International Journal of Medical Science and Health Research

Vol.10, No.01; 2026

ISSN: 2581-3366

On Potential Association of COVID-19 Infection During Pregnancy and Epigenetic Regulation of Metabolic Pathways in Newborns

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doi: 10.51505/ijmshr.2026.10108 URL: http://dx.doi.org/10.51505/ijmshr.2026.10108

Received: Dec 20, 2025 Accepted: Dec 31, 2025 Online Published: Feb 10, 2026

Abstract

Background: The COVID-19 pandemic has highlighted the increased susceptibility of pregnant women to SARS-CoV-2 infection and its short-term consequences for maternal and neonatal health, while the longer-term implications for children remain unclear. Evidence from fetal programming and epigenetic research suggests that inflammatory exposures in utero, including maternal infection, may lead to enduring changes in gene regulation that could influence metabolic and immune health later in life.

Methods: We analyzed publicly available bulk and single-cell RNA sequencing (RNA-seq) datasets from cord blood of full-term neonates with and without in utero exposure to maternal SARS-CoV-2 infection. Gene expression data were normalized and examined using a pathway-focused gene set analysis approach. Applying the Linear Combination Test to curated KEGG and Reactome gene set catalogs, we aim to identify cell-type–specific epigenetic effects and potential alterations in metabolic pathways.

Results: Bulk RNA-seq analysis revealed alterations in innate immune–related gene expression in neonates exposed to maternal COVID-19, with univariate LCT identifying multiple KEGG pathways associated with innate immunity, immune regulation, and antiviral responses. Then, multivariate LCT identified eleven pathways consistently linked to these phenotypes. Complementary single-cell RNA-seq data analyses showed cell-type–specific differential expression of genes involved in epigenetic regulation, with marked heterogeneity observed

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ISSN: 2581-3366

across cell populations. Pathway-level comparisons restricted to epigenetically altered cell types further identified both unique and broadly shared KEGG pathways across cell populations.

Conclusion: Together, these findings indicate that maternal SARS-CoV-2 infection during pregnancy is associated with epigenetically mediated alterations in neonatal immune and metabolic pathways that may have lasting consequences for offspring health. By linking cell-type–specific epigenetic regulation to pathways implicated in metabolic disease, this study underscores the importance of long-term follow-up and highlights potential molecular targets for early risk stratification.

Keywords: COVID-19, epigenetic regulation, fetal programming, metabolic pathway, single-cell RNA sequencing, transcriptomics

1. Introduction

The COVID-19 pandemic has posed unprecedented challenges for maternal and child health worldwide. Pregnant women are more susceptible to COVID-19 due to physiological and immunological adaptations during pregnancy, which increase the risk of severe illness, hospitalization, and adverse neonatal outcomes, including NICU admission and respiratory complications (Alfaraj et al., 2019; Allotey et al., 2020; Dashraath et al., 2020; Norman et al., 2021; Wong et al., 2004). While maternal SARS-CoV-2 infection during pregnancy has been extensively studied in relation to vertical transmission, placental pathology, and short-term neonatal outcomes, little is known about its potential long-term consequences on offspring health. (Baergen & Heller, 2020; S. Chen et al., 2020; Mulvey et al., 2020; Patanè et al., 2020; Prabhu et al., 2020; Richtmann et al., 2020; Sharps et al., 2020; Vivanti et al., 2020). Immune imprinting of maternal inflammation due to infections like SARS-CoV-2 in a fetus may leave an immunological legacy on the newborn, potentially predisposing them to diseases later in life as well (Abu-Raya et al., 2016; Al-Haddad, Jacobsson, et al., 2019; Al-Haddad, Oler, et al., 2019; Apostol et al., 2020; Barcelos et al., 2021; H. Chen et al., 2020; Gee et al., 2021; Jeganathan & Paul, 2022; Johansson et al., 2016; Kleeman et al., 2022; Li et al., 2020a; Matute et al., 2022; Musa et al., 2021; Yazigi et al., 2017).

Emerging research suggests that adverse intrauterine environments—such as poor maternal nutrition, glucocorticoid exposure, or infection—can disrupt fetal development and program metabolic dysfunction, predisposing offspring to obesity and metabolic syndrome later in life. This phenomenon is primarily driven by fetal epigenetic programming, a pivotal mechanism in fetal metabolic programming that was first proposed by Barker and Hales in 1998 (Barker, 1998; Barrès & Zierath, 2016; Bošković & Rando, 2018; Grundy, 2005; Huypens et al., 2016; Sun et al., 2018; Tamashiro & Moran, 2010; Tang et al., 2016; Zhu et al., 2019). Epigenetic mechanisms are heritable changes in gene expression that occur without altering the DNA sequence itself, such as mutations or translocations (Buenrostro et al., 2013; Harvey et al., 2018; Heard & Martienssen, 2014; Waddington, 2012; Zilbauer, 2014). During pregnancy, epigenetic processes like DNA methylation and histone modification are essential in managing the complex

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interactions between the mother and fetus. These mechanisms are sensitive to both internal and external environmental influences, including infections, and can significantly impact gene expression. The dysregulation of epigenetic pathways during gestation has been associated with a spectrum of defects in both the fetus and adolescents. Evidence from recent clinical studies has revealed the role of epigenetic mechanisms through which prenatal immune activation due to virus and bacterial infections can contribute to various complications such as schizophrenia, congenital heart defects, Type-1 Diabetes (T1D), asthma, and metabolic syndrome (Barua & Junaid, 2015; Bermick & Schaller, 2022; Bohacek & Mansuy, 2015; Fong et al., 2020; Gluckman et al., 2007, 2009, 2010; Kleeman et al., 2022; McCullough et al., 2017; Pradhan et al., 2023; Solomons, 2009; Szyf, 2015). Experts have raised concerns that children born to mothers who contracted COVID-19 during pregnancy may face long-term health complications and emphasized the need for life-course studies to monitor and assess the potential long-term health effects on these offspring (McCarthy et al., 2021). However, most existing studies have concentrated on individual gene-level analyses or immune profiling, leaving the wider functional impact of maternal infection on epigenetically regulated gene networks largely unexamined.

Advances in transcriptomics allow for large-scale analysis, helping to identify key pathways and targets. These studies are especially important for understanding how maternal infections, such as COVID-19, may impact fetal gene expression and long-term health (Hill et al., 2023; Taylor et al., 2003). Traditional single-gene expression studies often face challenges such as high variability and increased false discovery rates due to their limited focus on individual genes without accounting for functional interrelationships. Gene Set Analysis (GSA) addresses these limitations by evaluating groups of genes within biological pathways, thereby reducing false positives and utilizing existing biological knowledge to improve statistical power and mechanistic understanding. This pathway-focused approach enhances the detection of subtle yet biologically significant gene expression changes, facilitating biomarker identification and therapeutic advancements. Among GSA methods, the Linear Combination Test (LCT) is particularly effective, as it accounts for correlations within gene sets and outcomes, reducing Type II errors and increasing sensitivity. LCT is also computationally efficient, adaptable to diverse study designs, and capable of handling binary, univariate, and multivariate outcomes in complex biological systems (Dinu et al., 2013, 2021; Song & Black, 2008).

Overall, despite recognizing pregnant women as a high-risk group for SARS-CoV-2 infection, current literature lacks comprehensive data on the long-term and transgenerational implications of maternal COVID-19 infection. In this study, we aimed to address this gap by investigating epigenetic regulation in specific cord blood cell types from neonates born to SARS-CoV-2–infected mothers. Using a gene set–level analytical approach, we assessed coordinated changes in biologically relevant pathways, particularly those associated with metabolism and health, in multiple datasets from real clinical studies. By integrating cell-type–specific insights with pathway-level analyses, our study provides a comprehensive evaluation of how epigenetically maternal COVID-19 infection may influence offspring metabolic programming and highlights potential targets for early preventive interventions.

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2. Method

2.1 Dataset Description

2.1.1 Gene Expression Datasets

To investigate modulation in metabolic pathways of neonates born to COVID-19-infected mothers without vertical transmission, we used two publicly available RNA sequencing (RNA-seq) datasets accessed from the Gene Expression Omnibus (GEO) database. For simplicity, we refer to the GSE195938 dataset as the "bulk RNA-seq dataset" and GSE165193 as the "single-cell RNA-seq dataset."

Bulk RNA-seq dataset:
The gene expression dataset GSE195938 involves genome-wide profiling conducted with Human Clariom S gene chips on total RNA extracted from cord blood cells of 16 full-term newborns. Among these, 8 were exposed to COVID-19 in utero, while the remaining 8 served as controls: 6 born prior to the pandemic and 2 born to mothers who tested negative for COVID-19 antibodies (Gayen Nee' Betal et al., 2022).

Single-cell RNA-seq dataset:
The gene expression dataset GSE165193 utilized droplet-based single-cell RNA-seq and T-cell receptor sequencing on cord blood mononuclear cells (CBMCs) collected from six full-term infants (gestational age >37 weeks). Three of these infants were born to mothers with mild SARS-CoV-2 infection, as defined by the NIH 2020 criteria (i.e., cases), while the other three were non-exposed controls born during the same period. The dataset includes 25,970 cells with high-quality single-cell transcriptomes, 14,748 cells from cases, and 11,222 cells from controls. The visualization of the cell population composition was achieved by uniform manifold approximation and projection (UMAP). Additionally, the identification of cell types was determined by analyzing cluster-specific canonical marker genes (Matute et al., 2022).

2.1.2 Curated Databases of Gene Sets and Pathways

KEGG LEGACY: KEGG is a comprehensive database that supports the study of biological systems across multiple levels, from cellular processes to whole organisms, by integrating molecular information from genome sequencing and high-throughput technologies. It consists of sixteen interconnected databases covering systems, genomic, chemical, and health-related data. The KEGG PATHWAY module provides curated maps of molecular interactions and reactions, organized into seven clusters representing major biological and cellular functions. (Kanehisa & Goto, 2000) We used KEGG LEGACY, consisting of 186 gene sets accessible in the Human Molecular Signatures Database (MSigDB)(Mootha et al., 2003; Subramanian et al., 2005).

Reactome: Reactome is an open-access pathway database developed through careful manual curation, expert authorship, and peer review by biologists. Its annotations are generated in collaboration with the Reactome editorial team and cross-validated against multiple bioinformatics resources to ensure accuracy. For example, the

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"Reactome_Epigenetic_Regulation_of_Gene_Expression" gene set, which includes 187 genes involved in epigenetic regulation, was compiled by Bruce May and G. Gopinathrao and reviewed by Lisa Matthews, reflecting Reactome's emphasis on high-quality, reliable data (Milacic et al., 2024). We used the Reactome pathway database because it integrates all identified epigenetic pathways along with their contributing genes, capturing the relationships between genes within and across pathways. This approach allows us to assess coordinated epigenetic processes systematically, avoiding the potential bias of analyzing individual pathways separately and overlooking the interconnections and interactions between genes. By considering these relationships, Reactome provides a more biologically meaningful and comprehensive view of epigenetic regulation.

2.2 Method Description

We chose the LCT (Dinu et al., 2021) to effectively address the high dimensionality problem inherent to our datasets, and the correlation of expression measurements present across gene sets and/or biological pathways via a shrinkage covariance matrix estimation approach. It is superior in terms of power and computational efficiency when compared to other gene-set analysis methods in both simulations and real data analysis.

2.2.1 LCT Statistical Framework

Take into consideration gene expression data from n subjects, n_I cases and n_2 controls. The null hypothesis that needs to be tested is that two phenotype groups exhibit equal expression of a predefined gene set consisting of K genes, $\{X_l, \ldots, X_K\}$. One univariate expression for this multivariate hypothesis that can be used as a null hypothesis is H_0: $X_l, \ldots, X_K$ does not have a linear combination linked to the phenotype. Let $Z(\boldsymbol{\beta}) = \beta_l X_1 + \ldots + \beta_K X_K$ be a linear combination of $X_l, \ldots, X_K$. The following univariate model can be used to determine whether or not the combination $Z(\boldsymbol{\beta})$ is linked to the phenotype for a given vector β of combination coefficients: $Z_{ij}(\boldsymbol{\beta}) = \mu_i + e_{ij}$, where $e_i j \sim N(0, \sigma^2), j$ indicates subjects $1, \ldots, n_i$ of groups $i = 1,2$ and μ_1 and μ_2 are the mean gene expressions for the cases and controls, respectively. The Student t-test, $T(\boldsymbol{\beta})$, following a t-distribution, or equivalently $T'(\boldsymbol{\beta})$ following a F-distribution, are frequently used test statistics for this type of traditional two-sample test problem. The most-significant linear combination of $\{X_l, \ldots, X_K\}$, or the linear combination with the highest statistical significance among all potential linear combinations, is taken into consideration for the H_0test. We have

$$\beta^* = \underset{\beta}{\operatorname{argmax}} T^2(\beta), \qquad (1)$$

as the most important linear combination's coefficients. The two-sample t-test squared gives us

$$T^2(\boldsymbol{\beta}) = \frac{\left(\overline{Z}_1 - \overline{Z}_2\right)^2}{S^2 z_1 z_2 \left(\frac{1}{n_1} + \frac{1}{n_2}\right)}, \qquad (2)$$

where $\overline{Z}_1 = \boldsymbol{\beta}^T \overline{X}_1$, $\overline{Z}_2 = \boldsymbol{\beta}^T \overline{X}_2$, $S^2_{Z_1 Z_2} = \boldsymbol{\beta}^T \widehat{\boldsymbol{\Omega}} \boldsymbol{\beta}$, $\widehat{\boldsymbol{\Omega}}$ is a pooled covariance matrix over the two groups of the phenotypes, and $\overline{X}_1$ and $\overline{X}_2$ are the K-sample averages of gene expressions within cases and controls, respectively, and with the hh'-th entry being

$$\omega_{hh'} = \frac{1}{n-1} \sum_{l=1,n} (x_{hl} - \overline{x}_h)(x_{h'l} - \overline{x}_{h'}). \quad (3)$$

As a result,

$$T^2(\boldsymbol{\beta}) = \frac{\boldsymbol{\beta}^T (\overline{X}_1 - \overline{X}_2)(\overline{X}_1 - \overline{X}_2)^T \boldsymbol{\beta}}{\boldsymbol{\beta}^T \left(\frac{1}{n_1} + \frac{1}{n_2}\right) \widehat{\boldsymbol{\Omega}} \boldsymbol{\beta}}. \quad (4)$$

The optimization problem mentioned above can be expressed as

$$\boldsymbol{\beta}^* = \text{argmax}_{\boldsymbol{\beta}} \frac{\boldsymbol{\beta}^T A \boldsymbol{\beta}}{\boldsymbol{\beta}^T B \boldsymbol{\beta}}, \quad (5)$$

where $B = \left(\frac{1}{n_1} + \frac{1}{n_2}\right) \widehat{\boldsymbol{\Omega}}$, and $A = (\overline{X}_1 - \overline{X}_2)(\overline{X}_1 - \overline{X}_2)^T$. Accordingly, $T^2(\boldsymbol{\beta}*)$ is the corresponding eigenvalue and the maximal eigenvector $A\mathbf{B}^{-1}$ is the solution to this optimization problem (Johnson and Wichern, 2002).

The matrix **B** is singular when the size of the gene set is larger than the sample size, or K>n. A potential solution to the singularity is to use a shrinkage covariance matrix $\widehat{\boldsymbol{\Omega}}^*$, as suggested by Schafer and Strimmer (2005), to replace the singular covariance matrix $\widehat{\boldsymbol{\Omega}}$, much like Tsai and Chen (2009) did when they adjusted the modified Hotelling's T^2. This can be done by using the formula $\omega^*_{hh'} = \rho^*_{hh'} \sqrt{\omega_{hh} \omega_{h'h'}}$, with shrinkage coefficients $\rho^*_{hh'} = 1$, if $h = h'$, and $\rho^*_{hh} = \rho_{hh} . \min\{1, \max(0, 1 - \hat{\lambda}^*)\}$, if $h \neq h'$, where $\rho_h h'$ is the pooled sample correlation between the h -th and h'-th genes, and the formula $\hat{\lambda}^* = \sum_{h \neq h'} V\,ar(\rho_{hh'}) / \sum_{h \neq h'} \rho^2_{hh'}$ can be used to estimate the ideal shrinkage intensity $\hat{\lambda}^*$. This method of adding the covariance matrix estimator to the test statistic has a very high computational cost. We use a group of normalized orthogonal bases in place of the original observation vectors in order to solve the computational efficiency issue. First, we decompose the shrinkage covariance matrix into its eigenvalues, $\widehat{\boldsymbol{\Omega}}^* = \mathbf{UDU}^T$. A set of orthogonal basis vectors is then obtained as follows: $(Y_1, \ldots, Y_K) = (X_1, \ldots, X_K)\mathbf{UD}^{-1/2}$. One way to rewrite the square of the two-sample test statistic is as

$$T^2(\gamma) = \frac{\gamma^T (\overline{Y}_1 - \overline{Y}_2)(\overline{Y}_1 - \overline{Y}_2)^T \gamma}{\gamma^T \gamma}, \quad (6)$$

where $\boldsymbol{\gamma} = \mathbf{D}^{1/2}\mathbf{U}^T\beta$, and the K-sample averages within cases and controls are denoted by $\overline{Y}_1$ and $\overline{Y}_2$, respectively. According to a matrix algebra calculation (Johnson and Wichern, 2002), the mean-vector difference between the two groups,$\boldsymbol{\gamma}^*$ c $(\overline{Y}_1 - \overline{Y}_2)$, provides the coefficients of the most significant combination. Once the orthogonal transformation is applied, this LC test statistic is proportional to the L_2- norm of the mean-vector difference between the two groups:

$$T^2(\gamma^*) = c \parallel \bar{Y}_1 - \bar{Y}_2 \parallel^2, \qquad (7)$$

where c is a constant. With this test statistic, we can use a permutation test (permuting phenotype labels) to assess the statistical significance against the null hypothesis. In the permutation test, the constant c can be disregarded. The computational advantage of this method is that, unlike permutations in the modified Hotelling's T^2 approach, $\widehat{\boldsymbol{\Omega}}^* = \mathbf{UDU}^T$ is evaluated only once

2.3 Data Analysis

Bulk RNA-seq dataset:
For the preprocessing of the bulk RNA-seq dataset, quantile normalization was applied to the data to reduce systematic biases and technical variability, thereby enhancing the accuracy of downstream analyses. LCT was employed to investigate the transcriptional enrichment of predefined gene sets from the KEGG LEGACY catalogue. Both univariate and multivariate LCT approaches were utilized to examine associations between these gene sets and three innate immune phenotypes: general immune function, immune response regulation, and antiviral response. Additionally, Fisher's Ratio (FR) was used to rank genes based on their discriminative power between phenotypes, aiding in the development of predictive models.

Single-cell RNA-seq dataset:
The analysis aimed (a) to determine whether genes associated with epigenetic regulation show differential expression in umbilical cord blood cells from infants born to mothers with COVID-19 compared to unexposed controls, and (b) to subsequently identify which biological pathways are perturbed within specific cell types exhibiting altered epigenetic regulation.

Before analysis, zero inflation in the gene expression data was addressed to enhance analytical accuracy. For approximately 14,000 genes with zero expression values, the base R function 'jitter' was applied, adding small random deviations to these zeros. This adjustment maintains values near zero while mitigating the bias introduced by excessive zeros, thereby enabling more robust and reliable downstream analyses. (J. M. Chambers et al., 2018; J. M. Chambers & Hastie, 2017)
All statistical computations were conducted utilizing R software (version 4.3.2). https://www.r-project.org/

The LCT was first applied to assess differential expressions within a Reactome gene set comprising 187 genes related to epigenetic mechanisms. Cell types with a p-value <= 0.01 are

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considered significant. Hematopoietic stem and progenitor cells (HSPCs), lacking distinct clusters, are excluded from subsequent analysis. Subsequently, the KEGG gene set collection was used to identify the biological pathways impacted in these cell types. The overall objective was to uncover potential metabolic disturbances linked to maternal immune activation resulting from SARS-CoV-2 infection.

Fisher's method, or Fisher's combined probability test, combined the results of nine independent comparisons (nine p-values for each KEGG gene set) into a single overall p-value (Fisher & Tippett, 1928). This combined p-value indicates the strength of evidence against the null hypothesis when considering all nine LCT results for each KEGG gene set together.

3. Results

Bulk RNA-seq dataset:
The univariate LCT revealed significant associations between several KEGG gene sets and immune system functions. Specifically, 30 gene sets were significantly associated with innate immune system pathways, 8 gene sets with the regulation of innate immune response, and 3 with antiviral innate immune response. By applying the multivariate LCT to assess gene set expression across these three immune phenotypes, eleven gene sets were consistently associated with the combined phenotypes, including KEGG pathways related to asthma, systemic lupus erythematosus, aminoacyl-tRNA biosynthesis, folate biosynthesis, and glycosaminoglycan metabolism (Table 1). These results indicate a potential immune system modulation in neonates due to maternal COVID-19 infection, which could have long-term implications.

Table 1: Multivariate LCT results for KEGG LEGACY gene sets.

Gene set	Gene set size	p-value
Asthma	25	0.011
ABC transporters	42	0.014
Folate biosynthesis	11	0.026
Valine, leucine, and isoleucine biosynthesis	10	0.026
Glycine, serine, and threonine metabolism	31	0.027
Aminoacyl tRNA biosynthesis	40	0.031
Glycosaminoglycan biosynthesis keratin sulfate	15	0.033
Lysine degradation	43	0.033
Glycerolipid metabolism	47	0.036
N glycan biosynthesis	46	0.037
Systemic lupus erythematous	123	0.039

Gene sets from the KEGG LEGACY catalog were analyzed using multivariate LCT combining three phenotypes: innate immune system, regulation of innate immune response, and antiviral innate immune response. Only gene sets with p-value < 0.05 are shown.

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Single-cell RNA-seq dataset:

Our findings revealed that genes involved in epigenetic mechanisms show differential expression in specific cell types of umbilical cord blood from COVID-19-infected mothers, indicating their susceptibility to epigenetic modulation. This susceptibility may contribute to the complex outcomes associated with alterations in epigenetic mechanisms like neurodevelopmental disease and metabolic syndrome, as discussed previously. Another intriguing finding was the presence of heterogeneity within cell types, such as B cells. Specifically distinct results were observed within a particular cluster of B cells, referred to as B cell cluster 2, compared to B cell cluster 1 and 3. This variability was also evident within other cell types, including CD14 monocytes, NK cells, and T cells (Table 2).

Table 1: LCT results using Reactome catalog and samples stratified by cell type

Cell type and clusters	p-value	q-value
B Cells 1	<0.001	<0.001
B Cells 2	0.31	0.41
B Cells 3	<0.001	<0.001
CD14 Monocytes 1	<0.001	<0.001
CD14 Monocytes 2	0.002	0.003
CD14 Monocytes 3	0.01	0.02
CD14 Monocytes 4	0.41	0.45
CD14 Monocytes 5	<0.001	<0.001
NK Cells 1	<0.001	<0.001
NK Cells 2	0.49	0.49
T Cells 1	0.36	0.43
T Cells 2	<0.001	<0.001
T Cells 3	<0.001	<0.001
HSPCs	0.005	0.009
CD16 Monocytes	0.02	0.04
Megakaryocytes	0.64	0.83
Conventional DCs	0.74	0.83
Plasmacytoid DCs	0.86	0.86

Next, to further assess which KEGG LEGACY pathways are differentially expressed considering epigenetic modulation, we performed pairwise comparisons based on "Reactome epigenetic regulation of gene expression" collection results only in cases. In detail, considering the KEGG collection, we performed LCT comparing B cell 2 (as control) with B cell 1 and 3 in cases, thus finding which KEGG gene sets are differentially expressed in B cell 1 and 3 (which had epigenetic signatures) compared to B cell 2 (which did not have any epigenetic alteration). We did a similar comparison for other cell types. Then, for each gene set in the KEGG collection, we obtained nine p-values from nine pairwise comparisons. To consolidate p-values into a single

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summary statistic, we used Fisher's sum of log *P* method to obtain combined p-values and created a jitter plot (Figure 1). The dots represent the 186 gene sets, Y[g] is an indicator of combined p-values, and X[g] is the number of comparisons with p-value < 0.01. We designated the color of dots as the comparison number (1 to 9) for which the LCT p-value is minimum. Gene sets depicted on the left side of the figure exhibit differential expression in specific cell clusters regarding pairwise comparison. Conversely, gene sets on the right side of the figure demonstrate differential expression across almost all comparisons. This allows for prioritization for further investigation and interpretation of the results into unique as well as biologically relevant gene sets while deprioritizing those with common patterns of differential expression (Figure 1 and Table 3).

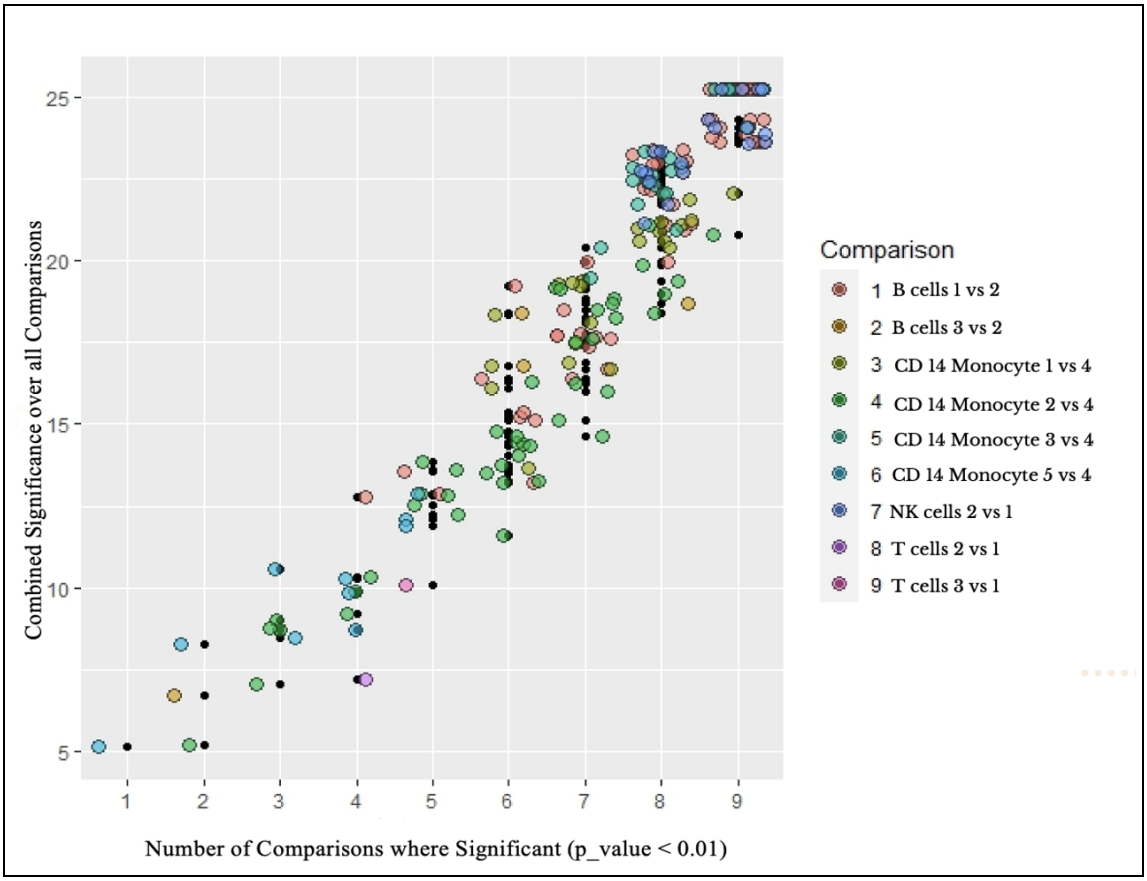

Figure 1: Scatterplot of KEGG gene set significance across cell type comparisons.

Each point represents one of 186 KEGG gene sets. The x-axis shows the number of significant pairwise cell type comparisons (p < 0.01) for each gene set. The y-axis shows the negative log of the combined p-value (jittered for visual clarity). Color indicates which cell type comparison yielded the smallest p-value for that gene set. Black dots represent gene sets without significant comparisons.

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Table 2: Differentially enriched KEGG LEGACY gene sets.

KEGG LEGACY gene set (g)	X_g	Y_g	Cell type	Clusters
Taurine and Hypotaurine Metabolism	1	5.13	CD14 monocyte	5 vs 4
Non-Homologous End Joining	2	6.74	B cell	3 vs 2
			T cell	3 vs 1
Glycosphingolipid Biosynthesis Globo Series	2	5.21	CD14 monocyte	2 vs 4
			CD14 monocyte	3 vs 4
			CD14 monocyte	5 vs 4
Sulfur Metabolism	2	8.301	T cell	3 vs 1
Aminoacyl tRNA Biosynthesis	3	8.99	T cell	2 vs 1
			CD14 monocyte	2 vs 4
			CD14 monocyte	5 vs 4
Glycosphingolipid Biosynthesis Ganglio Series	3	8.73	T cell	3 vs 1
			CD14 monocyte	2 vs 4
			CD14 monocyte	5 vs 4
Limonene and Pinene Degradation	3	8.78	B cell	1 vs 2
			CD14 monocyte	2 vs 4
			CD14 monocyte	5 vs 4
Pantothenate and CoA Biosynthesis	3	7.05	T cell	3 vs 1
			CD14 monocyte	2 vs 4
			CD14 monocyte	5 vs 4
Citrate Cycle TCA Cycle	3	10.58	T cell	2 vs 1
			T cell	3 vs 1
			CD14 monocyte	5 vs 4
Valine Leucine and Isoleucine Biosynthesis	3	8.46	T cell	2 vs 1
			T cell	3 vs 1
			CD14 monocyte	5 vs 4

For each gene set (g), Xg is the number of significant comparisons ($p < 0.01$), and Yg is the combined p-value across all comparisons. Comparisons were performed between clusters within each cell type.

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4. Discussion

Our findings suggest that maternal COVID-19 infection during pregnancy may contribute to an increased risk of metabolic syndrome in offspring later in life. We identified distinct epigenetic regulatory signatures in specific cord blood cell populations from neonates born to SARS-CoV-2–infected mothers compared with uninfected controls. Subsequent pathway analysis using KEGG annotations revealed perturbations in biological processes associated with oxidative DNA damage, as well as amino acid and lipid biosynthesis and metabolism—pathways previously linked to the development of metabolic and cardiovascular diseases (Bhattacharya et al., 2022; Dehghanbanadaki et al., 2023; Falk, 2006; Guan & Miao, 2020; Ji et al., 2023; Laderoute, 2020; Lai et al., 2018; Liu et al., 2016; Thomas et al., 2020; Verschuren et al., 2013). The differential regulation of these metabolic pathways suggests a potential mechanistic connection between maternal infection, epigenetic modulation, and the offspring's susceptibility to metabolic disease. We understand that our study has certain limitations. Although gene expression data provide valuable insights into transcriptional dynamics, they cannot fully encompass the complexity of epigenetic mechanisms that may contribute to the development of metabolic syndrome in neonates. While epigenetic regulation is responsive to environmental influences, transcriptomic profiling offers only a partial perspective on the broader regulatory landscape. Moreover, epigenetic modulation during pregnancy is shaped by numerous maternal factors—including nutrition, psychological and socioeconomic stress, environmental exposures, lifestyle, preexisting health conditions, maternal age, and gut microbiome composition—many of which are difficult to match or control across study groups (Barua & Junaid, 2015; Brown & Derkits, 2010; Heijmans et al., 2008; Roseboom et al., 2001; Salisbury et al., 2011; Suter et al., 2011) . In addition, as the placenta serves as the critical interface between mother and fetus, the absence of a strategy to exclude maternal-derived cells from umbilical cord blood mononuclear cells (CBMCs) may have introduced maternal transcriptional signals into the dataset, potentially influencing the observed gene expression profiles. Finally, several limitations inherent to the primary datasets should be acknowledged, including a relatively small sample size, lack of representation across the full spectrum of maternal SARS-CoV-2 infection severity, variability in the infection-to-delivery interval, and the presence of maternal comorbidities such as thyroid dysfunction, obesity, and gestational diabetes.

Yet, our study also has a number of strengths including its analytical framework. In contrast to the primary analysis, which focused predominantly on individual gene-level differences, we applied a gene set–level analysis approach. This strategy captures the coordinated activity of genes within molecular pathways, offering greater biological interpretability, thus enhancing statistical robustness of our findings. Through our multivariate gene set-level approach, we identified significant dysregulation within KEGG-defined metabolic pathways that may more accurately represent the complex biological responses to maternal SARS-CoV-2 infection. Notably, it also revealed heterogeneity within specific immune cell populations that was not apparent in the original datasets. For example, among B cell clusters identified in prior analyses, we observed distinct epigenetic pathway signatures in one cluster compared with the others, suggesting functional diversity among phenotypically similar cell types. This cell-type

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ISSN: 2581-3366

heterogeneity may reflect variable immune and metabolic adaptations to maternal infection, with implications for immune maturation, metabolic programming, and long-term disease susceptibility in offspring. By integrating gene set–level and cell-type–specific analyses, our study offers a more nuanced understanding of the molecular consequences of maternal COVID-19 exposure and highlights potential pathway-level targets for preventive and therapeutic strategies. Collectively, these findings emphasize the importance of continued investigation into the long-term effects of maternal COVID-19 infection on offspring health—an area at risk of being overlooked as global focus shifts beyond the pandemic.

Previous studies examining the impact of maternal SARS-CoV-2 infection on fetal and neonatal outcomes have largely focused on vertical transmission, placental pathology, IgG/IgM antibody detection, immune dysregulation, and short-term clinical outcomes (Barcelos et al., 2021; C. Chambers et al., 2020; H. Chen et al., 2020; Dashraath et al., 2020; Dong et al., 2020; Gee et al., 2021; Groß et al., 2020; Jeganathan & Paul, 2022; Levy et al., 2008; Li et al., 2020b; Mirbeyk et al., 2021; Musa et al., 2021, 2021; Prochaska et al., 2020; Wu et al., 2020; Zeng et al., 2020). Our findings are broadly consistent with these reports demonstrating alterations in immune and gene expression profiles in neonates born to infected mothers (Gee et al., 2021; Kleeman et al., 2022; Zeng et al., 2020). However, they extend current understanding by emphasizing the involvement of epigenetic regulatory pathways that may mediate the relationship between maternal infection and long-term metabolic risk in offspring. Consistent with the observations of Hill et al. (2023), we also identified putative dysregulation of epigenetic pathways; yet, through our two-step gene set–level analysis, we further uncovered differential expression within specific KEGG pathways, suggesting potential long-term metabolic consequences of maternal COVID-19 infection in newborns. In contrast, Kocher et al. (2023) found no significant epigenetic differences associated with maternal infection during pregnancy and proposed that observed neonatal epigenomic variations were more likely driven by the broader environmental and societal conditions of the COVID-19 pandemic rather than by direct maternal infection effects.
Future research should integrate multi-omics technologies with large-scale population datasets and longitudinal cohort studies to comprehensively elucidate how maternal COVID-19 infection influences the long-term health of offspring. Longitudinal designs are particularly valuable, as they enable continuous monitoring of child health outcomes and allow adjustment for key confounding factors such as genetics, socioeconomic status, and lifestyle. Complementary experimental validation through both in vitro and in vivo models will further strengthen causal inference. Studies using animal models of maternal SARS-CoV-2 infection can provide mechanistic insights into future cardiovascular and metabolic consequences in such offspring by combining molecular, epigenetic, and physiological assessments. Moreover, applying pharmacological and genetic interventions in these models could help delineate the contribution of specific pathways to disease development and identify potential therapeutic or preventive targets.

In conclusion, taking into account the strengths and limitations of our study, alongside the existing literature, further research is warranted to clarify the role of epigenetic regulation in

determining long-term metabolic outcomes in offspring of mothers affected by COVID-19 during pregnancy.

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