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Using Residual Newborn Blood Spots to Investigate CpG Methylation in Relation to Air Pollution and Congenital Heart Defects

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Abstract

Background: The prevalence of tetralogy of Fallot (TOF), a common congenital heart defect, has increased over the last two decades.

Methods: Genome-wide CpG methylation patterns were assessed and analyzed in relation to gestational air pollution exposure among 24 infants with TOF and 24 sex-matched control infants without a birth defect from a North Carolina population-based, case-control study of major structural birth defects (2006–2011). Air pollution exposure during obstetric weeks 3–8 was assigned based on self-reported residence. DNA was extracted from residual newborn blood spots and DNA methylation levels were measured using the Illumina EPIC Array.

Results: Cases had higher exposure to both PM_{2.5} and O₃ compared to controls. No specific CpG loci were statistically significantly associated with TOF status or air pollution exposure, however, we observed associations between TOF case status and DNA methylation at specific genomic regions with genes enriched for functions in metabolism. Additionally, there were significant regions that displayed differential DNA methylation in relation to air pollution exposure within genes involved in apoptosis, necrosis, inflammation, and immune response pathways.

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Conclusions: Generally, air pollution exposure and TOF were associated with differential DNA methylation in distinct genes. These results highlight suggestive links between the environment, epigenome, and TOF to be further investigated in larger studies.

Keywords

Birth Defects; Dried Blood Spots; Epigenetics; DNA Methylation; Air Pollution

Introduction

Congenital heart defects (CHDs) are the most common class of birth defects, with an estimated worldwide prevalence of 9.4 per 1000 births (Liu et al., 2019). Despite improvements in treatment and reductions in mortality, the prevalence of CHDs has remained relatively stable over the last 3 decades (Wu et al., 2020). Previous research suggests only a small portion of CHD cases are explained by known factors such as single-gene deletions and mutations (Boyd et al., 2022; Moreau et al., 2019).

Tetralogy of Fallot (TOF) is the most common complex CHD, with an estimated prevalence of approximately 0.5 per 1000 births in the US (Stallings et al., 2024). TOF consists of four distinct malformations: i) a narrowing of the pulmonary valve, i.e. pulmonary stenosis; ii) a thickening of the wall of the right ventricle; iii) a displaced aorta that overlaps the right ventricle; and iv) a ventricular septal defect. The pulmonary stenosis restricts the amount of blood that can flow out of the right ventricle, causing this blood to go into the left ventricle and into the displaced aorta. As a result, blood bypasses the pulmonary circulation, fails to become oxygenated and leads to cyanosis. TOF requires surgical correction, typically in the first six months of life (O'Brien & Marshall, 2014). The birth-prevalence of TOF has slightly increased over the last two decades, with prevention efforts hindered by a lack of knowledge on contributing causes (Stallings et al., 2024). Research into potential etiologic agents for TOF is important to address the increase in prevalence.

Epigenetic regulation has been hypothesized to play a role in the occurrence of CHDs not explained by chromosomal or Mendelian disorders. For example, Bentham and Bhattacharya point to disruptions in epigenetic regulation as a potential explanation for observed variability in CHD phenotypes resulting from single gene mutations in animal research (Bentham & Bhattacharya, 2008). Others have suggested the role of folate, an important source of methyl groups, in reducing birth defects as supporting the hypothesis that epigenetic regulation plays a role in CHDs (Bahado-Singh et al., 2020; Serra-Juhe et al., 2015). As such, there has been a growing interest in determining if disruptions to DNA methylation may be related to the occurrence of CHDs. DNA methylation, a form of epigenetic regulation, refers to the addition of a methyl group to the cytosine base on DNA. Cells have specific DNA methylation patterns that influence gene and protein expression, and this DNA methylation is maintained during cell replication. While dependent on genomic location, DNA methylation is typically associated with repressed gene expression, potentially by blocking promoters where transcription factors can bind; however, instances where DNA methylation is associated with increased gene expression are also common. Previous research has found that disruptions to DNA methylation contribute to chromosomal

instability, altered gene expression, abnormal cellular differentiation, and apoptosis during embryogenesis (Robertson, 2005).

Epidemiologic studies have observed differential methylation when comparing cases of CHDs as a whole and specific phenotypes, such as TOF, to infants without a birth defect. These studies have used various designs and assessed methylation in various tissues of both mothers of affected offspring and the offspring themselves. For example, Chowdhury et al. (2011) found associations between measures of maternal LINE-1 DNA hypomethylation in blood and having offspring with a CHD (Chowdhury et al., 2011). Similarly, Sheng et al. (2012) reported evidence of LINE-1 hypomethylation in heart tissue samples from infants with TOF when compared to autopsy controls. In subsequent analyses, they reported differential methylation between TOF cases and controls in promoter regions that resulted in both up and down regulation of genes (Sheng et al., 2014). Bahado-Singh et al. (2020) took a different approach and measured genome-wide methylation within placental tissue of TOF births and controls and identified 165 differentially methylated CpG loci. A recent study (Nelson et al., 2022) demonstrated similar DNA methylation profiles in venous blood, heart tissue samples, and buccal cells among TOF cases, but few studies have utilized residual newborn bloodspots. Richards et al (2022) utilized newborn dried bloodspots from eight children with both childhood lymphoma and a CHD and observed associations with molecular changes in the genome at birth and the risk of lymphoma among individuals born with a CHD.

Individual studies of air pollution, specifically ozone (O₃) and fine particulate matter (PM_{2.5}), and TOF have reported both positive and null associations. However, Michel et al. (2023) systematically reviewed published review studies of prenatal exposure to criteria air pollutants and CHDs, including TOF. They identified three systematic reviews of PM_{2.5} and TOF (Hall & Robinson, 2019; Hu et al., 2020; Yu et al. 2021), all of which reported positive associations between PM_{2.5} exposure and TOF, with meta-analytic odds ratios (ORs) ranging from 1.09 to 1.16 per 10 µg/m³ increase. The systematic review by Yu et al. (2021) included the largest number of individual studies examining association between PM_{2.5} and TOF (n=7) and reported a summary OR (95% confidence interval (CI)) of 1.09 (1.00–1.18) per 10 µg/m³ increase. Michel et al. (2023) identified one systematic review of O₃ and TOF; Hu et al. (2020) included six individual studies examining associations between O₃ and TOF and reported a summary OR (95% CI) of 1.08 (0.96–1.22) per 10 ppb increase. Because prenatal air pollution exposure can be risk factor for TOF, and air pollution has been shown to modulate DNA methylation (Rider & Carlsten, 2019), considering air pollution exposure during early gestation may help to explain differences in DNA methylation between TOF cases and controls.

The objective of this research was to (1) compare genome-wide methylation patterns among infants with TOF (cases) and sex-matched infants without a birth defect (controls) and (2) to examine if gestational air pollution exposure could contribute to any observed differences in methylation patterns between cases and controls. In this exploratory study aimed at identifying the contribution of air pollution exposure to epigenetic risk factors for TOF, we leverage previously collected residual newborn blood spots (NBS), an innovative and efficient source of DNA, extensive clinical and epidemiologic data on participants in the

National Birth Defects Prevention Study (NBDPS) from North Carolina, and data on air pollution exposures.

Methods

Study Population

The NBDPS is a population-based, multi-center case-control study of risk factors for major structural birth defects. Study details have been previously published (Reefhuis et al., 2015). Briefly, eligible cases (livebirths, stillbirths, or terminations) with one of over 30 types of birth defects were identified by statewide or regional birth defects registries in multiple US states, and further verified and systematically classified for the NBDPS by clinical geneticists and pediatric cardiologists (Botto et al., 2007; Rasmussen et al., 2003). Infants without a birth defect were sampled as controls using birth certificate records reflecting the same temporal (year) and geographic (state) population as cases. Mothers of cases and controls were invited to participate in the NBDPS protocol, which included an hour-long telephone interview on various demographic, clinical, behavioral, and other pregnancy-related factors.

In some states, residual newborn bloodspots from local Newborn Screening programs were obtained as part of the NBDPS protocol (Wong et al., 2022). For nearly all infants born in the United States, bloodspots are collected via heel stick during the early neonatal period to screen for metabolic, endocrine, and genetic disorders. Some Newborn Screening programs retain any portion of the bloodspot that is left-over after screening activities are complete. With maternal consent, residual bloodspots for NBDPS participants were obtained from the North Carolina State Laboratory of Public Health within three years after delivery and stored in freezers at the University of North Carolina at Chapel Hill.

The eligible population for our study was the subset of interviewed NBDPS participants from North Carolina who delivered a liveborn infant between 2006 and 2011 and for whom bloodspots were available (n=326; 30 cases and 296 controls). We excluded mothers with missing DNA/buccal samples (n=104; 0 cases and 104 controls), mothers with pre-existing diabetes (n=2; 1 case and 1 control), and mothers for whom no geocoded residence was available for the period of time between one month before conception through the end of the first trimester (based on self-reported residential history collected during the interview) (n=9; 1 case and 8 controls). From the remaining 28 cases and 183 controls, we randomly sampled 48 participants for this exploratory study: 24 case infants with tetralogy of Fallot, and 24 control infants matched on infant sex.

This research was approved by the University of North Carolina at Chapel Hill's Office of Human Research Ethics (#21-1717). The EPA's Human Subjects Research Officer also reviewed and approved this work.

Exposure to Air Pollution

Daily average concentrations for fine particulate matter (measuring 2.5 micrometers or less in diameter (PM_{2.5})) and ozone (O₃) were estimated using EPA's Community Multiscale Air Quality (CMAQ) model. Details on the data processing from the CMAQ model are

described elsewhere (Berrocal et al., 2010). Briefly, in its most general form, the model links observed pollution values from EPA monitoring sites with deterministic chemistry/meteorology model output from the CMAQ modeling system through a spatially and temporally varying regression coefficients model. The full spatial-temporal coverage of the CMAQ output allows for prediction of air pollution concentrations in locations and on days without recorded measurements from a fixed-site monitor, while the varying coefficients allow the form of the bias in the CMAQ output (with respect to monitoring data) to change across space and time. The CMAQ Downscaler model estimates a daily average concentration for the centroid of each census tract (US EPA, 2021). Daily pollution estimates for each 2010 census tract were approximated by assigning the model estimated concentration at the centroid of the census tract to geocoded residential addresses within the census tract.

Specifically, air pollution exposure was assigned by geocoding maternal addresses for each case and control and assigning daily, census tract-level, exposure values of PM_{2.5} and O₃ from the CMAQ Downscaler Model to each day of gestation. We were specifically interested in air pollution exposure during obstetric weeks 3 through 8 as this encompasses the critical window of susceptibility for congenital heart defects, including TOF. Therefore, a 6-week average exposure for PM_{2.5} and a 6-week average exposure for O₃ during 3–8 weeks of gestation was calculated. The exposure estimate for PM_{2.5} is a 6-week average of the daily 24-hour average concentration of PM_{2.5} (in micrograms per cubic meter (µg/m³)) during weeks 3–8 of pregnancy for the census tract containing the geocoded maternal address(es) during that period. The exposure estimate for ozone is a 6-week average of the daily 8-hour maximum concentration of O₃ (in parts per billion (ppb)) during weeks 3–8 of pregnancy for the census tract containing the geocoded maternal address(es) during that period.

DNA Methylation

DNA was extracted from newborn blood spots and DNA methylation was assayed using the Illumina EPIC array v.1, which measures the percent methylation at over 800,000 loci across the genome. Quality control of DNA methylation results was determined using an established procedure implemented by the Fry Laboratory (Clark et al. 2022; Eaves et al. 2023; Santos et al. 2023; Supplemental Material).

Resulting DNA methylation values were assessed for associations with TOF and air pollution using candidate gene/loci approaches as well as genome-wide approaches as detailed below.

Methylation Data Quality Assessment and Quality Control

Preprocessing of the DNA methylation data consisted of evaluating and removing sex mismatches, removing failed probes, removing outliers, and evaluating and controlling for batch effects using the minfi R package (Aryee et al., 2014). Sex was estimated based on the median values of measurements on the X and Y chromosomes, respectively. Sex mismatches were identified using this estimation along with demographic data. Failed probes were identified using detection p values. Detection p values were calculated by comparing the total DNA signal (methylated + unmethylated) for each position to the background signal

level. The background is estimated using negative control positions. A probe was considered a failed probe if the detection p value was >0.01 . Any samples with detection p-values >0.01 in more than 15% of probes were removed. Any probes with detection p-values >0.01 in more than 10% of samples were also removed. Normalization was done using the normal-exponential out-of-band (noob) correction method and functional normalization using the minfi R package. Principal components analysis and visualizations techniques were used to identify any batch effects. The ComBat function from the sva (v3.38.0) R package was used to remove any batch effects. Finally, methylation β values were generated for statistical analyses. Analyses described here were conducted using R (version 4.1.0; Vienna, Austria), unless otherwise noted. Regression models were run using the RegParallel package (Blighe & Lasky-Su, 2023) and regional analyses were conducted using DMRcate (Peters et al., 2021).

Cell-type estimation

Cell type proportions were estimated as a potential way to control for the variation in DNA methylation levels across cell types. These were estimated using the FlowSorted.Blood.EPIC R package (v1.8.0) for neonatal blood data utilizing the cord blood-derived reference library. Proportion estimates were center log transformed, using the compositions R package (v2.0.2). Principal components analysis was then utilized where the first three principal components, explaining 89% of variation, were calculated to be used for cell type adjustment in statistical analyses.

Statistical Analyses

Epigenome-wide association study (EWAS) models were fit to assess the association between percent methylation (methylation beta value) and TOF cases and controls. Specifically, logistic regression was performed where TOF status was the dependent variable and DNA methylation beta values were the independent variable. All regression models were adjusted for maternal age, infant year of birth, and the previously described three principal components estimated from cell type proportions. We used the Benjamini-Hochberg false discovery rate (FDR) adjustment for p-values as the multiple test correction method.

Regional analyses, which often have greater power than individual loci analyses, were conducted to identify genomic regions where DNA methylation across the region may differ between TOF cases and controls. The DMRcate R package was used to calculate results from regional analyses with a minimum of two CpGs to define a region. The DMRcate package (Peters et al. 2021) required gaps ≥ 1000 base pairs between significant CpG sites to designate separate regions. Models were similarly adjusted for maternal age, infant sex, infant year of birth, and cell type proportion-derived principal components. A p-value cutoff of $1e-05$ was used to determine statistically significant regions.

Linear regression models were fit to examine the relationship between air pollution exposure and methylation loci. Air pollution was measured as exposure to either $PM_{2.5}$ or O_3 from weeks 3 to 8 during pregnancy. CpG methylation was transformed to M-values and modeled as the dependent variable and air pollution exposure as the independent variable. Region-

based analyses were used to identify potentially significant regions with differential DNA methylation, using the same parameters for DMRcate as used in the TOF analysis. Any regions that were identified in association to air pollution exposure were compared with regions observed to have differential DNA methylation associated with TOF status. As with previous analyses, regression models were adjusted for relevant confounders including for maternal age, infant year of birth, and cell type proportion-derived principal components. Additionally, the other air pollution exposure was included as a confounder. P-value cutoffs and adjustments were applied as previously described.

Sensitivity analyses were conducted to assess the impact of high or low variation within DNA methylation beta values on our results. In a study of both the Illumina 450K and EPIC it was observed that loci with low variability often had low correlation between replicates and would thus be unlikely to produce replicable results (Logue et al., 2017). Therefore, any loci with low variation in the methylation beta value (<0.05) were removed. Pathway analysis, protein-protein interactions, and assessment of functional annotations were conducted for all significant differentially methylated regions (DMRs) for TOF case-control status, PM_{2.5}, and/or O₃ that mapped to known protein-coding genes. To better understand the biological context of the observed associations, CpGs with differential DNA methylation were compared to known genes linked with TOF. The potential functional significance of CpGs was evaluated based on regulatory region location as annotated in the Illumina manifest files.

To identify significant protein-protein interactions within the gene sets, String (v12) was used (Szklarczyk et al., 2019). String uses available evidence to compute a score for each interaction representing the level of confidence of there being an association. These analyses were run separately for each gene set.

To identify whether there was enrichment of specific biological pathways in our gene sets, Ingenuity Pathway Analysis was used (IPA, v01–23-01, QIAGEN Inc.). Specifically, a Core Analysis was used to identify pathways and their significance. The software identified significant canonical pathways using a right-tailed Fisher's Exact Test that calculates the p-value of overlap (QIAGEN, n.d.). The significance represents how likely it is that the association between our genes and the identified pathway is only chance (QIAGEN, n.d.). Analyses were run separately for the three gene sets. Pathway networks were generated for each gene set and identified networks that included multiple genes in our list.

To identify whether any genes identified in these analyses are also associated with birth defects, the Comparative Toxicogenomics Database was used (CTD, v12) (Davis et al., 2023). The MyGeneVenn function was used to compare the three gene sets to genes associated with TOF and two heart-related disease categories defined in CTD, congenital abnormalities and cardiovascular abnormalities. Congenital and cardiovascular abnormalities were selected in addition to TOF because of their connection as heart-related birth defects. In CTD, congenital abnormalities as a category includes all types of congenital abnormalities, including cardiovascular abnormalities, while the category of cardiovascular abnormalities is restricted to those related to defects in the cardiovascular system (CTD, n.d.-a, n.d.-b). The analyses were run separately for each combination of the three gene sets and the three

disease outcomes. All associations that were found were inferred associations rather than curated associations. An inferred association is indicated between a chemical and a disease when there is a CTD-curated association between a chemical and a gene and between that gene and a disease.

Results

Descriptive Statistics

The final study sample included 48 total subjects, with 24 TOF cases (11 female, 13 male) and 24 controls, that were matched on infant sex. General descriptive characteristics are shown in Table 1. On average, mothers of cases were slightly older than mothers of controls. There were no substantial differences between cases and controls in birth year or smoking during pregnancy. Cases were more likely to have reported a race/ethnicity other than non-Hispanic white. Finally, cases tended to have higher exposure to both PM_{2.5} and O₃ during gestational weeks 3 to 8 when compared to controls.

Main Associations

TOF status. An EWAS at the CpG level was conducted to assess the relationship between TOF and DNA methylation levels. From these initial models, no CpG loci displayed DNA methylation that was significantly associated with TOF status. Then, potential associations between differentially methylated regions and TOF status were investigated. A total of 23 significant DMRs were associated with TOF case-control status; these mapped to 19 genes (Table 2; Table S6). Among the genes that were identified to have differential methylation were Glutathione S-Transferase Mu 1 (*GSTM1*), Glutathione S-Transferase Mu 2 (*GSTM2*), and Glutathione S-Transferase Mu 5 (*GSTM5*), where DNA was hypomethylated in cases relative to controls. DNA was hypomethylated by -7.6% (*GSTM1*, *GSTM2*) and -7.3% (*GSTM5*).

An analysis focused on biological pathway enrichment for this gene set highlighted 24 significant canonical pathways (Table S1). The 13 most significant pathways included two or more *GSTM* genes. The two most significant pathways overall included glutathione-mediated detoxification ($p=3.42E-06$) and xenobiotic metabolism AHR signaling pathway ($p=4.19E-05$). Significant pathways that were enriched among other genes in the list included cardiomyocyte differentiation via BMP receptors ($p=0.021$), which included the gene SMAD Family Member 9 (*SMAD9*), and carboxyterminal post-translational modifications of tubulin ($p=0.035$), which included Tubulin Tyrosine Ligase Like 8 (*TTL8*).

In an analysis focused on identifying known protein-protein interactions (Figure 1; Table S2), interactions were observed between *GSTM1* and *GSTM2* (score 0.999), Calcitonin Related Polypeptide Alpha (*CALCA*) and Calcitonin Related Polypeptide Beta (*CALCB*) (0.973), *GSTM2* and *GSTM5* (0.951), *GSTM1* and *GSTM5* (0.870), and Ankyrin Repeat And SOCS Box Containing 3 (*ASB3*) and G Protein-Coupled Receptor 75 (*GPR75*) (0.646), with higher scores indicating greater confidence in the evidence supporting a linkage between the genes (von Mering et al., 2005). Notably, the three *GSTM* genes in the first and

third highest-scoring interactions were also in the most significant pathways identified in String pathway analysis. Additionally, several genes were identified during pathway analysis as being part of one or more larger gene networks (Table S3). Significantly enriched protein domains were identified and associated with genes (Table S4).

An analysis using CTD that compared the identified genes to those with inferred associations with TOF, cardiovascular abnormalities, or congenital abnormalities highlighted several connections. All 19 genes from the significant DMRs in Table 2 had inferred associations with cardiovascular abnormalities and with congenital abnormalities, and 14 genes had inferred connections with TOF (Table S5). The 14 genes identified as having inferred connections to all three outcomes include *GSTM5*, *GSTM2*, *GSTM1*, *GPR75*, and *CALCA*, which were identified as having significant interactions with each other in protein-protein interaction analyses.

Air Pollution. Similar to TOF status, the relationships among individual CpG loci and regions were assessed relative to both PM_{2.5} and O₃ exposure during weeks 3–8 gestation. Again, no individual CpG loci were identified to be statistically significantly associated with either air pollutant exposure. However, differentially methylated regions were identified that were significantly different between the exposure groups. Specifically, for the PM_{2.5} analysis, 11 regions were identified with differential methylation (Table 3). These DMRs mapped to eight genes (Table S6). For the ozone analysis, 34 regions were identified with differential methylation (Table 4). These regions mapped to 25 genes (Table S6). Interestingly, these regions had some overlap across the air pollution exposures (n=3 regions), though, no overlap with any of the regions found in the TOF analyses.

Pathway analyses of the genes that had statistically significant DMRs for PM_{2.5} (Table 3) were mapped and identified four significant canonical pathways (Table S1). Three pathways were related to Nuclear Receptor Subfamily 5 Group A Member 2 (*NR5A2*) and one to Tenascin R (*TNR*). The top pathway with *NR5A2* was regulation of beta-cell development (p=0.0138). The pathway with *TNR* was extracellular matrix organization (p=0.0346).

When comparing the genes with statistically significant DMRs for PM_{2.5} to genes within CTD that have inferred association with cardiovascular abnormalities, congenital abnormalities, and TOF, eight genes (*ADGB*, *AKR1E2*, *ANKRD30B*, *NR5A2*, *TBC1D22A*, *TMEM176A*, *TNR*, *TRAM1L1*) had inferred connections to cardiovascular and congenital abnormalities and six genes (*ADGB*, *AKR1E2*, *NR5A2*, *TBC1D22A*, *TMEM176A*, *TNR*) had inferred connections with TOF (Table S5). Additionally, all eight genes were part of a larger network identified during pathway analysis (Table S3).

Analyses of the 25 genes that showed significant DMRs for O₃ exposure (Table 4) identified 41 significant canonical pathways (Table S1). The pathways with highest significance were neuroinflammation signaling (p=0.000296), *RIPK1*-mediated regulated necrosis (p=0.0005), antigen presentation pathway (p=0.000744), and TNF signaling (p=0.00183). Overall, genes that were enriched in the most significant pathways often included Major Histocompatibility Complex, Class II, DP Alpha 1 (*HLA-DPA1*), Major Histocompatibility Complex, Class II, DP Beta 1 (*HLA-DPB1*), and CASP8 And FADD Like Apoptosis Regulator

(*CFLAR*). Across all significant pathways, Protein Phosphatase 3 Catalytic Subunit Alpha (*PPP3CA*) was also frequently present. Notable pathways connected to other genes include methylmalonyl pathway (p=0.00413; Methylmalonyl-CoA Mutase (*MMUT*)) and regulation of beta-cell development (p0.0426; *NR5A2*).

Protein-protein interaction analyses (Figure 2) of the genes identified interactions between four gene pairs (Table S2). The top two were *HLA-DPA1* and *HLA-DPB1* (score 0.999) and *CFLAR* and Ubiquitin Conjugating Enzyme E2 L3 (*UBE2L3*) (0.503). Notably, *CFLAR*, *HLA-DPA1*, and *HLA-DPB1* were also connected to the most significant pathways identified in pathway analysis. Additionally, four larger networks were identified during pathway analysis that contained at least one of the genes (Table S3).

An analysis comparing the significant genes to genes associated with cardiovascular abnormalities, congenital abnormalities, and TOF identified prominent overlap. All but one of the 25 genes had inferred connections with cardiovascular abnormalities, all 25 genes had inferred connections with congenital abnormalities, and 19 genes had inferred connections with TOF (Table S5). The genes connected to all three outcomes include *CFLAR*, *HLA-DPA1*, and *HLA-DPB1*.

Discussion

The underlying causes for birth defects are complex and include genetic, environmental, as well as other factors. The associations among the environment, the epigenome, and birth defects are understudied. In this exploratory study, we evaluated the relationship between genome-wide DNA methylation patterns and an individual congenital heart defect, TOF, as well as DNA methylation in relation to air pollution exposure using newborn dried blood spots that were collected for participants of the NBDPS in North Carolina. In doing so, we sought to compare genome-wide DNA methylation patterns among infants with TOF (cases) and infants without a birth defect (controls) to determine if there were any differences between the two groups. Additionally, we also examined if prenatal air pollution exposure was associated with any of the observed differences in DNA methylation patterns. There were two key findings of the present study. First, in relation to TOF case status, there were associations between DNA methylation at specific genomic regions with genes enriched for functions in metabolism. Second, when analyzed in the context of air pollution, there were significant regions that displayed differential DNA methylation in relation to either ozone or PM_{2.5}. These regions were enriched with genes linked to functions in inflammation and immune response pathways, in addition to apoptosis or necrosis pathways. Findings from this exploratory study highlight potential links between the environment, epigenome, and birth defects to be investigated in future studies.

Genes with differential methylation that were associated with TOF status were identified including several glutathione transferases, namely *GSTM1*, *GSTM2*, and *GSTM5*. These genes play important roles in metabolism and detoxication of a wide range of compounds (NIH NCBI, 2024b, 2024c, 2024d). These genes displayed hypomethylation in cases relative to controls in the present study. Importantly, *GSTM1* has previously been found to be related to birth defects. First, in a study of children with the congenital condition esophageal atresia

(EA) and their mothers, *GSTM1* deficiency was significantly higher among children with EA and among mothers of children with EA (Filonzi et al., 2010). Second, genetic variation in both *GSTM1* and *GSTT1* has been associated with higher risk of CHD in the children of parents exposed to toxicants (Cresci et al., 2011). Third, *GSTM1* variants have been found to be influential on the relationship between maternal smoking and birth defects (Lammer et al., 2005; Li et al., 2015), though results indicating no effects have also been reported (Carmichael et al., 2006; Cresci et al., 2011; Hartsfield et al., 2001). Notably, in mouse models, glutathione has been shown to have protective effects against congenital heart defects (CHD) associated with maternal pregestational diabetes (Moazzen et al., 2014). Specifically, Moazzen et al. (2014) found that treatment of pregnant diabetic mice with N-Acetylcysteine (NAC) to replenish glutathione levels resulted in significantly decreased CHDs in offspring compared to the offspring of untreated diabetic dams (Moazzen et al., 2014). This is hypothesized to occur through reduction of reactive oxygen species (ROS) and associated oxidative stress (Moazzen et al., 2014). Though this sample excluded those with diabetes, the results suggest a role for this pathway in TOF status. Additional research will be useful in determining if this hypothesized pathway is specific to those with diabetes, or if it is generalizable to those without diabetes as well.

We identified genomic regions that displayed differential DNA methylation in relation to air pollution exposure during early pregnancy. Genes that were associated with PM_{2.5} included *NR5A2* and *TNR*, which were hypermethylated in individuals with high levels of exposure in the present study. These genes were involved in pathways including regulation of beta-cell development (*NR5A2*) and extracellular matrix organization (*TNR*). Notably, *NR5A2* is involved in embryonic development (Gassler et al., 2022) and *TNR*, present only in the nervous system, may play a role in several neurologic diseases, including multiple sclerosis and brain tumors (Anlar & Gunel-Ozcan, 2012). In relation to O₃, genes included *CFLAR*, *HLA-DPA1*, and *HLA-DPB1* in our study. *HLA-DPA1* and *HLA-DPB1* are associated with immune system function (NIH NCBI, 2024e, 2024f). Of interest, *CFLAR* is associated with apoptosis regulation and may be important for embryonic development (NIH NCBI, 2024a; Sakamaki et al., 2015). In mouse models, *CFLAR*-deficient embryos had abnormal heart formation, and none survived to birth (Yeh et al., 2000). Our results suggest a role for these pathways as potential linkages between the environment, DNA methylation, and health.

While we are aware of a single previous study of DNA methylation and congenital heart defects that used newborn blood spots (Richards et al. 2022), the results of the present study are generally consistent with those reported in a recent review of DNA methylation measured in other tissues and congenital heart defects (Cao et al. 2021). In their review, Cao et al. (2021) highlighted studies that evaluated DNA from both maternal (e.g., maternal blood, placenta) and newborn (e.g., newborn blood, cardiac tissue) samples. They characterized the association of both hypermethylation and hypomethylation of certain genes with congenital heart defects, and TOF specifically. Hypermethylated DMRs (~14,000) outnumbered hypomethylated DMRs (~1400) in TOF cases (N = 8) (Grunert et al. 2016). Hypermethylation in the promoter regions of the *SCO2* gene and the CpF islands of *NKX2-5* and *HAND1* genes was observed in the cardiac tissue of TOF patients (Sheng et al. 2012). Hypomethylation of maternal LINE-1a and in the promoter CpG island of *TBX20* was associated with non-syndromic congenital heart defects (Sheng et al. 2012). Though

there were no genes or regions identified in these studies that overlap with those in our study, these results provide plausibility for DNA methylation to play a key role in TOF development.

In a study similar to this one, Radhakrishna et al. (2018) conducted a genome-wide methylation assay in newborn blood samples from 24 TOF cases and 24 matched controls and identified 64 differentially methylated genes known or plausibly linked to heart development or postnatal heart disease (e.g., *ABCB1*, *CREM*, *LHX9*, *PPP2R5C*, *RUNX*, *SELL*, *SCN3A*, *TLR1*). Several of the genes identified by Radhakrishna et al. (2018) are in the same or similar families as genes identified in our analyses, specifically *GPR123* and *GPR75*.

The observed DNA methylation changes associated with air pollution exposure point to a potential role for epigenetic regulation in birth outcomes. This aligns with research indicating the importance of epigenetics in other health outcomes and the relatively small existing body of literature on DNA methylation and birth outcomes. Epigenetic regulation plays an essential role in normal development and health via the differentiation and maintenance of cell fate, but altered epigenetic regulation is also associated with diseases (Hussain 2012). Although the role of DNA methylation in some common health outcomes, such as cancer, autoimmune diseases, metabolic disorders, and neurological disorders, has been documented (Jin and Liu, 2018), relatively few studies have investigated the association with birth defects. The existing literature suggests several pathways by which epigenetic regulation could affect birth defects. Differentially methylated genes in the mitogen-activated protein kinase (MAPK) pathway are associated with cardiac development (Cao et al. 2021). Differential DNA methylation of genes may lead to variation in transcriptional regulation and contribute to TOF pathogenesis (Sheng et al. 2013). Both hypermethylation and hypomethylation may contribute to CHDs; down regulation of *SCO2* caused by hypermethylation can lead to glycolysis and eventually delay terminal differentiation, resulting in cardiomyopathy (Gunert et al. 2016). Conversely, hypomethylation in promoter regions of *TDGF1* has been observed in TOF patients. The *TDGF1* gene plays an important role in maintenance and differentiation of early cardiac progenitors (Grunert et al. 2016). The addition of epigenetic analyses related to birth defect phenotypes contributes to the overall evidence of birth defect etiology and may provide information for risk assessment (Rozek et al. 2014).

While the results highlight some associations between both TOF and air pollutants and DNA methylation, this study is not without limitations. As an exploratory study, the sample size was comparatively small (24 cases and 24 controls). Dried blood spots were used as a DNA source rather than a target organ tissue, and the DNA source was collected at birth, not during the relevant embryonic period for TOF. Considering that the tissues/sources of genetic materials used to generate DNA methylation profiles vary across studies, and that the methylation profile measured at birth may not be entirely representative of the profile during the early weeks of pregnancy, identifying epigenetic changes that are not impacted by the source cell type or that are impacted after the relevant exposure window have been identified as a limitation (Campbell et al. 2020; Jin and Liu et al. 2018). Additionally, only DNA methylation was assessed and not gene or protein expression. Thus, it is not possible

to assess whether hypo- or hyper-methylation is associated with increases or decreases in gene or protein expression. Future research could incorporate assessment of gene expression and protein expression from blood spots. Air pollution was assessed as a linear construct based on well-documented evidence for linear, no-threshold relationships between PM_{2.5} and ozone with morbidity and mortality (U.S. EPA 2019; U.S. EPA, 2020). Few studies have conducted concentration-response analyses with air pollutants and birth outcomes, but there is no evidence to suggest that the shape of the relationship would be different from the linear relationship with other types of morbidity outcomes. Finally, this study assesses methylation only for liveborn cases with TOF, but there may be differences in DNA methylation between cases that result in fetal loss (e.g., miscarriage or stillbirth) and those that survive to delivery. Differences could reflect severity, co-occurring anomalies or other conditions, maternal factors, exposure status, or other factors. The risk of fetal loss for TOF is not negligible (Garagiola & Thorne, 2023), presenting an unknown potential for selection bias due to differences among those that survive and are live born, vs. those that do not.

There are several strengths to this study. DNA methylation was measured in newborn blood collected near the time of delivery. This allows for a focus on epigenetic changes within the fetus due to gestational exposures. Cases and controls were identified from an active population-based surveillance system, with defects identified up to 1 year post-delivery. As part of NBDPS, cases were also reviewed by clinical geneticists and pediatric cardiologists. An additional strength is the rich interview data on various relevant pregnancy-related factors, including residential history. Finally, here we look at epigenetic factors and air pollution concentrations, both of which can influence DNA methylation. However, this study cannot separate the effects of these potential causes. In cases where both genotype and environmental exposures can contribute to the etiology of the outcome, DNA methylation can be a mediator (i.e., an intermediary in disease etiology), modifier (i.e., impact the association between genotype or environmental exposure and the outcome), or even a consequence of the disease (i.e., gene-environment interaction) (Jin and Liu et al. 2018). A limitation of this study is that we cannot distinguish between these pathways given the small sample size of this exploratory study.

Conclusion

This study is among the first to assess relationships among air pollutants, DNA methylation and TOF case status using residual newborn bloodspots. This exploratory work sets the stage for larger studies to investigate these relationships.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability

The process for accessing the data used in this study is described at <https://www.cdc.gov/nbddd/birthdefects/nbdps-public-access-procedures.html>. Please contact the corresponding author for further information.

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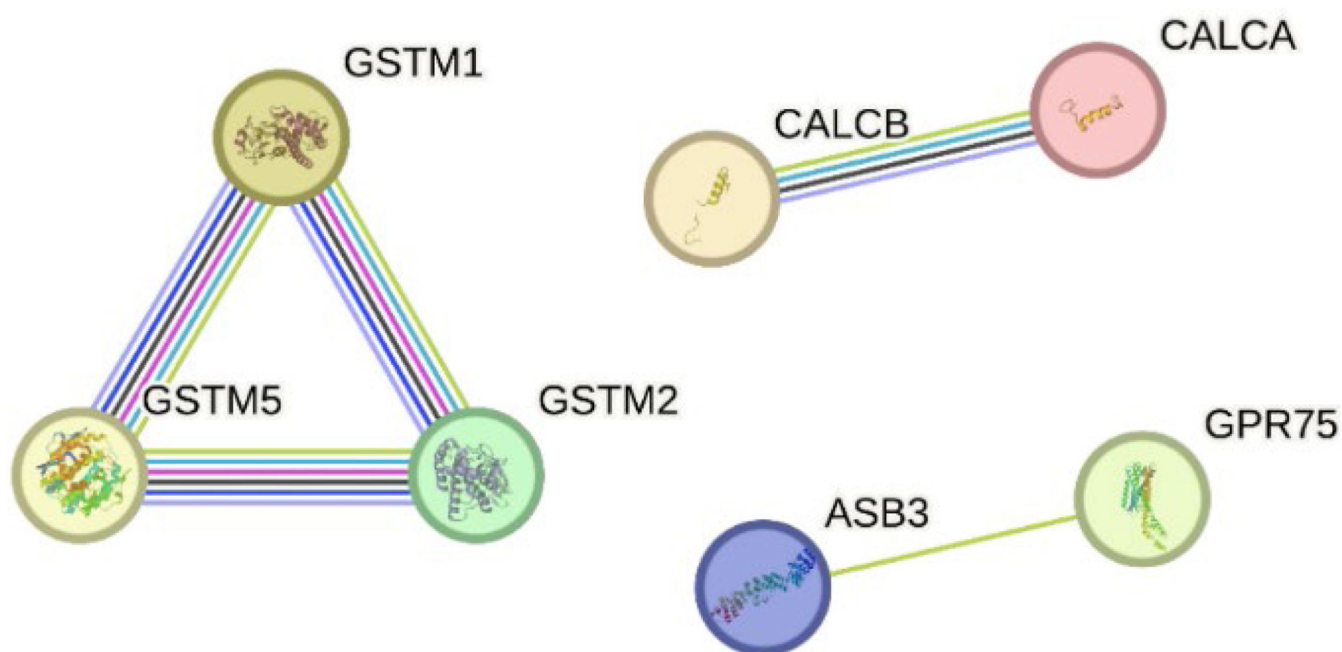


Figure 1. Network analysis of genes that displayed differentially methylated regions between TOF cases and control. Of 19 genes, 7 displayed known protein-protein connections.

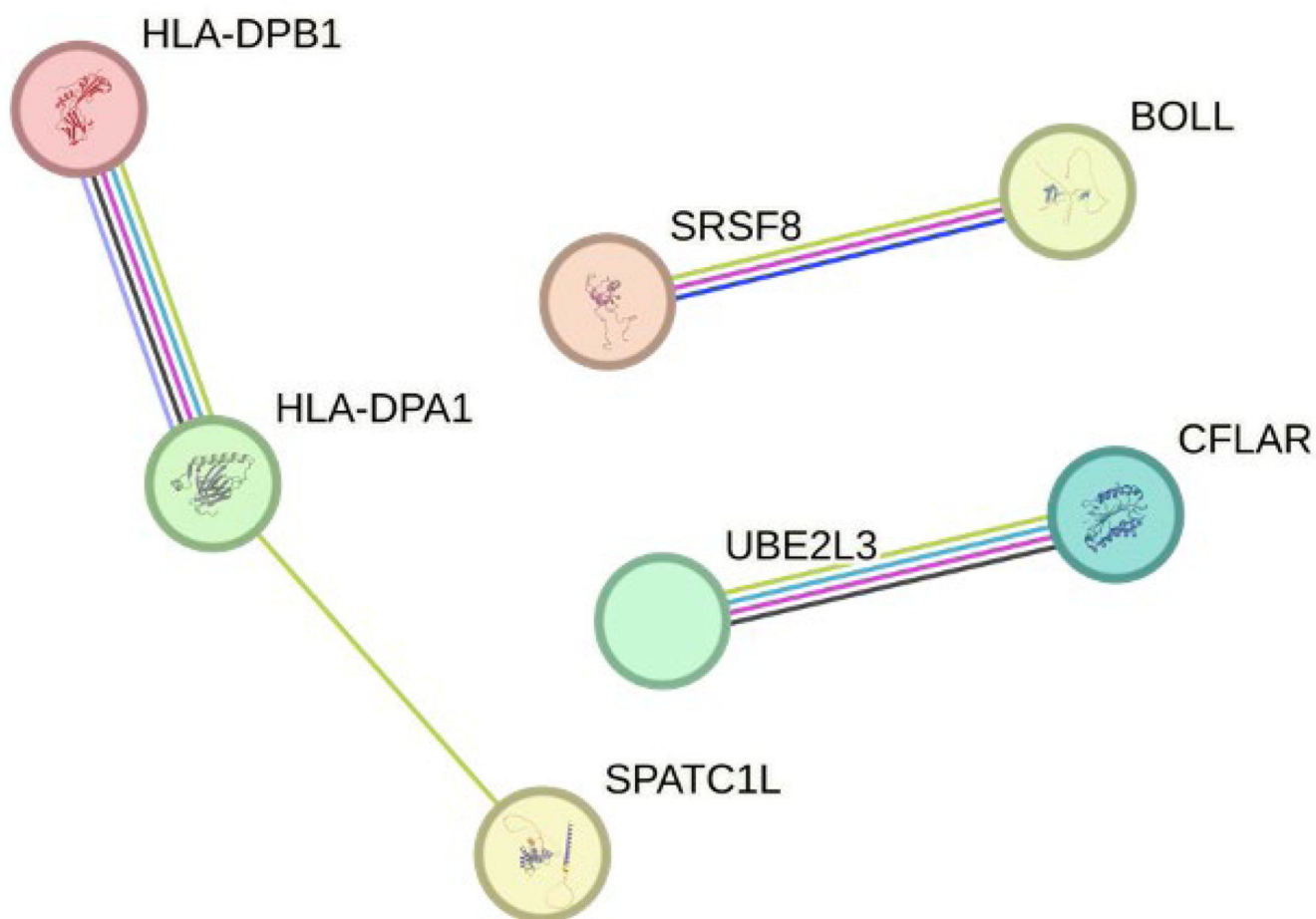


Figure 2.
Network analyses of 25 genes in O₃-associated differentially methylated regions. 18 genes with no connections are hidden.

Table 1.

Demographic characteristics and air pollution exposure for the total analytic sample, controls and TOF cases, National Birth Defects Prevention Study, North Carolina 2006–2011.

Demographics	Total Sample (n=48)	TOF Controls (n=24)	TOF Cases (n=24)	P value
Maternal age	29.1 (6.97)	29.5 (7.66)	28.7 (6.34)	0.668
Race/ethnicity				0.038
Asian	4 (8.33%)	0 (0.00%)	4 (16.7%)	
Hispanic	4 (8.33%)	3 (12.5%)	1 (4.17%)	
Non-Hispanic Black	9 (18.8%)	2 (8.33%)	7 (29.2%)	
Non-Hispanic White	28 (58.3%)	17 (70.8%)	11 (45.8%)	
Other	3 (6.25%)	2 (8.33%)	1 (4.17%)	
Infant sex				1.000
Female	21 (43.8%)	11 (45.8%)	10 (41.7%)	
Male	27 (56.2%)	13 (54.2%)	14 (58.3%)	
Maternal Cigarette Smoking				
Any during pregnancy	7 (14.6%)	2 (8.3%)	5 (20.8%)	0.416
None during pregnancy	41 (85.4%)	22 (91.7%)	19 (79.2%)	
NBS Storage Time (Months)	14.9 (5.89)	14.1 (5.46)	15.8 (6.30)	0.333
Year of birth				0.913
2006	8 (16.7%)	3 (12.5%)	5 (20.8%)	
2007	2 (4.17%)	1 (4.17%)	1 (4.17%)	
2008	13 (27.1%)	8 (33.3%)	5 (20.8%)	
2009	7 (14.6%)	4 (16.7%)	3 (12.5%)	
2010	12 (25.0%)	5 (20.8%)	7 (29.2%)	
2011	6 (12.5%)	3 (12.5%)	3 (12.5%)	
PM _{2.5} (µg/m ³ ; weeks 3–8)	11.9 (3.18)	11.5 (3.26)	12.4 (3.08)	0.340
O ₃ (ppb; weeks 3–8)	42.2 (10.7)	40.3 (10.4)	44.4 (10.9)	0.219

Significance calculated by Fisher's exact test for categorical variables (race, infant sex, year of birth, smoking) and by T-test for continuous variables (maternal age, storage time, PM_{2.5}, O₃).

Table 2.Significant ($P < 1 \times 10^{-5}$) differentially methylated regions by TOF case-control status (n=23).

Chromosome	Start	End	Width	Number of CpGs	Max difference	Mean difference	Overlapping Genes	FDR P-value
chr9	136435523	136435554	32	2	-0.0631483	-0.0531526	<i>ADAMTSL2</i>	1.20E-06
chr1	249152151	249152158	8	2	-0.0168385	-0.0165364	<i>ZNF692</i>	5.46E-06
chr2	43328122	43328266	145	2	-0.0421617	-0.0354037	<i>AC093609.1</i>	5.46E-06
chr14	54080827	54080861	35	3	0.0299524	0.01826126	<i>AL163953.3</i>	9.53E-06
chr12	49687369	49687715	347	4	-0.0182801	-0.0179543	<i>PRPH</i>	3.36E-06
chr22	50482925	50483545	621	5	0.05596103	0.03087602	<i>TTLL8</i>	4.83E-09
chr14	22538566	22538812	247	5	0.01979446	0.01508949		3.13E-06
chr7	26897253	26897714	462	6	-0.044352	-0.0294638	<i>SKAP2</i>	2.18E-08
chr2	38978794	38979602	809	6	-0.0400273	-0.0209204	<i>GEMIN6</i>	9.40E-08
chr1	110230252	110230633	382	7	-0.1334722	-0.0763039	<i>GSTM2, GSTM1</i>	4.56E-07
chr13	37453429	37453966	538	7	0.0575953	0.02193386	<i>SMAD9</i>	7.10E-13
chr5	1867978	1868738	761	8	0.09507388	0.06284422		1.51E-08
chr1	146551194	146552552	1359	9	-0.1137311	-0.041036	<i>RNVU1-8, NBPFI3P</i>	6.62E-11
chr1	201708419	201708932	514	9	0.06417973	0.02551943	<i>NAV1, IPO9-AS1</i>	5.25E-09
chr16	53406901	53407808	908	11	-0.0923198	-0.052404	<i>RP11-44F14.2</i>	2.97E-13
chr16	66613096	66613407	312	9	0.03847422	0.01376966	<i>CMTM2</i>	1.85E-06
chr1	110253977	110255096	1120	12	-0.1230843	-0.072957	<i>GSTM5</i>	1.15E-13
chr2	54086854	54087469	616	11	-0.0429559	-0.0271681	<i>GPR75-ASB3, ASB3, GPR75</i>	2.44E-07
chr13	51417469	51418221	753	11	-0.0359191	-0.0168451	<i>DLEU7-AS1, DLEU7</i>	8.13E-09
chr7	27225342	27226329	988	13	-0.0347705	-0.0152066	<i>HOXA11-AS</i>	7.31E-11
chr21	45705175	45706101	927	14	-0.0494954	-0.0235081	<i>AIRE</i>	1.30E-13
chr11	14993378	14994561	1184	19	-0.029758	-0.0149515	<i>CALCB, CALCA</i>	1.08E-12
chr10	135050619	135052004	1386	26	-0.0428241	-0.0138815	<i>VENTX</i>	1.03E-19

Table 3.Significant differentially methylated regions for PM_{2.5} exposures during weeks 3–8 gestation (n=11).

Chromosome	Start	End	Width	Number of CpGs	Max difference	Mean difference	Overlapping Genes	FDR P-value
chr7	150498493	150498594	102	2	0.01228218	0.01153193	<i>TMEM176A</i>	7.69E-06
chr1	175474437	175474533	97	3	0.0094231	0.00684129	<i>TNR</i>	6.56E-06
chr1	200010626	200010788	163	3	0.00582022	0.00392098	<i>NR5A2</i>	2.25E-08
chr18	14748285	14748439	155	3	0.00505611	0.00296389	<i>ANKRD30B</i>	6.23E-06
chr22	47169227	47169976	750	10	0.03026724	0.01086435	<i>TBC1D22A</i>	5.78E-12
chr10	82295394	82296516	1123	7	0.01247541	0.00826464	<i>RP11-137H2.4</i>	3.27E-11
chr10	4867773	4869088	1316	13	0.01337207	0.00493959	<i>AKR1E2</i>	2.12E-17
chr4	118006405	118007225	821	11	0.00477445	0.00203747	<i>TRAM1L1</i>	1.50E-10
chr5	8457124	8458392	1269	11	0.01257789	0.00806287	<i>MIR4458HG</i>	6.63E-14
chr6	146919633	146920295	663	11	0.00335742	0.00138386	<i>ADGB</i>	8.05E-11
chr8	104152244	104152754	511	5	0.01310041	0.00565269	<i>C8orf56</i>	1.17E-06

Table 4.

Significant differentially methylated regions for ozone exposures during weeks 3–8 gestation (n=34).

Chromosome	Start	End	Width	Number of CpGs	Max difference	Mean difference	Overlapping Genes	FDR P-value
chr15	25322128	25322131	4	2	0.00148509	0.00148202	<i>SNHG14</i>	9.88E-06
chr1	76189707	76189770	64	2	-0.0065008	-0.0053193		5.69E-06
chr3	36422059	36422061	3	2	0.00067205	0.00039544	<i>STAC</i>	9.87E-06
chr5	14872551	14873039	489	4	0.00097454	0.00031337	<i>CTB-40H15.4</i>	5.70E-09
chr6	163569844	163570371	528	3	0.00509157	0.00316469	<i>PACRG</i>	2.70E-06
chr8	79577618	79577796	179	3	0.00610689	0.00524688		7.61E-07
chr11	13508769	13509032	264	3	0.00404646	0.00376971		3.79E-07
chr14	36003826	36004142	317	3	0.00033849	0.00018768	<i>INSM2</i>	3.24E-07
chr7	150498493	150498670	178	3	0.00373151	0.00288803	<i>TMEM176A</i>	2.39E-06
chr1	200010283	200010788	506	4	0.00152403	0.00085127	<i>NR5A2</i>	5.70E-09
chr13	112547341	112547653	313	4	0.00462807	0.00232649		2.83E-06
chr4	135122912	135123107	196	4	0.00097751	0.00072912		5.21E-06
chr22	21922038	21922638	601	2	8.43E-05	6.60E-05	<i>UBE2L3</i>	1.44E-06
chr14	106330121	106330538	418	5	0.00300359	0.00222402	<i>IGHJ4</i>	2.55E-06
chr11	94800095	94800316	222	4	0.00044398	0.00012747	<i>SRSF8</i>	2.16E-06
chr8	104152194	104152754	561	6	0.00323176	0.001482	<i>C8orf56</i>	6.24E-09
chr12	85305677	85306021	345	4	0.00017884	0.00013089	<i>SLC6A15</i>	1.52E-06
chr22	47169227	47169667	441	6	0.00650117	0.00300422	<i>TBC1D22A</i>	4.16E-06
chr21	47604052	47604986	935	8	0.00658587	0.00467308	<i>SPATC1L</i>	1.92E-08
chr1	248902767	248903467	701	8	0.00236789	0.00169506	<i>LYPD8</i>	8.85E-07
chrX	47077168	47077524	357	10	0.00158377	0.00081652	<i>CDK16</i>	4.51E-06
chr2	43454447	43454628	182	6	0.00020798	0.00010333	<i>THADA</i>	7.45E-06
chr4	122853464	122854532	1069	11	0.00231406	0.00137962	<i>TRPC3</i>	6.59E-14
chr5	8457124	8458392	1269	11	0.00343719	0.00244691	<i>MIR4458HG</i>	8.79E-18
chr5	74062240	74062846	607	8	-0.0005857	-2.60E-05	NSA2, GFM2	2.72E-06
chr4	102268075	102268854	780	11	0.00161221	0.00028384	<i>PPP3CA</i>	3.24E-07
chr10	134600463	134600680	218	8	0.00093179	0.00017357		8.14E-06
chr6	31465958	31466798	841	12	0.00418009	0.00102374	<i>MICB</i>	9.60E-09
chr6	49430837	49431231	395	9	0.00022164	0.00010947	<i>CENPQ, MMUT</i>	5.85E-06
chr2	198650603	198651590	988	16	0.00155345	0.00079075	<i>BOLL</i>	2.96E-09
chr6	33048086	33048760	675	17	0.0063917	0.00296538	<i>HLA-DPB1, HLA-DPA1</i>	2.08E-06
chr15	40074520	40075521	1002	15	-0.0009148	-4.03E-05	<i>RP11-7C7.3, FSIP1</i>	2.44E-08
chr2	201980638	201981146	509	13	0.00044039	0.00014956	<i>CFLAR</i>	2.70E-06
chr17	41277847	41278655	809	21	-0.0034912	-0.0018279	<i>NBR2</i>	4.38E-09