table 2

5 CpG loci significantly associated with infant toenail Mn tertile, passing Bonferroni threshold of $p < 1.3 \times 10^{-7}$.

Illumina CpG Designation	Genomic Position (Build 37)	UCSC Gene Symbol	CpG Location	p-Value for the Association with Toenail Mn Tertile
cg16063747	Chr 10: 119294173	EMX2OS	Island	3.15E-08
cg08192560	Chr 2: 24149860	ATAD2B	Island	3.48E-08
cg26692097	Chr 16: 53738201	FTO; RPGRIP1L	Island	8.69E-08
cg07419575	Chr 2: 119605959	EN1	N Shore	1.26E-07
cg22284422	Chr 18: 74240476	LOC284276		1.29E-07

Table 3

Placental methylation status of Mn-associated cg22284422 within the *LOC284276* gene region is associated with infant birth weight in a multivariable linear regression model controlled for potential confounders.

Covariate	Coefficient Estimate on Birth weight in g (Standard Error)	p-Value
10% change in cg22284422 methylation status	−293.44 (120.80)	0.018*
Gestational age (weeks)	194.21 (68.20)	0.0062*
Maternal age (years)	−25.11 (20.87)	0.23
Infant gender		
Female	Ref	
Male	266.52 (158.96)	0.099