Three Years Follow-Up Study Showed Prenatal Methamphetamine Exposure May Cause Chronic Abnormalities In Transcriptomic Pattern

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Abstract

Background: The high rate of methamphetamine use disorder among young adults and women of childbearing age makes it imperative to clarify the long-term effects of Methamphetamine exposure on the offspring. Behavioral and cognitive problems had been reported in children with parental Methamphetamine exposure (PME). The present study aimed to assess the acute and chronic effects of PME in molecular regulations and gene expression profiles of children during their first years of life.

Methods: All subjects were recruited before birth, and sampling was conducted from the first ten days of birth, twelve months, twenty months, and thirty-six months of age. Finally, 2658 children with PME and 3573 normal children had been finished the follow-up. RNA extraction was operated from blood samples and gene expression profiling was conducted by using the Affymetrix GeneChip Human Genome U133 plus 2.0 Array Platform. Gene expression data were confirmed by Real-time PCR.

Results: Gene expression profiling during thirty-six months showed several constant mRNA level alterations in children with PME compared with normal. These genes are involved in several gene ontologies and pathways involved with the immune system, neuronal functions, and bioenergetic metabolism. It seems that Methamphetamine use disorder before and during the pregnancy period may affect the expression profile of children, and these changes could remain years after birth. Affected genes have some similarities with the gene expression patterns of addiction, psychiatric disorders, neurodevelopmental disabilities, and immune deficiencies.

Conclusion: Findings may shed light on the molecular effects of prenatal methamphetamine exposure and may lead to new psychological and somatic caring protocols for these children based on their potential abnormalities.

Background

Methamphetamine (MA) is a psychostimulant, neurotoxic drug that causes chronically psychological dependence in the use disorder. Methamphetamine (MA) use disorder is a global problem, and its use disorders are more than cocaine and opiates combined (1). Gender analysis showed that female first-time users of methamphetamine are more than male first-time users (2). On the other hand, only less than fifty percent of those women are seeking treatment. In the United States, about seven percent of MA dependents that were referred to clinics for treatment were pregnant women (3). In Iran, Methamphetamine use is not longer than three decades, but unfortunately, the prevalence of Methamphetamine use disorder is fast growing. Also, while the total percentage of women addicts from all Iranian addicts are about 10 percent, the percentage of women with Methamphetamine use disorder from all Methamphetamine users are about 40 percent. Taken together, prenatal methamphetamine exposure could be a major health concern in Iran's future.

Previous studies about the effects of prenatal methamphetamine exposure on child development showed an increase in gestational size at birth and decreased length through three years. Deficiency in movement

and arousal along with increased stress signs in the newborn period and poor grasping ability at one to three years old children were reported(4). Further studies in the age of three to five years have been shed light on attentional issues such as inhibitory control, aggressive behavior, and adjustment issues that could be related to abnormalities in the development of executive function in prenatal methamphetamine exposure children(5, 6).

Acute and chronic methamphetamine use disorder and dependents show severe behavioral problems, including addictive behaviors (such as craving, salience) and psychotic symptoms (such as aggressiveness, hallucination, and dilution). Also, individuals with methamphetamine dependence have a higher risk of schizophrenia (7). The workgroup recommendations for DSM-5 revisions suggested combining use disorder and dependence criteria into a single substance use disorder (2). While it is accepted that methamphetamine is a highly addictive substance with widespread and constant effects in the central nervous system (CNS), its molecular mechanisms on adults and fetuses are not clarified. Methamphetamine increased blood-brain barrier (BBB) permeability by rearrangement of F-actin cytoskeleton and reduction of tight junction (TJ) proteins (8). Mouse model experiments were reported that increased total DNA methylation in the mPFC could be associated with mPFC malfunction and long-term cognitive decline (9).

While there is a lack of information about the molecular mechanisms of methamphetamine and its effects on the development of CNS, neuroimaging studies provide valuable evidence about the role of prenatal methamphetamine exposure in functional and structural changes in the brain (10). Methamphetamine use disorder in adult persons could decrease the metabolism in the insula and frontal cortex and increase in basal ganglia (11). However, diffusion tensor imaging (DTI) studies have reported that prenatal exposure to methamphetamine is more associated with white matter microstructure alterations (12). Alteration such as diffusion measures in white matter connections and reduction of fractional anisotropy in several connections between the striatum and midbrain (12, 13).

Based on some studies, mood difficulties, cognitive deficiencies, and chronic behavioral abnormalities are increased in PME children in childhood and maybe adolescence (13). Early detection of vulnerable children and psychological caring may prevent severe social and psychiatric problems in the next generation. That is why a general pattern of all alterations caused by prenatal methamphetamine exposure in molecular, neuroanatomical, and behavioral levels is highly needed (13).

The present study aimed to comprehensively evaluate the acute (or early) and chronically (or long-standing) effects of prenatal methamphetamine exposure in gene expression profile, behavior, and neuronal activities in the brain.

Material And Methods

We followed up a large sample size of children with prenatal methamphetamine exposure from date of birth to thirty-six months of age by using a functional genome-wide profiling approach.

Subject selection

Participants were recruited from a national cohort project of methamphetamine addiction (NCPM) in Iran, with over twenty thousand methamphetamine-dependent subjects. Infants were referred from female subjects of NCPM to PME group based on voluntary maternal self-report of methamphetamine use disorder during pregnancy, confirmed by positive meconium screen, and gas chromatography or mass spectroscopy confirmation. PME infants and mothers were matched to a normal group for maternal race, age of mother and father in pregnancy time, birth-weight category (<1500 g, 1500–2500 g, >2500 g), health care insurance situation, and education in comparison of infant-mother pairs.

Sociodemographic and prenatal substance use details obtained from interview with mothers (4). Study assessments and sampling were conducted five times when infants were 1, 12, 24, 30, and 36 months of age. Mothers with less than twenty years of age, with any records of opioid, or any other use disorder during the pregnancy, any records of severe emotional disorders or cognitive dysfunctioning, or psychotic abnormalities, or antipsychotic usage were excluded from the study. Children with exclusion criteria including critical illness, multiple gestations, life-threatening congenital anomaly, chromosomal abnormality, overt clinical evidence of intrauterine infection, or a sibling previously enrolled in the study. The occurrence of any severe somatic or psychological disease or changes in lifestyle such as immigration, adaptation, or parents' divorce during the thirty-six months of follow-up were lead to the exclusion of subjects from the study. Maternal use of alcohol and tobacco and smoking during pregnancy was considered as background variables in both PME and normal comparison groups. Heavy methamphetamine exposure was defined as maternal use at least three days per week across pregnancy. The pattern of use, according to trimester and was showed in Table 1. Indicated overall decline and quitting methamphetamine use throughout the pregnancy. The level of exposure to other drugs of use disorder was calculated as cigarettes per day, ounces of absolute alcohol per day.

Written consent form filled by all mothers after explaining the aim of study for each subject in private. The study was approved by the local ethical committee based on Helsinki declaration obligations.

Blood sampling and Microarray Analysis

Blood samples (5 ml, PAXgene vacutainer blood RNA-tubes; PreAnalytiX, Hombrechtikon, Switzerland) were collected between 10.00 and 11.00 AM. Total RNA was extracted from peripheral blood samples immediately after sampling by column Purification kit (GeneJET™ RNA Purification Kit#K0732, Thermo scientific, Latvia). RNA quality and integrity were examined by Agilent 2100 Bioanalyzer (Agilent Technologies) before the beginning of the microarray process. Gene expression profiling analyses were conducted by using the Affymetrix GeneChip Human Genome U133 plus 2.0 Array Platform containing probes representing 39,000 genes. Preparation of labeled and fragmented aRNA targets, hybridization, and scanning was carried out according to the manufacturer's protocol (Affymetrix Santa Clara, CA). A hundred nanograms of total RNA for each sample were processed using the GeneChip 3' IVT Express Kit. RNA was reverse transcribed and converted to double-stranded cDNA before biotin labeling during in vitro transcription. In next step, fifty micrograms of labeled aRNA were fragmented, and quality control (QC)

was carried out using the Agilent Bioanalyzer. Fragmented aRNA was hybridized on GeneChip Human Genome U133 Plus 2.0 arrays for sixteen hours at 45°C. Finally, arrays were washed and stained by using the GeneChip Hybridization, Wash, and Stain Kit on the GeneChip Fluidics Station 450. Then chips were scanned using the Affymetrix GeneChip Scanner 3000, and all arrays passed the QC criteria examination (14).

The GeneChip analysis was performed in Genesis 2.0 (Gene Logic Inc.) and with Microarray Analysis Suite (MAS) 5.0, Data Mining Tool 2.0, and Microarray Database software (available at http://www.affymetrix.com) (14). All of the genes represented on the GeneChip were globally normalized and scaled to a signal intensity of a hundred. Genes that passed MAS 5.0 criteria for false-positive results reduce included to further assessment. Expression data were analyzed using Genesis 2.0 (GeneLogic Inc., Gaithersburg, MD, USA) and DAVID software (Strand Genomics, Redwood City, CA, USA). Gene expression values were floored to 1, and then log2 transformed. Analysis of variance test (ANOVA) was performed for each gene to identify significant gene expression alteration. Genes with statistical significance were subjected to a post hoc t-test for evaluation of contrasts groups one by one for identification of differences between groups. Two criteria were used in all subjects to determine whether a gene was differentially expressed; a Fold change greater than 2 and a P value less than 0.05. Then P values adjusted for multiple testing correction.

Pathways and gene ontology analysis

Functional enrichment was conducted on differentially expressed data used for all comparisons. For functional annotation, gene ontology analysis was performed for differentially expressed genes with enrichment algorithms integrated into the online Database for Annotation, Visualization, and Integrated Discovery (DAVID 6.8) (15, 16). To enrichment analysis of the differentially expressed genes (DEGs) and calculated the enrichment factor (EF) for each term or pathway, Kyoto encyclopedia of genes and genomic (KEGG) pathway onloine data base were used. The P values were calculated via hypergeometric tests and go with a correction (17). Only those GO terms or KEGG pathways with enrichment of corrected $P < 0.05$ and $EF > 2.0$were included.

Biomarker assessment based on the k-nearest neighbor algorithm

Detection of most reliable and potential predictor biomarkers and footprint of prenatal methamphetamine exposure effects that could be potentially used for clinical and prognostic aspects are highly desired. Microarray data had analyzed by using Bioconductor and GeneFilter package from R programming language for the removal of low-intensity features. The GeneFilter package was used to filter and select genes from the microarray dataset according to a variety of different filtering mechanisms. Then the k-nearest neighbor (KNN) classification was performed using leave-one-out cross-validation. Commonly, the KNN algorithm assumes that similar things exist in close proximity (18). This is a supervised machine learning algorithm that recently draws attention to use for classification and regression models in molecular biology. First, to the exclusion of any false positive and false negative results DEGs that were meet the criteria (Fold change > 2, multiple comparisons corrected P-value < 0.05, and intensity threshold

> 2000) in all four-time points were selected. The function returns the predicted classifications as its returned value. To achieve the most corrected predictive model*, the K range between 6 to 12 and* Area Under the curve (AUC) performance between 0.6 to 1.00 in receiver operating characteristic (ROC) assumed as selective criteria. only one model meets these criteria which included 17 genes from all 26 genes with DEGs criteria mentioned above. These 17 genes were considered for confirmation with qPCR. classification conducted based on previous studies (16).

Confirmation by quantitative Real-Time PCR

To testing the reproducibility of the predictive PME biomarkers model, and also for the regular confirmation of array data's reliability, mRNA levels of 17 selected genes (fold change>2 and p<0.05) were quantified by quantitative RT-PCR in all subjects. Blood sampling, RNA extraction, and cDNA synthesis were repeated with the same method used for DNA microarray. Primers for all selected genes were designed by "oligo7" software and blasted on the NCBI website to check the specificity. Quantitative PCR was performed by using SYBR green (Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2X) #K0221, Fermentas, Latvia). Triplicate method performed for Quantitative Real-time PCR by using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). GAPDH is used for normalization as an endogenous reference gene.

Statistical analysis

Statistical analysis analyzed by SPSS, version 26. Descriptive data are expressed as mean ± SD (range), and the level of statistical significance was set at P < 0.01. Compliance with normal distribution for continuous variables was assessed via the Kolmogorov-Smirnov test. One-way ANOVA analysis was used for statistical differences in multiple group comparisons. Pearson correlation test was used for the evaluation of relations between gene expression data and clinical results. Pearson correlation analysis was conducted to determine the relationship between variables. Bonferroni correction was used for multiple comparison corrections. Body mass index (BMI), RNA quality and concentration, cDNA synthesis quality, plates/runs of qPCR, and primer efficiency were added as covariates, and persistence of the significant difference in the main effect between groups was assessed by ANCOVA to control any potential confusion.

Results

Maternal and neonatal characteristics were examined for significant differences between the PME group and the normal group (Table 1). No difference was found in the baseline and demographic characteristics between groups. At the beginning of the study, 3560 PME infants, including 2217 boys and 1343 girls, and 4120 normal infants, including 2252 boys and 1868 girls, were recruited. At the end of the 36 months follow-up in the PME group, only 2658 children, including 1287 boys and 930 girls, and in the normal children group, 3573 children including 2064 boys, and 1509 girls remained in the project and were participated in all terms of sampling and testing. Only these 2658 children in the PME group (1287 boys /

930 girls) and 3573 normal children (2064 boys / 1509 girls) were analyzed for the study, and other children were excluded from the project.

DNA microarray results

Subjects of each time point were considered separately and DEGs were selected based on at least 2fold change differentiation and corrected p-value less than 0.05. Initial filtering with MAS 5.0 based on significance, fold change, and the effect size was showed 1234 genes differently expressed (626 up-regulated genes,608 down-regulated genes) in PME infants vs. normal infants. In twelve months sampling 1103 genes (572 up-regulated genes,531down-regulated genes), in twenty-four months sampling 976 genes (561 up-regulated genes,415 down-regulated genes), and thirty-six months sampling 1006 genes (628 up-regulated genes,378 down-regulated genes) were differently expressed in PME children vs. normal children. Final results were calculated after FDR correction ($p<0.01$), the ANOVA, and post hoc analysis ($p<0.01$) along with Bonferroni multiple-comparison correction. Details list of differentially expressed genes in microarray in all comparisons between PME groups and normal subjects have been presented at supplementary data. There were 826 genes whit altered expression during the whole 36 months of life in PMEs. Gene lists and venn plots of DEGs in each sampling during the follow-up are shown in supplementary file table S1 and part A of figure 1.

DAVID and KEGG analysis

To reveal the molecular and cellular functional characteristics and pathways affected by PME, all of the DEGs were subjected to enriched gene ontology calculations, pathway analysis by DAVID and KEGG pathways. Most of the constant DEGs during the follow-up period were involved in eighteen gene ontologies and three pathways including immunodeficiency, synapse formation, and respiratory chain complexes. DAVID gene ontology findings were presented in supplementary file table S2 and part B of figure 1. KEGG pathways findings were presented in supplementary file table S3 and part B and C of figure 1 and also figure 2.

Potential biomarker prediction analysis

QPCR confirmation was conducted on 17 DEGs to validate changes in gene expression from the microarray analysis. Genes were selected based on fold changes, p-value, intensity threshold, and KNN classification algorithm. Real-time PCR confirmations in these significantly altered genes were showed the same alterations in the same directions as those observed in the microarray. All genes in this list were showed the same direction in expression in all follow-up samplings. The comparison of the fold change obtained from qPCR and microarray determined a significant correlation between techniques ($P < 0.0001$,$r = 0.874$) that shows confirmations by qPCR indicated a strong reproducibility of gene expression results. Results of biomarker assessments showed a shortlist of candidate genes with high sustained during the follow-up, which was determined in supplementary file table S4 and figure 3. Also, ROC analysis of 17 biomarkers was presented in supplementary file table S5. Dopamine receptors, dopamine

degradations, and FOXP2 as human accelerator genes were showed the most sensitivity and specificity during the follow-up samplings. Primers of analyzed genes in qPCR are presented in table 2.

Discussion

The present study aimed to shed a light on the big picture of molecular changes in prenatal methamphetamine exposure during the first three years of life. Findings showed that three molecular pathways, including Primary immunodeficiency, Apoptosis, and Parkinson's disease are mostly affected by PME. Also, abnormalities in the bioenergetic, dopaminergic, and synaptic processes were detected at all time points. Based on expression profiling data a gene list of biomarkers was selected by machine learning to use as a potential model to diagnosis the PMEs.

Recently studies have focused on the effects of prenatal exposure to CNS stimulant drugs (19). Although there are several reports about the molecular, cognitive, and brain structure of methamphetamine use disorder, there is a great lack of information about PMEs. Few animal studies reported the PME effects on molecular pathways in the brain (20, 21, 22). In addition, some psychological studies evaluated the cognitive effects of PME in children at the age of six or seven or adolescence (23, 24).

Genome expression profiling on methamphetamine-treated animal model studies showed changes in critical genes for brain functions, including neuronal plasticity, mitochondrial energy metabolism, and immune response (25). METH treatment induction on mitochondrial energy metabolism and associated glutamate receptor alterations as a neurotoxic response in the brain (including the amygdala, prefrontal cortex, hippocampus, and striatum) of rats accompanied with a significant behavioral sensitization had reported (26, 27).

The present study may suggest that PME may induce chronic bioenergetic deregulations in children that, in turn, could increase the possibility of severe psychiatric disorders caused by mitochondrial dysfunction. Mitochondrial dysfunction especially in complex I of the respiratory chain are biomarkers for depressive mode (28,29) as well as psychosis and several cognitive dysfunctions in schizophrenia and bipolar disorder (30).

Neuron projection, which is the most affected cellular component during the follow up is any process extending from a neural cell, such as axons or dendrites, and is related to addiction tendency and stress problems, and changes in the brain circuitry (31, 32). Synaptic transmission and transmission of nerve impulses are two affected biological processes that are essential for normal cognitive functions such as learning and memory (33). Dysfunction in synaptic transmission and transmission of nerve impulses had been reported in several psychiatric disorders such as schizophrenia and autism (34, 35). Findings may explain the learning and cognitive problem (36, 37) and even anatomical changes in the brain of PMEs, such as reduced caudate and thalamus volume (38).

Probably the major and severe primary immunodeficiency is the most worrying effect of PME observed in the present study. METH treatment may cause an inflammatory response that plays a potential role in

METH-induced neuronal injury and deregulation of cellular immune responses (39, 40). METH also could increase blood-brain barrier (BBB) permeability that may explain similarities between PMEs and methamphetamine use disorders (8), but taking together with primary immunodeficiency it could be considered as a risk factor for CNS infections (41). Immunodeficiency caused by methamphetamine can get escalated when accompanied by mitochondrial damage and increased levels of oxidative stress (42). By the way, primary immunodeficiency diseases (PIDs) are heterogeneous disorders, and many PIDs patients are diagnosed late. Due to poor prognosis, many cases suffer from complications by the burden of chronic infections, irretrievable end-organ damage, or even death before the definitive diagnosis (43, 44). Results revealed that PMEs are born at risk of lethal immunodeficiency disorders, but the bright side is that the targeted prognosis of PMEs may lead to timely diagnosis and appropriate treatment of those PMEs who may affect by PIDs.

Recent epidemiological cohort studies showed that methamphetamine use disorder increases the risk for developing Parkinson's disease (PD) and there are similar neurodegenerative processes in PDs and Meth use disorders that target the nigrostriatal system (45,46). Like addiction tendency, anxiety, and low-stress resilience, Parkinson's disease pathway also is mostly involved with dopaminergic and GABAergic pathways (47). Constant and severe deregulation of Parkinson-related genes in PMEs provide evidence that prenatal methamphetamine exposure can cause long-lasting disabilities or degeneration of dopaminergic cell bodies, and it may increase the risk of PD in PMEs as well as stress disorders and addictive behaviors. Also, these potential risks for PD can be detected in neonates.

The number of deregulations caused by methamphetamine, including reactive oxygen species activation and mitochondrial dysfunctions may lead to death receptor and ER pathways of apoptosis (48, 49). Methamphetamine-induced apoptosis pathways are mostly mitochondria-mediated (50), but also the footprints of the mTOR signaling pathway were detected (51). Several genes from both mitochondrial and mTOR were differentially expressed in PMEs. mTOR pathway is also associated with autistic-like behaviors and locomotor activity problems (52).

Based on our assessment to find predictive biomarkers for PME, we short-listed a gene list of seventeen genes. five genes of the biomarker list are involved in the dopamine pathway, dopamine receptors (D1, D2, and D3), brain-derived neurotrophic factor (BDNF), and MAOA. The similarity of expression pattern in PME and methamphetamine use disorder in adults may support the evidence that methamphetamine can pass through the blood-brain barrier of infants. MAOA down-regulation that observed almost constantly during all-time points may suggest that PME children could be at high risk for psychiatric disorders, also may explain the stress and violence problem of PMEs (4,5,53,54).

Genes related to the immune system that meet the criteria of biomarkers are all involved in neurodevelopmental disorders, especially ASD. Cytokines participate in normal neural development and function, and abnormal cytokine activity caused by meth neurotoxic effects may lead to several neurological dysfunctions. It may explain some of the similar symptoms of ASDs and PMEs, such as language impairments and lack of communication (4, 5,55).

Synaptogenesis dysfunctions are shared between PMEs and ASDs. Lack of function in SHANK2 may cause ASD-like behaviors such as reduced social interaction and social communication and repetitive behaviors (56, 57,58).

Transcription factors in the PMEs biomarker list, CREB1, DLG4, MECP2, EGR2, and FOXP2 are essential in memory formation, language function, and cognition. Findings may suggest that PMEs are vulnerable to affect by psychiatric disorders as well as syndromic and heterogeneous disabilities in executive functions such as memory abnormalities and language impairments (59, 60).

CYP2E1 is an essential protein for the cytochrome P450 mixed-unction oxidase system that is involved in the metabolism and degradation of drugs, toxic environmental chemicals, and carcinogens along with several basic metabolic reactions such as fatty acid oxidations, ethanol, and glucose metabolism (61). It seems that abnormal activity of the immune system in PMEs could cause constant effects on xenobiotic metabolism and several digestive pathways related to glucose and lipids. The expression level of interlukins (IL1B and IL8), CDK4, and TGFB1 were previously reported as modulators of humoral immunity via transcriptional programs and suppressed cellular energetics of both glycolysis and oxidative phosphorylation (62). Deregulation of these genes may lead to a number of autoimmune disorders.

These 17 DEGs may use as biomarkers to predict PME-associated disease in children. Constant up-regulation of dopamine receptors and down-regulation of dopamine degradation gene (MAOA) strongly suggest that PMEs could be high risk for psychotic disorders such as schizophrenia and bipolar disorder. Several transcription factors and synaptogenesis genes related to neurodevelopmental disorders could provide evidence that PME could be a risk factor for language impairments, ADHD, and Autistic like behaviors. It seems that the most PME associated potential diseases would be severe psychiatric disorders and autoimmune diseases.

Limitations

Lack of accessibility to the expression profile of brain tissue may reduce the accuracy of pathway interpretations. Also, neuroimaging and psychological assessments are missing in this study and we need to continue the follow-up to add these evaluations to the study.

Conclusion

Findings suggest that PMEs may face psychological and somatic problems during their lifetime. The severity of these problems could be different subject to subject and could be related to time and dosage of exposure. Finding out the nature of these health concerns along with early detection of affected pathways may lead to better and targeted preventive health behaviors. Further functional and epigenetic studies may explain some of the complexity that was detected in the expression pattern during our follow-up. It seems that future follow-up studies along with psychological testings in different ages,

including children at the school age, after the synaptic pruning period, adolescence, and even adulthood may help to better understanding PME effects on brain functions. It is planned to continue the follow-up until adolescence age and synaptic pruning that may shed light on the long-term effects of PME in the young generation.

Abbreviations

PME: Parental methamphetamine exposure.

CNS: Central nervous system.

METH: Methamphetamine.

ASD: Autism spectrum disorder.

NCPM: National cohort project of methamphetamine addiction.

QC: Quality control.

MAS: Microarray analysis suite.

KEGG: Kyoto encyclopedia of genes and genomic.

DEGs: Differentially expressed genes.

EF: Enrichment factor.

KNN: k-nearest neighbor.

BMI: Body mass index.

Declarations

Ethics approval and consent to participate:

All processes of the study were performed based on Helsinki declaration obligations. Written consent form filled by all mothers after explaining the aim of the study. The study was approved by the central ethical committee of Islamic Azad University.

Consent for publication:

All authors are informed and consent about the submission of manuscript and order of authors.

Availability of data and materials

All data are fully available without restriction in the Arvin Gene Company database. The datasets generated and/or analysed during the current study are available in the "https://github.com/gaolongs/arvin" repository, and "GSE255077" accession in GEO database.

Competing interests:

The authors declare that they have no competing interests.

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Authors' contributions:

AH is the head of the research team and he participated in study design, laboratory procedure, data analysis, and manuscript writing. SS and PR participated in laboratory procedure and data analysis and preparation of parts of the manuscript. NR and RG participated in a laboratory procedure.

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Tables

Table 1. groups' comparison for socio-demographic differences of mothers and children (at the time of birth)

Characteristics	Prenatal methamphetamine exposure children	Normal children	p-value
Maternal/demographic			
Public insurance	2498(93.98)	3466(97.0)	0.17
No partner	1776 (66.81)	2112(60.9)	0.13
Education (who didn't complete 12 years of education)	1262(47.5)	1511(42.3)	0.14
Maternal age	22.56±1.3	23.34±1.2	0.18
Prenatal marijuana use	422(15.9)	403(11.3)	0.08
Joints/d across pregnancy	0.066± 0.24	0.052 ±0.16	0.12
Prenatal alcohol use	(15.6)	23 (8.4)	0.09
Absolute alcohol/day (oz) across pregnancy	0.016± 0.29	0.011 ±0.08	0.05
Prenatal tobacco exposure	2068 (77.8)	2443(68.4)	0.18
Cigarettes/d across pregnancy	2.94± 6.3	1.86 ±4.8	0.06
Neonatal/demographic			
Birth weight, g	3366±522	3596±447	0.16

Low birth weight	760(28.6)	468(13.1)	0.04
Length, cm	50.46±4.4	51.06±3.7	0.12
Head circumference, cm	31.23±2.1	34.06±1.9	0.03
Gestational age, wk	38.1±2.2	39.2±1.2	0.22

Data are presented as n (%) or mean ± SD.

Table 2. Primer sequences of 17 differentially genes and GAPDH gene for Real-time PCR

Gene	Forward Primer	Reverse Primer
DRD1	5'GGCTCTCGAAAGGAAGCCAA3'	5'ACTAGACCCCGGCTAAGGG3'
DRD2	5' CATGGACCACTCACACCCC 3'	5' GAACGAACAAACACACACGGG 3'
DRD3	5' GAACCCCACGAATGTTTCAGG 3'	5' GAGTTGTCAGGTCCCCATCAG 3'
BDNF	5'AAGCTCCGTAGTGCAGGAAG3'	5' ACATCCAGTTGTCCTTCGGG 3'
FOXP2	5'CCTAGGACTCCGTTTCAAGGT3'	5'TCTGTCGCAGATTCCTGCAT3'
TGFB1	5'ACCTCATCCAGGAAGTCCCC3'	5'CAGAGGAACGTCAATGCAGG3'
IL1B	5'CCAAACCTCTTCGAGGCACA3'	5'TTCAGACACCTAGTTGTAAGGAAGA3'
IL8	5'AGCACTCCATAAGGCACAAACT3'	5'TGGTTCCTTCCGGTGGTTTC3'
CDK4	5'GGAGTCTGTGATTGTAGGGTCTC3'	5'TAGGCACCGACACCAATTTCA3'
UPF3B	5'CAGGTCGATTCTGGTGGCAA3'	5' CCTTCCTGGAGAGGGTACACA3'
EGR2	5'AATAACACTACACCAGCAACTCC3'	5'CCCAACTCCCTCGCTACTC3'
CREB1	5'GCTTGAAATTCTGTGACTCTTCCC3'	5'TGGACTTGAACTGTCTGCCC3'
CYP2E1	5'TTCCGATGTTGAATTTTCCTTCTGG3'	5'GGTGACTCCGAGGGCAGA3'
MECP2	5'TATAGTTCCCATCAGGAGCCGT3'	5'CACATCAAAGCAGGAACTGGTG3'
MAOA	5'TAGAAGGGTCCTTCCCACCC3'	5'CACCTCCGATCACGACTACG3'
DLG4	5'CCATCCCCAGAAATACCGCT3'	5'CTCGGTCCCGTTCACATATCC3'
SHANK2	5'TTGTACCCCTTGTGCCAACC3'	5'TCCAAGTTGCAAGACTGGGC3'
GAPDH	5'CACTAGGCGCTCACTGTTCTC3'	5'AAATCCGTTGACTCCGACCT3'

Figures

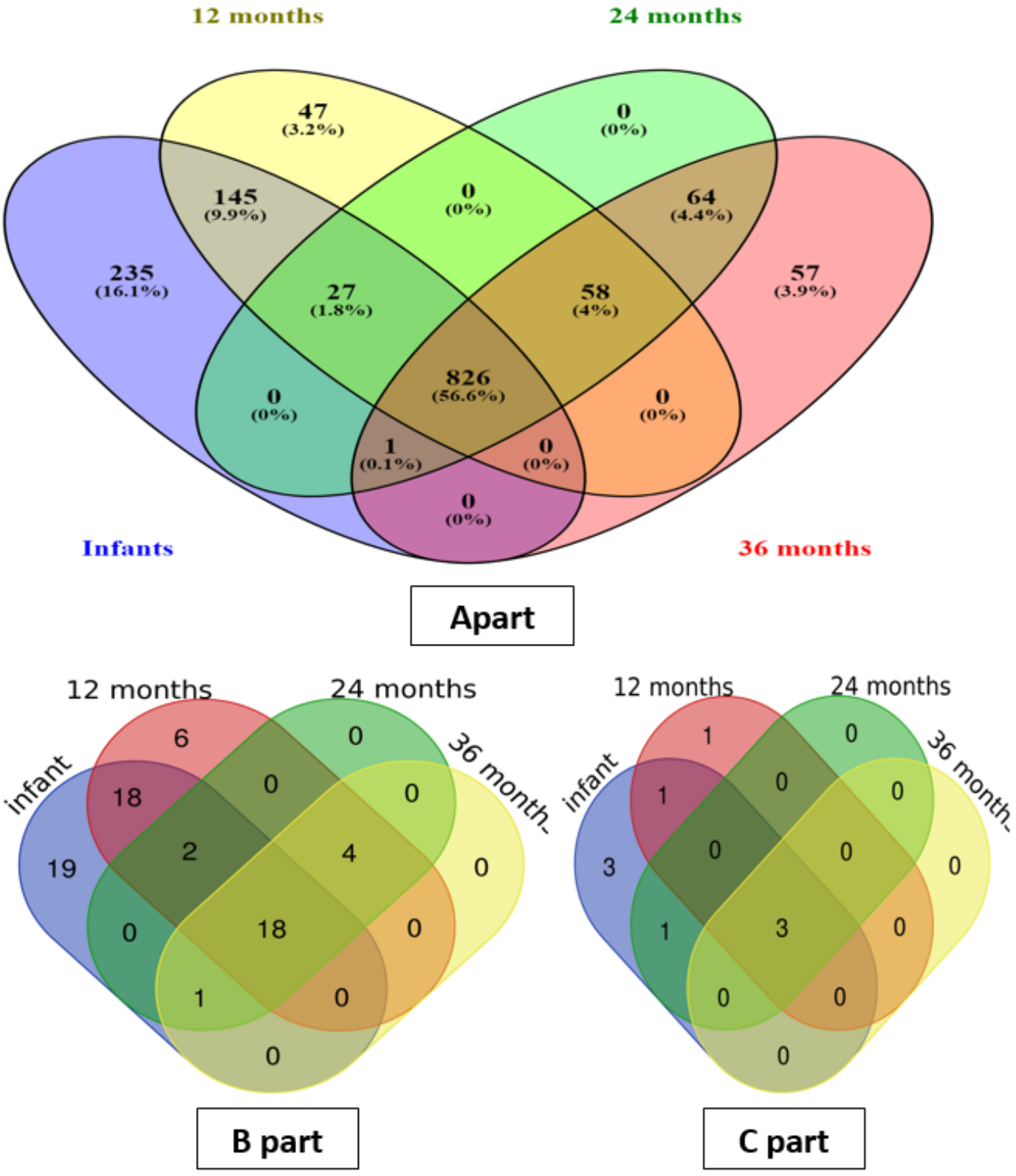

Figure 1

Part A: DEGs in response to prenatal meth exposure. Part B: Altered gene ontologies in response to prenatal meth exposure. Part C: Altered pathways in response to prenatal meth exposure. Venn diagrams showed the common set of DEGs, gene ontologies, and pathways in subjects with prenatal METH exposure during the follow-up. None of the shared genes showed different directionality of expression changes.

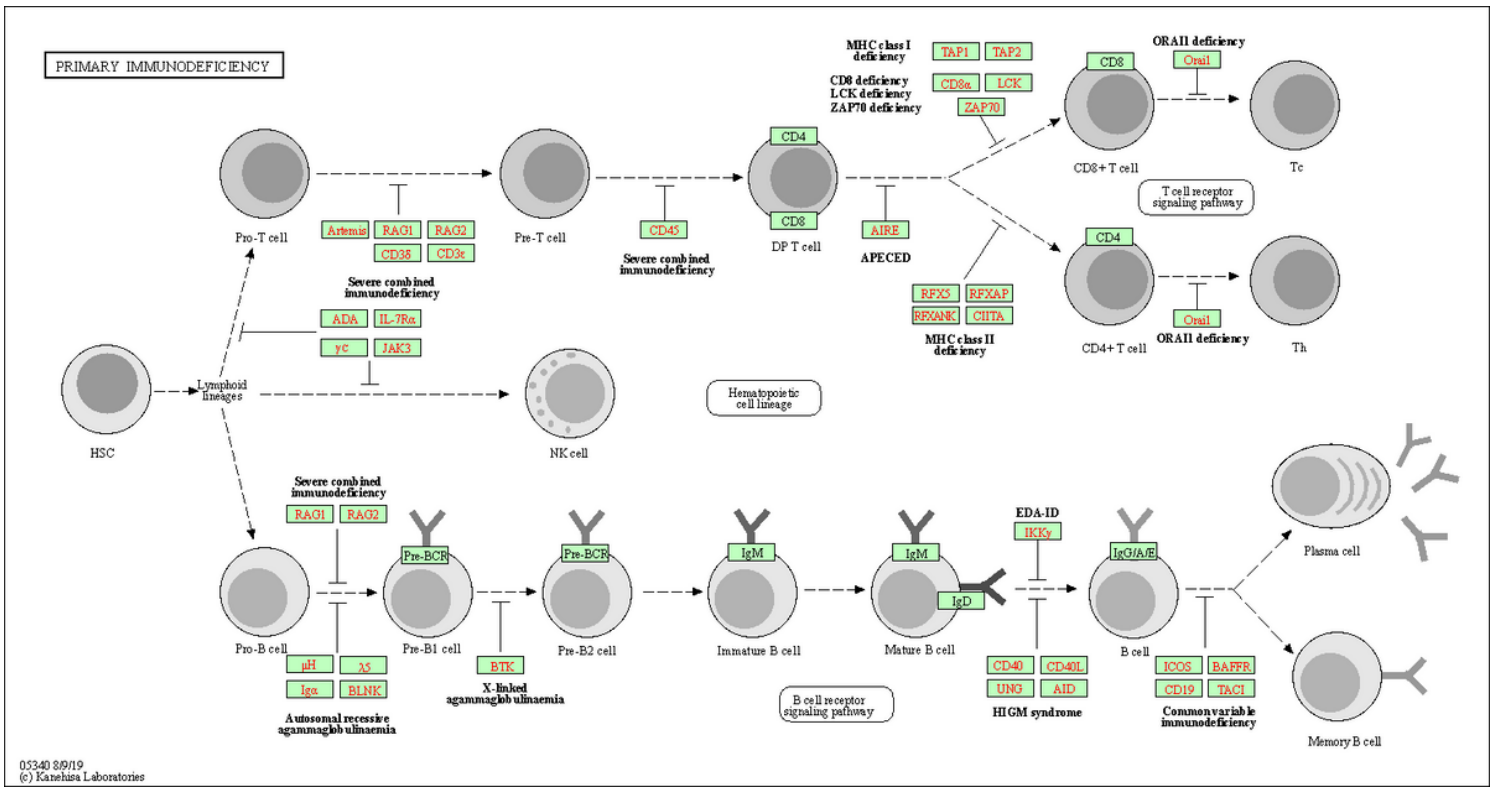

Figure 2

KEGG pathway analysis. The most significantly related pathway which was involved during the whole follow-up was primary immunodeficiency. The image is from the Kyoto encyclopedia of genes and genomes. Red genes were differentially expressed in all four samplings in PMEs compared to normal children.

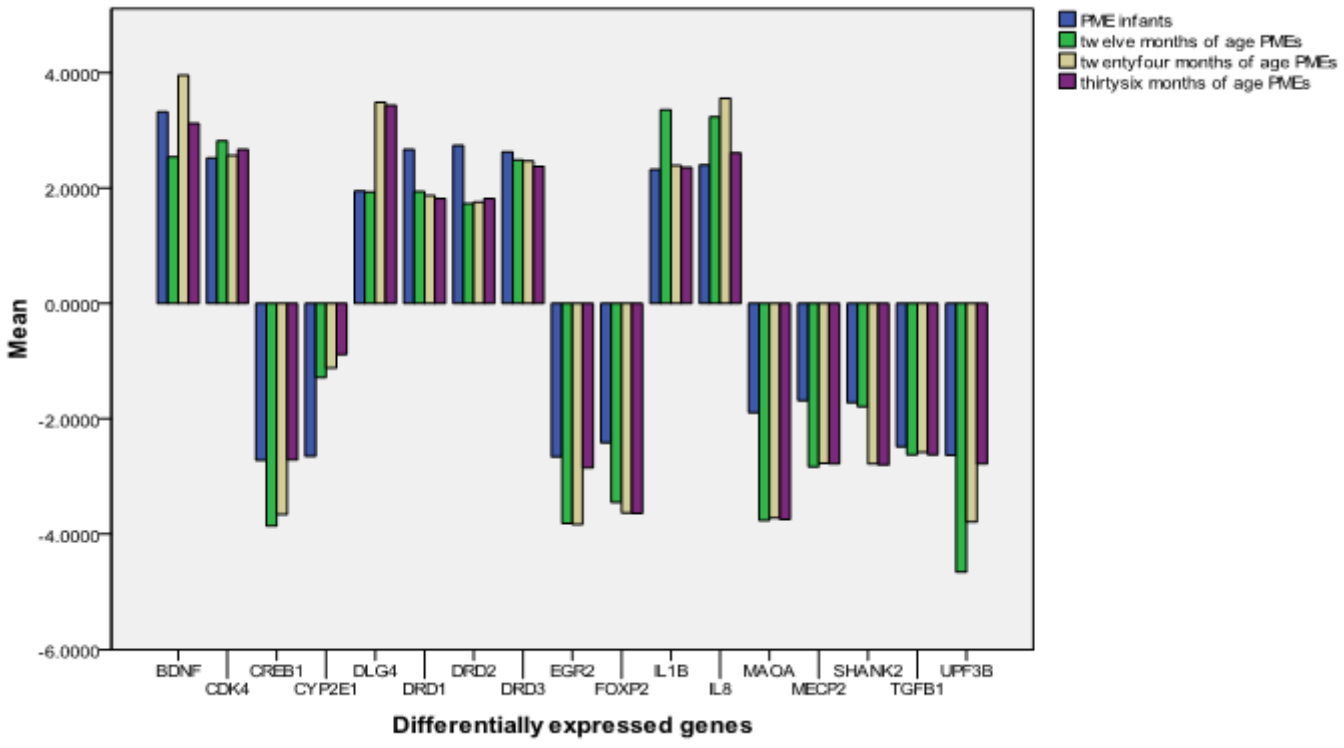

Figure 3

Potential biomarkers log2 fold change. Significant gene expression changes were identified through qPCR in PME children during four-time samplings. Expression of normal children presumed as one and revealed as the black line. Fold changes of PMEs groups calculated in comparison with normal children from matched age group. Blue bars are for infant PMEs, red bars are for twelve months of age PMEs, green bars are for twenty-four months of age PMEs and yellow bars are for thirty-six months of age PMEs.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SUPPLEMENTARYFILEPMEtable12345.xlsx