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# Maternal Cannabis Use is Associated with Suppression of Immune Gene Networks in Placenta and Increased Anxiety Phenotypes in Offspring

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**Maternal cannabis use is associated with suppression of immune gene networks in placenta and increased anxiety phenotypes in offspring**

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**ABSTRACT**

While cannabis is among the most used recreational drugs during pregnancy, the impact of maternal cannabis use (mCB) on fetal and child development remains unclear. Here, we assessed the effects of mCB on psychosocial and physiological measures in young children along with the potential relevance of the *in-utero* environment reflected in the placental transcriptome. Children (~3-6 years) were assessed for hair hormone levels, neurobehavioral traits on the behavioral assessment system for children (BASC-2) survey, and heart rate variability (HRV) at rest and during auditory startle. For a subset of children with behavioral assessments, placental specimens collected at birth were processed for RNA-sequencing. Hair hormone analysis revealed increased cortisol levels in mCB children. In addition, mCB was associated with greater anxiety, aggression, and hyperactivity. Children with mCB also showed a reduction in the high frequency component of HRV at baseline, reflecting reduced vagal tone. In the placenta, there was reduced expression of many genes involved in immune system function including type I interferon, neutrophil, and cytokine signaling pathways. Finally, several of these mCB-linked immune genes organized into co-expression networks that correlated with child anxiety and hyperactivity. Overall, our findings reveal a relationship between mCB and immune response gene networks in placenta as a potential mediator of risk for anxiety-related problems in early childhood.

**Keywords**: maternal cannabis use, anxiety, cortisol, heart rate variability, placenta, immune system, cannabinoid receptor 1

**SIGNIFICANCE STATEMENT**

Cannabis use is becoming more prevalent, including during developmentally sensitive periods such as pregnancy. Here we find that maternal cannabis use was associated with increased cortisol, anxiety, aggression, and hyperactivity in young children. This corresponded with wide-spread reductions in immune-related gene expression in placenta which correlated with anxiety and hyperactivity. Future studies are needed to examine the effects of cannabis on immune function during pregnancy as a potential regulatory mechanism shaping neurobehavioral development.

## INTRODUCTION

Along with the progressive legalization of recreational cannabis across the world, there is a prevalent misconception that cannabis use is without significant health risks. In line with this softening public perception, cannabis has emerged as one of the most consumed recreational drugs of abuse during pregnancy, with past month cannabis use ranging from 3 to 16 % among pregnant women (1, 2). In addition, the concentration of the psychoactive component in cannabis, tetrahydrocannabinol (THC), has increased consistently over the past decade, further increasing its potential harm (3). Gestational development is highly vulnerable to environmental factors which are transduced by the mother through the placenta to impact fetal health (4). Indeed, THC freely passes from the maternal blood to the fetus via the placenta (5). Thus, although cannabis is considered to provide relief from symptoms such as nausea during pregnancy (6), it may also present a significant xenobiotic challenge to the developing fetus (7).

THC targets the endocannabinoid system in both the placental tissue and developing brain (8, 9), highlighting the vulnerability of the developing fetus to prenatal cannabis exposure. Maternal cannabis use during pregnancy is associated with fetal growth restriction (7, 10), low birth weight (7, 11) and preterm birth (12). Ex-vivo treatment of placenta with THC (40 μM) increasing levels of the endocannabinoid anandamide (13). Moreover, rodent studies have demonstrated that THC exposure during gestation induces growth restriction and alters placental vasculature (14). Neurobiological consequences are also evident. Cannabis use during pregnancy is associated with reduced dopaminergic receptor expression in fetal brain (15, 16), altered cognitive development (17), increased incidence of autism (18) and depression and psychosis symptomology (19, 20). Preclinical studies directly examining the effects of THC and cannabinoid

(CB) receptor agonists during pregnancy also report adverse effects on adult offspring such as anxiogenic phenotypes (21), altered learning behavior (22), and increased drug-seeking behavior (23). Collectively, this evidence suggests that cannabis use or THC during pregnancy may exert long-lasting effects on neurobehavioral development.

Despite the abundance of evidence associating maternal cannabis use with fetal health and child development independently (24), few studies have concurrently investigated the role of the *in-utero* environment. This is noteworthy as many clinical and preclinical studies have elucidated an important role for the placenta in the neurodevelopmental effects associated with prenatal exposures such as chronic stress (25). Therefore, in the current study, we examined the effect of maternal cannabis use on the placental transcriptome in relation to offspring development, leveraging a longitudinal cohort of mother-child dyads from the New York metropolitan area. The results provide a unique multimodal interrogation of the impact of maternal cannabis use.

## RESULTS

### *Demographics*

The present study utilized longitudinal assessments from a total cohort of 322 mother-child dyads (see **Table 1** for demographics and **Figure S1** for consort flow chart). Maternal cannabis use (mCB) was associated with reduced maternal and paternal age and more single-mother pregnancies, state anxiety, trait anxiety, depression, and cigarette smoking. There was also a significant difference in the race demographic including slightly more African American (25.4 vs 19.1%), and less Caucasian (12.7 vs 21.5%), and Asian (4.2 vs. 10.4%) mCB cases vs. non-

cannabis users (non-CB). As a result, each of these presented confounds were adjusted for in all subsequent analyses. Notably, there was no difference across all measures of perinatal health or frequency of pregnancy complications for mCB vs. non-CB cases.

*Steroid hormone analysis*

Hair samples (taken close to the scalp from the posterior vertex of the head) collected from young children were used to evaluate stable steroid hormone concentrations accumulated over recent months (26) (see upper panel of **Table 2**). The results revealed increased cortisol levels in mCB children ($F_{(1,220)}$=15.67, adj. $p$<0.0001). There were no significant group differences for cortisone, DHEA, or cortisol:DHEA ratio.

*Neurobehavioral profile: clinical and adaptive behaviors*

To assess neurobehavioral traits in early childhood, we utilized the Behavior Assessment System for Children-2 (BASC-2) (27) as reported in the lower panel of **Table 2**. The BASC-2 is a well-standardized, multidimensional evaluation of the behavior of young children which measures clinical dimensions of behaviors. The instrument produces standardized T-scores, with 50 being the mean and 10 being the standard deviation, which is normalized for the sex and age for the category. Here, we found that mCB was associated with increased anxiety ($F_{(1,298)}$=16.73, adj. $p$=0.002), aggression ($F_{(1,298)}$=10.07, adj. $p$<0.001), and hyperactivity ($F_{(1,298)}$=8.50, adj. $p$<0.001). In addition, when we evaluated neurobehavioral problems meeting a clinically significant threshold (>60 on BASC-2), mCB was associated with significantly increased risk for clinical-level problems with aggression, anxiety, and hyperactivity (**Table S1**). Next, we evaluated BASC-2 measures for mCB × child sex interaction effects (**Table S2**). There was a significant mCB × sex interaction for

aggression ($F_{(1,296)}$=4.96, adj. $p$=0.027) and univariate post-hoc tests revealed a significant increase in aggression specifically in mCB females ($F_{(1,135)}$=20.85, adj. $p$<0.001) and not males ($F_{(1,163)}$=1.87, adj. $p$>0.05).

*Heart rate variability*

Heart rate variability (HRV) describes the change in time interval between heart beats. HRV measured in the high frequency (HF) domain is associated with active engagement of the parasympathetic nervous system (28). Reductions in HF-HRV are linked with various anxiety-related disorders in both adults and children (29). Here we examined HRV during three sequential one-min testing phases (pre-startle, intermittent auditory startle, and post-startle). As reported in **Table 3**, when we evaluated HF-HRV normalized to low frequency (LF)-HRV (reflecting parasympathetic/sympathetic balance), there was a significant main effect of mCB ($F_{(1,144)}$= 5.67, $p$=0.02) and significantly reduced HF-HRV both pre-startle ($F_{(1,146)}$=4.30, $p$=0.04) and during startle ($F_{(1,146)}$=6.37, $p$=0.01) for mCB children. There were no differences in heart rate or startle response magnitude/amplitude between mCB vs. non-CB groups (data not shown). For the subset of subjects with both BASC-2 and HRV measures (N=126 subjects), there was no relationship between behavioral traits such as anxiety and HF-HRV or normalized HF-HRV (see **Table S3**).

*Maternal cannabis use and the placenta transcriptome*

Placental biopsies were collected at the time of birth (gestational age: 39.13 +/- 2.63 weeks, µ+/-SD) and processed for RNA sequencing to carry out gene expression analysis (see

**Table S4** for demographics and **Dataset S1 and Figure S2** for RNA-seq quality metrics). Differential expression analysis revealed 480 significant genes (359 decreased, 121 increased) in mCB placentas at $p<0.05$ (**Figure 1A, Figure S3, Dataset S2**) with no genes passing false discovery rate (FDR)-adjustment ($q < 0.05$). While the high stringency of accounting for numerous covariates limited our statistcal power to pass FDR-adjustment, we evaluated the function of significant unadjusted genes ($p<0.05$) which allows the possibility to identify potential transcriptome pathways to inform future validation studies. Among the most robustly differentially expressed genes (DEGs) were many proinflammatory cytokines and chemokines (e.g., *IL1B*, *CXCL8*) which were also reduced in mCB placentas (**Figure 1A**). Cannabinoid receptor 1 (*CNR1*) was among the significantly reduced genes and was inversely correlated with weekly cannabis use ($R= -0.64$, $p<0.05$; **Figure 1B**). Gene ontology analysis revealed that DEGs were significantly enriched for immune response functions including type I interferon pathway, cytokine-mediated signaling, and neutrophil migration (**Figure 1C**, **Dataset S3**). Using a more stringent significance criterion (>1.5 fold change), 86 genes remained differentially expressed with gene ontology enrichment for neutrophil-related functions **(Figure 1C)**. Examining immune cell-type marker genes previously identified with single cell RNA-seq in term placenta (30) (**Dataset S4**), Fisher's exact tests revealed significant enrichment of mCB DEGs among monocyte marker genes ($OR=6.30$, $p<0.001$; **Figure 1D**).

To further examine the functional organization of immune genes associated with mCB, we constructed gene co-expression modules using multiscale embedded gene network analysis (MEGENA)(31). This identified six hundred interconnected genes modules (i.e., one gene can be present in multiple modules) ranging from 10-1000 genes per module (**Figure 2A, Dataset S5**).

Among these networks, many of the key driver genes (i.e., those with the strongest intra-network correlations) were significantly reduced in mCB placentas including monocyte-marker genes (e.g., *S100A8)* and interferon-induced genes (e.g., *OAS1)* **(Figure 2A, Dataset S6)**. Furthermore, Fisher's exact tests revealed 23 modules enriched for mCB DEGs with most containing top gene ontology categories involved in immune signaling pathways (**Figure 2B, Dataset S6**).

*Placental immune gene networks are associated with behavioral traits in early childhood*

We next examined the relationship between the 23 mCB-associated placental gene networks and neurobehavioral traits. We found that module eigengene values for three mCB-linked networks were significantly associated with heightened anxiety **(Figure 2C, Figure S4, Dataset S6)**. Directly examining the mCB-linked DEGs within these networks, major histocompatibility complex genes (*HLA-A/B/F*) involved in adaptive immunity, as well as interferon-induced genes (*IFI-35*) supporting innate immunity, were among DEGs most associated with elevated anxiety **(Figure 2D, Dataset S7).** In addition, we found two mCB-linked networks with eigengenes significantly correlated with hyperactivity levels (**Figure S5A, Dataset S6**). Each network featured the differentially expressed proinflammatory cytokines *IL1B* and *CXCL8* which were significantly associated with hyperactivity levels (**Figure S5B,** Dataset S7).

**DISCUSSION**

The present study identified significant relationships between cannabis use during pregnancy and many facets of early childhood development. Collectively, we report increased hair cortisol levels, elevated anxiety, aggression, and hyperactivity and a reduction in normalized

HRV associated with maternal cannabis use (mCB). In placenta, mCB was associated with reduced immune-related gene expression including proinflammatory cytokines and immune cell-type markers. Using gene co-expression analysis, we revealed immune-related gene networks in placenta significantly correlated with anxiety problems and hyperactivity. Overall, the results indicate a novel association between the placental transcriptome and developmental trajectories related to *in utero* cannabis exposure.

In line with previous investigations reporting greater risk for psychiatric illness in mCB children with prenatal cannabis exposure (18, 32), the current study showed that mCB is associated with increased anxiety, aggression, and hyperactivity in young children. Moreover, along with several altered psychobehavioral traits, mCB children exhibited endocrine and physiological phenotypes consistent with aberrant stress and anxiety regulation. First, mCB children had increased hair cortisol, a biomarker for long-term HPA activity. Early life HPA dysregulation is a risk for mental health disorders over the lifetime (33). Second, mCB decreased HRV, a measure of cardiac modulation of the autonomic nervous system, suggesting attenuated parasympathetic tone in mCB children. Low resting HRV variability is associated with multiple anxiety-related disorders in both young children and adults and is a risk factor for cardiovascular disease (29). Moreover, low anticipatory HRV is associated with increased stress-induced cortisol (34). Thus, while we did not observe significant relationships across behavioral assessments for the subset of children evaluated across multiple assessments in the current study, more robust inter-subject assessments will be important for future studies. Altogether, the current multimodal assessment revealed several novel phenotypes associated with stress and emotional

dysregulation in mCB children that will be important measures for continued longitudinal and mechanistic evaluation.

The finding that maternal cannabis use is correlated with reduced *CNR1* expression in placenta may reflect an adaptive response of the fetal endocannabinoid system as CNR1 and brain cannabinoid (CB) receptor expression is normally reduced in human cannabis users (35). In rodents, prenatal THC reduces CNR1 protein levels in the gestational cortex (36). As *CNR1* is highly expressed in the brain, particularly in limbic regions like hippocampus and amygdala (37) which contribute to stress regulation and anxiety, fetal endocannabinoid disturbances have implications for neurodevelopment and emotional regulation. Thus, monitoring endcannabinoid signaling in the placenta may be a valuable non-invasive indicator of *in utero* cannabis exposure severity.

The current finding of significant gene expression alterations centered on the placental immune-related transcriptome of mCB cases is intriguing. Pregnancy requires well-coordinated immunological activity, promoting maternal tolerance, while maintaining innate immune activity to protect against pathogens and viruses (38). For example, this delicate balance is reflected by type I interferon-mediated signaling in placenta which is essential for preventing viral replication, yet can also lead to significant pathology and fetal mortality (39). The reduced immune-related gene expression observed in the placenta of cannabis users mirrors other studies and is consistent with an immunosuppressive role for cannabinoids. For instance, in preclinical studies, THC during pregnancy suppresses the cytokine-sensitive STAT3 signaling pathway in the placenta (40). Moreover, experienced cannabis users have reduced monocyte chemotaxis in response to CB1 receptor agonists (41) and CB2 agonists modulate monocyte migration via reduced

interferon-γ activation (42). Both interferon-signaling genes and several monocyte markers were prominently reduced in mCB placentas in the current study.

Within gene co-expression networks enriched for differentially expressed genes, those most linked with increased child anxiety were HLA-A/B-F which encode major histocompatibility complex (MHC)-proteins. MHC-proteins are cell-surface antigen-presenting proteins recognized by T-cells to promote adaptive immune activation. Induction of HLA gene expression can be triggered by the interferon-signaling pathway (43) which was among the gene ontologies associated with mCB use. Reduced basal expression of immune activating genes could potentially increase vulnerability to pathogens or viruses during pregnancy. For instance, the infection efficacy of Zika virus is mediated by suppression of the interferon-signaling pathway (44). Importantly, the immune system (and thereby immune-related gene expression) is intertwined with stress, metabolic, and microbial environments (45). In addition, the complete impact of mCB on immune function (i.e. activation, suppression) cannot be evaluated from placenta gene expression alone. It will be critical for future human studies to examine cytokines over the course of pregnancy and their associations with other signaling factors in other tissues such as maternal and fetal cord blood in addition to placentas. Overall, our findings suggest that the immune-modulating effects previously associated with cannabis and THC may be pertinent to gestational development.

The potential risks of immune system dysregulation during pregnancy to offspring development have been studied with both activating and suppressing exogenous immunomodulators. For example, abuse of non-steroidal anti-inflammatory drugs (NSAIDs) has been linked to miscarriage and altered fetal maturation (46). In addition, maternal immune activation in rodents increases anxiety and depression phenotypes in offspring (47). Here we

found that mCB-linked suppression of immune-related gene networks in placenta correlated with increased anxiety and hyperactivity phenotypes. Previously, socioeconomically-disadvantaged pregnant mothers were found to have reduced IL-8 levels in serum during third trimester that associated with increased neurological deficits (48). In rodents, prenatal stress induces placental inflammation linked to hyperactivity in males (49). While we observed a reduction in proinflammatory cytokine signaling genes like *IL1B* and *CXCL8* in association with increased hyperactivity, this finding may reflect the importance of balanced immune regulation during pregnancy. More mechanistic preclinical studies are needed to directly and concurrently evaluate the impact of cannabinoids on the placental immunotranscriptome and offspring neurobehavioral development.

The current report has important limitations. First, in order to reduce stigma, the accuracy of our self-reported cannabis use was not verified. Furthermore, there was not sufficient power to examine prenatal and postnatal cannabis use separately and thus the findings can only be interpreted broadly as maternal cannabis use. However, it is likely that some mothers reporting postnatal use were also prenatal users and the correlation evident between maternal cannabis use and placental *CNR1* expression shows that the self-report information is robust to detect expected biological changes. Another limitation relates to the fact that differentially expressed genes identified in mCB+ placentas did not pass false discovery rate adjustment for multiple comparisons. Thus, further studies aimed at validating key findings, such as reduced *CNR1* gene expression, will be necessary. Fourth, while we controlled for various confounds, maternal cannabis use had significant differences in demographic profile and it is difficult to rule out important interaction effects (e.g., cannabis × stress or cannabis × nicotine) or other potential

unmeasured confounds influencing child outcomes (e.g., parental investment) contributing to the main effects. Finally, while many assessments were made in the current study, most mother-child dyads did not undergo all evaluations. This may have limited our ability to detect robust correlations across different measures such as between HRV and behavioral traits.

In summary, maternal cannabis use is associated with anxiety-related traits during early childhood in addition to altered stress hormones levels and psychophysiological activity. The heightened anxiety associated with reduced placental gene expression within immune response coexpression networks suggests that child development may be linked to *in-utero* immunomodulatory effects of cannabis. These results have significant implications for defining mental health risks attributable to cannabis use during pregnancy.

## MATERIALS AND METHODS

### *Procedures and participants*

The study cohort was comprised of mother-child dyads from an on-going Stress In Pregnancy project (50). Expectant mothers were recruited from obstetrics clinics at Mount Sinai Hospital and New York-Presbyterian/Queens in New York City. Women were excluded based on HIV infection, maternal psychosis, maternal age < 15 years, life-threatening maternal medical complications, and congenital or chromosomal abnormalities in the fetus. Written informed consent was obtained from eligible women for all procedures. The Institutional Review Boards at the City University of New York, Icahn School of Medicine at Mount Sinai, and New York Presbyterian/Queens approved the study. Demographic and clinical information were obtained at an initial face-to-face evaluation with a social worker as previously described (50). Annual

assessments continued at CUNY Queens College. At each annual visit, children's neurobehavioral development and mother's psychological functioning were ascertained. [As child behavioral, hormonal, and psychophysiological assays were each carried out during separate evaluations with uneven participation, group sizes varied across analyses (see Tables 2 and 3).

*Hair hormone levels*

Cortisol, cortisone, and dehydroepiandrosterone (DHEA) were measured in hair samples collected in children at ~36 months. Briefly, a bundle of ~100 hairs was cut from the posterior vertex of the child's head, close to the scalp. Samples of 3 cm section were processed using methanol extraction and assayed (Kirschbaum's laboratory, Dresden, Germany)(51).

*Behavioral and emotional functioning during childhood*

Clinical dimensions of child behavior and emotions were ascertained by administering the Behavior Assessment System for Children-2 (BASC-2) to parents (27). Dimensions included Hyperactivity, Aggression, Anxiety, Depression, Somatization, Atypicality, Withdrawal, and Attention Problems. The standardized t-score assessment was generated based on the child's biological age and sex. The mean age (SD) at the time of the assessments was 44.9 (10.9) months. Furthermore, we tested the effect of maternal cannabis use on clinically significant and at-risk conditions; in accordance with the BASC-2 manual, scores $\geq$60 on the standardized dimensions reflect clinical diagnosis with a behavioral or emotional problem.

*Electrocardiogram analysis of heart rate variability*

Electrocardiogram (ECG) recordings were made using the BIOPAC MP150 system (Biopac Inc., CA) and ECG100C BioNomadix amplifier with three pre-jelled Ag-AgCl disposable vinyl electrodes placed at the upper left collarbone, upper right collarbone, and lower left ribcage with a bandpass filter of 60 Hz. ECG signals were visually inspected for artifacts and corrected for false or undetected R-waves in AcqKnowledge 4.4. Heart rate variability (HRV) was quantified by spectral analysis of the inter-beat (RR) interval series analyzed at both high-frequency (HF) (0.15-0.40 Hz) and low frequency (LF) (0.04-0.24 Hz) power. $Log_{10}$ transformation was applied to correct the skewed distribution of HF and LF measures. The normalized HRV was computed by calculating $log_{10}$(HF-HRV/LF-HRV).

The psychophysiological assessment was carried out in children between 48-72 months with mean (SD) age of 54.39 (1.95) months. The testing session began with a 1-min rest period during which children were instructed to sit still and be quiet, followed by a 1-min startle paradigm, presented on the computer monitor. The startle paradigm involved a calm video suddenly interrupted by six startle probes of tone burst at 90 dB. After the startle paradigm, there was another 1-min resting period with continued ECG monitoring. Throughout testing, the mother sat next to the child and could talk to the child to maintain the child's attention and stillness. Notably, during the trial, measurements of electrodermal activity (EDA) were also recorded. However, since useable data was recovered for only a small percentage (<25%) of the experimental cohort, we were not able to perform statistical analysis for startle magnitude and amplitude.

*Statistical Analysis*

Self-report of either prenatal or postnatal cannabis use were collectively considered "maternal cannabis use" throughout the present study. Parental age, education, marital status, prenatal substance use, child's sex, child's age, and race were *a priori* defined as confounders. Additionally, aspects of maternal stress including pregnancy during Hurricane Sandy in 2012 and measures of maternal state/trait anxiety (derived from the State Trait Anxiety Inventory) were controlled for in all statistical models.

For the statistical analysis, descriptive statistics were first conducted to evaluate mean and standard deviation (SD) of outcome variables. General Linear Model (GLM) was used as the primary analytical method. Univariate GLM was used for hair hormones. For neurobehavioral (BASC-2) outcomes, a multivariable GLM was used which adjusts for correlations among dependent variables. The effect of mCB on values of neurobehavioral outcomes (i.e., classifying children with at-risk and clinically significant problems) was tested with the ordinal logistic link function in GLM. The exponentiation of the β-coefficient was obtained to calculate the odds ratio (OR) and associated 95% confidence interval (CI). Prior to the analysis, each subscale was evaluated for normality. If the assumption of univariate normal distribution was violated, log-transformation was applied to achieve normality. Finally, the electrocardiogram assessments of HRV used a linear mixed-effects model with trial as repeated measure and subject as a random effect along with univariate GLM tests for each trial as presented in the results table. Age at the time of testing was also included as a covariate for this assessment.

*Placental Tissue Collection*

Placental samples were acquired and processed as previously described (50). Briefly, biopsies from the outer layer of the blastocyst, free of maternal decidua, were collected from each placenta quadrant midway between the cord insertion and the placenta rim within an hour of delivery. Specimens were frozen in liquid nitrogen and stored at -80°C.

*RNA sequencing data analysis*

Transcriptome analysis (RNA-sequencing; RNA-seq) was carried out on a representative subset (N=131) of the total cohort. RNA was extracted from pulverized placental tissue and processed to remove ribosomal RNA and prepare sequencing libraries (Ribo-Zero Kit and TruSeq commercial kits; Illumina Inc., San Diego, CA). Libraries were then paired-end sequenced (2 x 75 bp) at a depth of 28.0 +/- 0.3 million reads (conducted by Novogene Corp, Sacramento, CA). Fastq sequencing output was then mapped to the hg38 genomic assembly using the STAR read aligner with standard mapping parameters. Uniquely mapped reads (91.6 +/- 1.73 % (μ+/-SD) of total reads) were annotated to genomic features from the Ensembl genome database and quantified using the –quantMode argument in STAR. Uniquely mapped reads with minimal average expression (<0.3 Fragments per Kilobase Million) were removed prior to subsequent analyses. All samples were evaluated with principal component analysis (PCA) to confirm absence of technical outliers (Figure S2).

For differential gene expression analysis, all group comparisons were made using the R package DESeq2 for moderated estimation of fold-change and statistical analysis. All analyses were performed examining main effects of mCB on each gene with a general linearized model weighted for library sizes and gene-level variance. The model accounted for many covariates with

maternal age, paternal age, fetal weight, and maternal trait anxiety evaluated as continuous variables and pregnancy during Hurricane Sandy, cigarette smoking, alcohol use, fetal sex, marital status, race, and educational attainment as discrete variables. While FDR-adjustment was applied, significant genes with uncorrected p-values were also considered in order to elucidate gene-sets and networks that can be directly tested evaluated in future studies without false-discovery burden. Identified differentially expressed genes (DEGs) were used for gene set enrichment analyses using the curated transcriptional, pathway, and ontology libraries managed on the Enrichr web server. All gene ontology analysis utilized the databases from the Gene Ontology Consortium and statistical significance was determined with Fisher's Exact Test in the Enrichr R package (52). Placental immune subtype-specific genes were defined using marker genes previously identified with single-cell RNA-seq in human term placenta (30).

Multiscale embedded gene co-expression analysis (MEGENA) was performed on all protein coding genes using the R package MEGENA. Briefly, Pearson correlation coefficients (PCCs) were computed for all gene pairs to identify all significant gene pair correlations (FDR-adj. $p < 0.05$). Next, planar filtered network (PFN) and multiscale clustering analyses were carried out to identify co-expression modules with 10/1000 set as min/max module sizes. Significance testing for overlap between DEGs and network module gene sets was performed using one-tailed Fisher's exact test with Benjamini & Hochberg false discovery rate adjustment ($q < 0.05$). Module eigengenes values were calculated with the moduleEigengenes function in the WGCNA R package (53). In calculating eigengenes, all gene members of each module were considered. Point-biserial or spearman correlations was used to associate eigengenes/genes with dichotomous and continuous neurobehavioral traits, respectively.

*Data availability*

The RNA-sequencing datasets generated and analyzed in the current study were submitted to the NCBI Sequence Read Archive (accession: PRJNA719417) and Gene Expression Omnibus (accession: GSE179930). All software used for transcriptomic analyses are publicly available.

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**CONFLICT OF INTEREST**

All authors have no financial relationships with commercial interests.

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**TABLE AND FIGURE LEGEND**

**Table 1: Demographics and reproductive characteristics of study participants.**

**Table 2: Maternal cannabis use in relation to steroid hormones levels and behavioral traits in early childhood.**

**Table 3: Maternal cannabis use (mCB) in relation to heart rate variability (HRV) in early childhood.**

**Figure 1: Maternal cannabis use (mCB) is associated with reduced immune-related gene expression in placenta. (A)** Volcano plot showing differentially expressed genes (DEGs) for mCB placentas (DEGs < 1.5 fold change (FC) = red points, DEGs $\geq$ 1.5 FC = blue points). Note: 1.5 FC ~ 0.58 $\log_2$ FC on x-axis. **(B)** Significant inverse correlation between self-reported prenatal cannabis use and placental *CNR1* gene expression ($R = 0.64$, $p < 0.05$). **(C)** Gene ontology analysis revealed that all DEGs (top) and DEGs > 1.5 fold change (bottom) were enriched for immune-related gene sets. **(D)** Log fold changes (mCB vs. non-CB) for immune cell-type marker genes in placenta (mCB DEGs in red (<1.5 FC) or blue ($\geq$1.5 FC) with bars depicting median). NK-cell = Natural killer cell.

**Figure 2: Maternal cannabis use is associated with placental gene co-expression networks related to immune function and heightened anxiety. (A)** Multiscale embedded gene co-expression network comprised of highly correlated gene modules. Arrows zoom in on two modules significantly enriched for mCB DEGs. Red nodes = DEGs. Diamond shape = network hub

genes. Darker edges between nodes represent stronger gene-gene correlations. **(B)** Bubble plot showing modules (blue circles) that are most significantly enriched for mCB DEGs are also enriched for immune-related gene ontology terms. **(C)** Heatmap of correlation coefficients between module eigengenes and child behavioral problems. Rows feature all modules enriched for mCB DEGs annotated with a unique color and top gene ontology term. *= $p<0.05$. **(D)** For each mCB DEGs in the teal, pink, and red (see panel C) modules correlated with anxiety, correlation coefficients with anxiety (x-axis) and the $-\log_{10}$ transformed p-values (y-axis) are depicted. Dash line represents significance threshold for gene-anxiety correlations.