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Placental Transcriptomic Signatures of Spontaneous Preterm Birth

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Abstract

Background: Spontaneous preterm birth accounts for the majority of preterm births and results in significant morbidity in the newborn and childhood period. This subtype of preterm birth represents an increasing proportion of all preterm births compared to medically indicated preterm birth, yet is understudied in 'Omics analyses. The placenta is a key regulator of fetal and newborn health, and the placental transcriptome can provide insight into pathologic changes leading to spontaneous preterm birth.

Objective: This analysis aimed to identify genes whose placental expression was associated with spontaneous preterm birth (including early preterm and late preterm birth).

Study Design: The ECHO PATHWAYS Consortium extracted RNA from placental samples collected from the Conditions Affecting Neurocognitive Development and Learning in Early childhood (CANDLE) and Global Alliance to Prevent Prematurity and Stillbirth (GAPPS) studies. Placental transcriptomic data were obtained by RNA sequencing. Linear models were fit to estimate differences in placental gene expression between term birth and spontaneous preterm birth (including gestational length subgroups defined by the American College of Obstetricians

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and Gynecologists). Models were adjusted for numerous confounding variables, including labor status, cohort and RNA sequencing batch. This analysis excluded patients with induced labor, chorioamnionitis, multi-fetal gestation, or medical indications for preterm birth. Our combined cohort contained gene expression data for 14,023 genes in 48 preterm and 540 term samples. Genes and pathways were considered statistically significantly different at FDR adjusted P<0.05.

Results: In total we identified 1,728 genes whose placental expression was associated with sPTB with more differences in expression in early preterm samples than late preterm samples compared to full term samples. 9 genes were significantly decreased in both early and late spontaneous preterm birth, and strongest associations involved placental expression of *IL1B*, *ALPL* and *CRLF1*. In early and late preterm samples, we observed decreased expression of genes involved in immune signaling, signal transduction, and endocrine function.

Conclusions: This study provides a comprehensive assessment of differences in the placental transcriptome associated with spontaneous preterm birth to date with robust adjustment for confounding. Results of this study are in alignment with known etiology of spontaneous preterm birth, as we identified multiple genes and pathways whose placental and chorioamniotic membrane expression was previously associated with prematurity, including *IL1B*. We identified decreased expression in key signaling pathways which are essential to placental growth and function, which may be related to the etiology of sPTB. We identified increased expression of genes within metabolic pathways associated exclusively with early preterm birth. These signaling and metabolic pathways may provide clinically targetable pathways and biomarkers. The findings presented here can be used to understand underlying pathological changes in premature placentas, which can inform and improve clinical obstetrics practice.

Keywords

Transcriptomics; Placenta; Spontaneous Preterm Birth; *IL1B*; *GABRP*; *ALPL*; chemokine signaling; placental metabolism; signal transduction

INTRODUCTION

Preterm birth is the leading cause of newborn death worldwide, is responsible for the majority of newborn morbidity, and represents a significant social and financial burden to caregivers and the healthcare system.[8] The majority (60%) of preterm births are spontaneous, arising from either premature membrane rupture (25%) or spontaneous preterm labor (35%).[9] The remaining 40% of preterm births occur due to multi-fetal pregnancies, fetal malformations, and medical indications such as preeclampsia or chorioamnionitis.[9] Characterizing the etiology of spontaneous preterm birth (sPTB) is paramount as neonatal and later life outcomes differ based on subtle shifts in delivery timing. Late preterm infants (born at 34–37 weeks) and early-term infants (born at 37–39 weeks) are at increased risk of adverse childhood health outcomes compared to full-term infants.[10–12] Consequently, the American College of Obstetricians and Gynecologists (ACOG) recommended more precise definitions of gestational length for research and clinical studies in order to best predict and prevent adverse outcomes.[1] Additionally, sPTBs comprise an increasing proportion of all preterm births, while the rate of medically indicated preterm birth is decreasing.[13,14]

The placenta is an essential regulator of the in-utero environment. It transports gas, nutrients, and waste, provides immunological surveillance for the developing fetus,[15] and produces neuropeptides, growth factors, and steroid hormones that are released into the maternal circulation to coordinate maternal adaptation to pregnancy.[16] The placenta is also involved in labor initiation via increased production of corticotropin-releasing hormone.[17] Perturbations in placental development across pregnancy may alter placental function and ultimately labor initiation. It has been long established that alterations in placental function (placental vasculopathy) is indicative of preterm labor independently of infection or premature membrane rupture.[18] Newer evidence suggests that most causes of spontaneous preterm labor are caused by placental insufficiency, a disorder characterized by abnormal nutrient transport, placental damage, and inappropriate development and vascularization.[19] Increased incidence of perturbed villous maturation (accelerated or delayed), vascular malperfusion, and chronic inflammation have been linked to spontaneous preterm labor.[20] Thus, identification of molecular differences in the placenta can indicate functional abnormalities and provide novel insight into mechanisms underlying spontaneous PTB.[21]

Transcriptomic data reflects gene expression and can reveal underlying functional differences at a molecular level. The majority of placental ‘omics research has focused primarily on medically indicated preterm birth, and few studies have investigated molecular changes relating to sPTB.[22] Previously, we identified a transcriptomic signature associated with PTB using aggregated microarray data.[23] Other RNA sequencing-based analyses using case-control study designs to investigate different subgroups of spontaneous preterm birth (including idiopathic spontaneous preterm labor, preterm premature membrane rupture, and chorioamnionitis/intra-ammonitic infection) have revealed differences in the transcriptome of key tissues including the placenta (villous tree)[5,24–26], chorioamniotic membranes[27,28], decidua basalis[26], and maternal blood[29,30] Single-cell sequencing based analyses have been used to characterize cell type specific changes that occur across the course of gestation,[31] as well as changes in the villous tree, decidual cells, and chorioamniotic membrane associated with preterm labor.[32]

The goal of our study was to identify genes whose placental expression is associated with sPTB and different gestational length subgroups (early preterm, late preterm, early term, and late term versus full term) in a retrospective cohort analysis with detailed covariate data allowing enhanced control for confounding variables. This is the largest such analysis of this type to date and is the first retrospective cohort analysis,[33] and our combined cohort is representative of a diverse group of participants from different parts of the country and includes under-represented populations. Importantly, our selection criteria were designed using established clinical guidelines to capture an important subgroup, “spontaneously premature” births, by excluding PTB due to medical conditions (e.g., induced labor, chorioamnionitis, preeclampsia and multi-fetal pregnancy).[11] This specific subtype of PTB is not as well studied and comprises the majority of preterm birth and is therefore an important target for clinical intervention if modifiable predictors are revealed, particularly via ‘omics analyses.[22] We hypothesized that the placental transcriptome would reveal underlying perturbations in placental function related to sPTB.

MATERIALS AND METHODS

1.1 Study Population

This is a retrospective cohort study to analyze the relationship between the placental transcriptome and sPTB. The ECHO PATHWAYS Consortium harmonized data from two pre-existing cohorts, the Conditions Affecting Neurocognitive Development and Learning in Early childhood (CANDLE), which enrolled 1,504 women from Shelby County, Tennessee and Global Alliance to Prevent Prematurity and Stillbirth (GAPPS) studies, which enrolled over 600 pregnant women and children from Seattle, WA and Yakima, WA. Each cohort had its own inclusion criteria. Inclusion criteria for the CANDLE study included individuals between 16–40 years of age with uncomplicated singleton pregnancies who planned to deliver at a participating study hospital. Inclusion criteria for the GAPPS study included: participant age of 18 years of age or older or medically emancipated and confirmed to be pregnant by self-test or by physician's medical testing. The ECHO PATHWAYS consortium sequenced a subset of placentas from each cohort (N= 289 GAPPS, N= 794 CANDLE). Our study specific exclusion criteria excluded patients with multi-fetal pregnancies, preeclampsia, placental abruption, chorioamnionitis (based on individual cohort collection information, see Section 1.2), recorded cervical insufficiency, and induced labor (Figure 1A). Data analyzed here represents a subset of each population with complete RNA sequencing data, complete covariate data and without the study specific exclusion criteria. (Figure 1A) All ECHO PATHWAYS research activities pertaining to these samples were approved by the University of Washington (UW) Institutional Review Board (IRB). All research activities for the CANDLE cohort were approved by the University of Tennessee Health Sciences Center IRB. The Seattle Children's Research Institute IRB approved all research activities for the GAPPS cohort.

1.2 Clinical Definitions

Preterm birth including gestational length subgroups were defined using the most recent ACOG guidelines (preterm birth<37 weeks, early preterm birth<34 weeks, late preterm birth 34–37 weeks, early term 37–39 weeks, full term 39–41 weeks, and late term >41 weeks).[1] In CANDLE, gestational length was calculated from medical record abstraction and self-report. 57.3% of CANDLE participants reported ultrasound dating as the primary method to calculate gestational length.[34] In GAPPS, gestational length was derived from clinical entered dates, which capture both the date of the last menstrual period, and first or second trimester ultrasound, in alignment with ACOG criteria.[35] Clinical information about the exclusion criteria was also gathered and defined by the retrospective cohorts based on medical record abstraction. In CANDLE, we excluded individuals with preeclampsia based on indication in the medical records of delivery indication for preeclampsia or hypertension, and chorioamnionitis based on medical record abstraction of confirmed clinical chorioamnionitis. No data on cervical insufficiency was available for CANDLE participants. In the GAPPS cohort, we also excluded individuals with preeclampsia, cervical insufficiency and chorioamnionitis based on medical record abstraction data. Premature PROM was defined as the rupture of membranes before labor onset in infants born before 37 weeks based on medical record abstraction. For both cohorts, phenotype data was

harmonized by applying a uniform data structure and consistent coding rules for phenotype variables.

1.3 Placental Collection

For placentas collected from the CANDLE study, one rectangular piece of placental villous tissue (approximate dimensions of 2 cm x 0.5 cm x 0.5 cm) was dissected from the middle of the parenchyma within 15 minutes of delivery as previously described.[36] The tissue cubes were placed in a 50 ml tube with 20 ml RNAlater and refrigerated at 4°C overnight, before being stored in RNAlater at −80°C. This tissue was manually dissected and cleared of maternal decidua. For placentas collected from the GAPPS biorepository, four 8-mm vertical tissue punches from the placental disc were collected from 2 sites separated by 7 cm on the placental disc, and placed in 5-ml tubes containing approximately 3 ml of RNAlater within 30 minutes of delivery and stored at −20 °C, as previously described.[37] Specimens then were shipped to the GAPPS facility and stored at −80 °C. The fetal villous tissue was manually dissected and cleared of maternal decidua using standard protocols developed by the GAPPS placental biorepository.

1.4 RNA Sequencing Processing and Analysis

RNA isolation and RNA sequencing quantification methods have been previously described for the CANDLE placental samples,[36] and the same protocols were used for GAPPS. Briefly, approximately 30 mg of fetal villous placental tissue was homogenized in using a TissueLyser LT instrument (Qiagen, Germantown, MD). RNA was isolated using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen). RNA integrity was determined with a Bioanalyzer 2100 using RNA 6000 Nanochips (Agilent, Santa Clara, CA), and only RNA samples with an RNA Integrity Number >7 were sequenced. RNA sequencing was performed at the University of Washington Northwest Genomics Center. Total RNA was Poly-A enriched, and cDNA libraries were prepared using the TruSeq Stranded mRNA kit (Illumina, San Diego, CA). Each library was sequenced to an approximate depth of 30 million reads on an Illumina HiSeq 4000 instrument. RNA sequencing quality control was performed using both the FASTX-tool (v0.0.13) and FastQC (v0.11.2) toolkits.[38] Transcript abundances were estimated by aligning to the GRCh38 transcriptome (Gencode v33) using Kallisto,[2] then collapsed to the gene level using the Bioconductor tximport package, scaling to the average transcript length.[39] Only protein-coding genes and long noncoding RNAs were included in this analysis. The transcriptomic results of the two cohorts were pooled together and then filtered to remove those genes that were unexpressed or expressed at an unreliably low level, defined as a mean log counts/million (logCPM) < 0. After filtering, 14,023 genes remained.

1.5 Identification of Differentially Expressed Genes (DEGs)

DEGs were identified by fitting a weighted linear regression using the “limma-voom” pipeline from the Bioconductor edgeR package (version 3.34.0), which converts gene counts into log counts/million counts (logCPM) and then uses observation-level weights to account for the mean variance relationship of the logCPM values. Comparisons between groups were made using empirical-Bayes adjusted t-statistics implemented in the Bioconductor limma package.[40] We selected potential confounders to be included in our analysis *a priori* through

a review of the literature surrounding preterm birth and gestational length, then constructed a directed acyclic diagram (Figure 1B). In all models, we adjusted for RNA sequencing batch, fetal sex, cohort, study site, labor status, premature PROM status, and maternal age, BMI, self-reported smoking status, self-reported alcohol consumption, income, education, race, and ethnicity (Figure 1B). We identified unmeasured confounding using surrogate variable analysis (SVA), and adjusted for these artifacts in our model.[7] For all analyses, genes were considered statistically significant with a false discovery rate (FDR) <0.05 using the Benjamini-Hochberg method.[41] Lists of DEGs were compared using the "UpSetR" R package.[42]

1.6 Pathway Enrichment Analysis

We identified differences in gene expression within KEGG (Kyoto Encyclopedia of Genes and Genomes) biological pathways[43] using the roast function in the Bioconductor limma package,[6] which compares the average t-statistic for each gene set to a null distribution estimated from random rotations of the residuals from the underlying model (10,000 rotations). We present results from the directional test, which first infers the direction of change for the gene set based on the preponderance of up or down-regulated genes, and then computes the average t-statistic based on the subset of genes in the set with the inferred direction of change. We included all KEGG Pathways[43] except disease pathways (KEGG release 98.1). Pathways were considered statistically significant at an FDR <0.05.

RESULTS

2.1 Clinical Characteristics

We identified differences in expression from placentas collected from 461 individuals from the CANDLE study and 127 individuals from the GAPPS cohort enrolled in the ECHO PATHWAYS Consortium study with complete RNA sequencing and covariate data (Table 1). Our combined cohort was 42.7% Black, and 44.7% of participants had a high school education or lower. This combined study included 48 preterm infants, 38.7% of which presented with preterm premature membrane rupture (N=24, based on medical record abstraction). 91.7 % of participants who delivered prematurely underwent labor. Our study also included 540 participants who were delivered at term, and 74.3% of these participants underwent labor. Our exclusion criteria eliminated all preterm and term infants with medically indicated causes of delivery based on medical record abstraction, including preeclampsia, multi-fetal gestation, as well as infants with chorioamnionitis or cervical insufficiency, and we adjusted for labor status and PPROM as confounders (Figure 1), leaving us with a transcriptional signature attributable to spontaneous preterm birth. Gestational length did not differ between CANDLE (Mean 38.89 weeks) and GAPPS (Mean 38.92 weeks) cohorts (p=0.9, T Test).

2.2 Global Assessment of Placenta Transcriptomic Signatures of Prematurity

We analyzed differences in placental gene expression first using a binary grouping of <37 weeks (preterm, N=48) versus ≥37 weeks (term, N=540). We observed broad differences in preterm transcriptomes, with 1,062 genes exhibiting statistically significant differences in expression (i.e Differentially Expressed Genes or DEGs). (Figure 2). We performed a

separate analysis of each gestational length subgroup as defined by ACOG guidelines (early preterm, late preterm, early term, full term, and late term),[1] with the reference group defined as full term infants (39–41 weeks). In this analysis, we observed more DEGs in early preterm infants compared to full term (1,359 DEGs), than late preterm infants compared to full term (27 DEGs) (Figure 2). There were no statistically significant differences in placental expression of early term or late term infants compared to full term. Within the group of preterm infants alone (N=48) we performed stratified analyses of transcriptomic differences associated with labor (N=44) vs. No labor (N=4), as well as PPROM (N=24) vs. No PPROM (N=38); but no genes were statistically significant for either analysis. (FDR adjusted <0.05) We also performed an analysis of fetal sex as a potential modifier of the relationship between placental gene expression and sPTB, but found no statistically significant instances of an interaction (FDR adjusted <0.05). Across all analyses, there were more DEGs with decreased expression than increased expression in preterm infants compared to term infants. (Figure 2, Figure 3A).

2.3 Assessment of Confounding by gestational age using Placental Maturation Signatures

As the placental tissues are not collected from the same timepoint in our preterm and term infants, our analysis is intrinsically confounded by developmental changes that occur related to gestational length, but it is not logistically feasible or ethical to collect placental samples from non-pathological placentas at the same gestational timepoint to act as a control group. To investigate how the gene expression changes we identified in association with prematurity might be attributable to normal developmental processes (i.e. placental maturation) related to gestational length, we created a curated list of 1,420 placental maturation signatures collected from maternal blood (N=522 genes)[3] cell free RNA across pregnancy (N=9 genes),[4] as well as genes whose placental protein expression was significantly different between 2nd trimester and term gestations[5] (N=954 genes) (Supplemental Figure 1A, Supplemental Table 1). A similar approach was used by Lien et al to disentangle gestational age related differences in their transcriptomic study of spontaneous preterm birth.[5] Overall, 101 of the DEGs associated with sPTB (vs. term birth), 115 of the DEGs associated with early preterm birth (vs. full term birth), and 5 of the DEGs associated with late preterm birth (vs. full term birth) were genes attributable to placental maturity based on this list (Supplemental Figure 1B). The results that were statistically significant but may be confounded by gestational length based on these maturation signatures are presented in Supplemental Table 2 and removed from all downstream analyses. The remaining genes that were statistically significant and used for all subsequent analyses are presented in Supplemental Table 3.

2.4 Shared and Distinct changes to the Placental Transcriptome between Prematurity Subgroups

We present shared and distinct DEGs between each preterm birth analysis subgroup (Figure 3A, B). 2 genes were significantly increased in all three analyses (*GABRP*, *KIF26B*), and 9 genes were significantly decreased across all three (with *1L1B* expression overall the most decreased, see Supplemental Figure S2). 468 DEGs were significantly associated with early preterm (compared to full term) and all preterm infants (vs. term). 9 DEGs were

significantly associated with late preterm (compared to full term) and all preterm infants (vs. term). Amongst the top five genes from each subgroup (Figure 3B), we observed increased expression of multiple chemokines (*CXCL8*, *IL1B*); and decreased expression of other immune signaling genes (*CRLF1, SAA1, PTX3*).

2.5 Comparative Analysis of Prematurity Signatures from Single Cell Sequencing Analysis

Complementary work by Pique Regi et al used single-cell RNA sequencing data collected from women undergoing term and preterm labor (N=3/group) to characterize transcriptomic differences within individual cell types related to prematurity.[32] 34 of these signatures overlapped with our transcriptomic signatures of early preterm vs. full term infants or preterm vs. term infants (Supplemental Figure S3). These signatures were predominantly related to changes identified in extravillous trophoblasts, cytotrophoblasts, and decidual cells.

2.6 Comparative Analysis of Prematurity Signatures derived from Chorioamniotic Membranes

As we did not generate transcriptome data on chorioamniotic membranes in this study, we compared the changes in expression we noted in the placenta with a previously published case-control study that identified 270 DEGs whose expression in chorioamniotic membranes was associated with prematurity using similar exclusion and inclusion criteria and study design as our own, including adjustment for PPROM as a confounder.[28] 62 of those 270 DEGs were also associated with preterm birth in our analysis. (Supplemental Figure S4). Two genes (*ALPL* and *IL1B*) were significantly decreased in the placenta across all three of our analyses, but were increased in the chorioamniotic membranes in relation to preterm birth. Pathway enrichment of these DEGs revealed that these 62 overlapping DEGs were significantly over-represented in 11 KEGG pathways (FDR adjusted $P < 0.05$). The top pathways based on p value included chemokine signaling, cytokine-cytokine receptor interactions, and osteoclast differentiation.

2.7 Identification of shared and distinct biological pathways

Pathway analysis was performed using self-contained gene-set testing. In the comparison of sPTB vs. term, we identified 133 down-regulated KEGG pathways and 16 up-regulated pathways. 120 pathways were down-regulated and 34 pathways were up-regulated in early preterm infants compared to full term infants. 72 pathways were down-regulated in late preterm infants compared to full term infants. Full results of the pathway enrichment analysis are presented in Supplemental Table 4. 65 pathways (36.3% of all significant pathways) contained genes with decreased expression from all three analyses (Figure 4A). These pathways were members of KEGG subgroups involving the immune system, signal transduction, signaling molecules, organismal systems, metabolism, and cellular processes (Figure 4B). The immune signaling keg subgroup was the largest (17 pathways), which contained the chemokine signaling pathway, which was the most significant pathway overall. The 2nd largest subgroup was signal transduction (14 pathways), and the most significant pathway here was Phospholipase D signaling pathway. The third largest subgroup

was endocrine system; a component of organismal systems (11 pathways), and the most significant pathway in this group was parathyroid hormone synthesis, secretion, and action.

No pathways were increased across all three preterm birth subgroups, but we noted several pathways with a higher proportion of genes that were increased in only late preterm infants (3 pathways, top pathway basal transcription factors or in early preterm infants (21 pathways, top pathway splicesome). Notably, two thirds (14/21) of pathways unique for early preterm infants involved metabolism, including carbohydrates (butanoate and glyoxylate), amino acids (alanine, aspartate glutamine, tryptophan selenocompounds), lipids, and co-factor and vitamin metabolism (porphyrin, vitamin B6, retinol and ubiquinone).

COMMENT

A. Principal Findings:

This is the largest and most robust assessment of differences in the placental transcriptome attributable to sPTB to date, which incorporates more nuanced measures, including gestational length subgroups. Our main findings were: (1) characterization of 1,728 genes associated with sPTB in our complete analysis, including shared and distinct genes associated with early and late sPTB, (2) increased expression of genes involved in KEGG immune, endocrine, and signal transduction pathways in both early and late preterm placentas compared to full term placentas, and (3) an overall trend of increased strength and significance for genes and pathways whose placental expression was associated with early preterm infants (vs. full term) compared to late preterm infants (vs. full term), including increased expression of genes involved in metabolic functions only in early preterm infants. Taken together, these findings yield novel insight into molecular perturbations related to sPTB.

B. Results in Context:

The findings from our study are in alignment with the known etiology of sPTB and prior transcriptomic analyses but expand on these by studying the placenta, which is a core regulator of the in-utero environment, and by using well phenotyped samples and adjusting for confounding variables. We observed a higher number and more significant differences in placental gene expression associated with early PTB (vs. full term) compared to late sPTB (vs. full term), suggesting broader differences in placental function in early sPTB. Other studies have identified unique molecular signatures in early preterm infants (both spontaneous and medically indicated).[44,45] A study involving biomarkers collected during first trimester screening identified a unique subset of biomarkers associated with early PTB.[44] Another multi-omics assessment of maternal-whole blood identified unique differences in the methylome and transcriptome associated with very early preterm labor (<28 weeks), which were not significantly associated with preterm vs. term labor or other preterm phenotypes.[45] We noted that 15 of the DEGs in this study were also significantly associated with differences in expression in monocytes and whole blood in relation spontaneous preterm labor in our previous study,[29] including the top DEG from the previous study, *ADAMTS2*, as well as several interleukin signaling genes which were demonstrated to be regulated by microRNAs in the context of preterm labor in a follow up

study.[46]Our study adds to this by providing the first comprehensive summary of genes and pathway reliably associated with early PTB within the placenta instead of maternal blood.

Placental expression of 30 of the DEGs associated with prematurity in our study were also associated with either iatrogenic and/or infection related preterm birth in Brockway et al, including genes related to angiogenesis (*VEGFA*,*FOXO1*), immune signaling (*IL1R2*), glucocorticoid signaling (*FKBP5*, *HSD11B1*), as well as *PTGS2*, which is essential for in labor initiation. Metabolic pathways exclusively associated with early sPTB in our analysis (including the TCA cycle, Vitamin B, fatty acids, and tryptophan metabolism) were also associated with sPTB in an integrated metabolomic and transcriptomic assessment.[5]

Beyond the placenta, the chorioamniotic membranes also play an important role in labor initiation in the presence/absence of infection, and changes to the chorionic membrane transcriptome have been identified in relation to prematurity.[28,47] Placental expression of 62 DEGs associated with prematurity in this study were also associated with spontaneous preterm birth in the chorioamniotic membranes,[28] with many of these shared DEGs involved in chemokine and cytokine signaling. There is a strong body of evidence, reviewed by Gomez-Lopez et al, that supports the role of signaling of key interleukin signaling in these membranes leading to premature labor through cervical ripening and rupture of the fetal membranes.[48] Our study demonstrates that some changes in gene expression can also be extended into the placenta. Future studies, particularly those studying phenotypes such as PPROM and chorioamnionitis, should consider matched collection of the chorioamniotic membranes and placental tissues, which is beyond the scope of this retrospective analysis.

C. Clinical Implications:

The topmost DEGs in all three analyses provide high confidence results that may be useful for future biomarker discovery, particularly for infant comorbidities related to sPTB. These signatures could also potentially serve as candidates for predictive markers of sPTB, which would need to be tested in maternal blood across pregnancy. Such analyses are beyond the scope of this study due to limitations in sample collection, but have successfully been used in several recent cell free RNA analyses.[49,50] Two of our most significant decreased genes- *IL1B* and *ALPL*- have already been established as biomarkers of sPTB. *ALPL* encodes the alkaline phosphatase gene, and maternal plasma levels of alkaline phosphatase have reliably demonstrated utility predictive markers of preterm birth in asymptomatic women.[51,52] While these studies demonstrated a positive relationship, it is important to note that ALPL is not the primary alkaline phosphatase produced by the placenta (*PALP*),[53] so our findings could reveal more complex regulatory mechanisms.

Interleukin 1 Beta (*IL1B*) is a cytokine produced from different reproductive cells (including trophoblasts)[54] in response to pro-inflammatory cytokines.[55] Within the placenta, IL1B promotes trophoblast motility in the first trimester extra villous trophoblasts,[56] as well as regulating cytotrophoblast metalloproteinase activity and invasion in vitro, by stimulating metalloproteinases such as MMP9.[57] IL-1B mediates the onset of premature labor in the context of infection,[58] and increased levels of IL-1B in the cervical, myometrium, and fetal membranes in the presence/absence of infection are associated with preterm birth,[55] and increased levels of IL-1B were identified in the decidual and placenta in relation to term

labor. [59] IL-1B1 has been proposed as a potential therapeutic target for preterm birth, but pre-clinical studies have shown reduced efficacy of IL-1B antagonists to prevent infection induced preterm birth, as reviewed by Nadeau-Vallee et al.[55] Our study demonstrates decreased expression of IL1B in the placenta in preterm infants compared to term infants, which is contrary to findings in other prenatal tissues. This decreased expression could reflect compensatory mechanisms or alternative signaling mechanisms occurring within the placenta. More work is needed to confirm these findings in an independent cohort and to understand *IL1B1* expression across multiple pregnancy tissues during preterm labor.

Our pathway analysis revealed decreased placental expression of genes within a variety of signal transduction, endocrine and immune pathways, which are clinically relevant because these pathways may provide modifiable targets for pharmacologic interventions. For example, many of the signal transduction pathways we observed decreased expression in are well studied in the context of cancer with well documented pharmaceutical treatments, including the MAP-kinase,[60], Ras1,[61] and JAK-Stat pathways.[62] These pathways are essential for placental functions, as discussed below, so future studies could explore these pathways as targets for clinical intervention.

We also observed increased placental expression of genes in of multiple metabolic pathways uniquely in sPTB placentas, which may provide clinically modifiable targets through dietary intervention. These included pathways involving metabolism of retinol, vitamin B6, pyruvate, and tryptophan. Notably, we observed increased expression of genes within the selenocompound metabolism pathway, which is responsible for converting selenocysteine to methylselenomethionine,[63] where it is involved in core biological processes that the placenta regulates, such as inflammatory processes and redox regulation.[64] Other studies have identified selenium deficiency as a modifiable risk factor for premature birth,[65,66] particularly in extremely preterm infants.[64] Genetic polymorphisms in the loci encoding selenocysteine tRNA-specific eukaryotic elongation factor, (participating in the incorporation of selenocysteine into selenoproteins), have been implicated in both gestational length and in spontaneous preterm birth.[67] The identification of the selenocysteine pathway suggests a role of maternal selenium micronutrient supplementation, which supports recently launched clinical trials in this space.[68,69] This study suggests that metabolism of these key micronutrients may be perturbed in preterm placentas based on differences in gene expression and contributes to a body of evidence that they may be modifiable risk factors for prevention of prematurity. Future studies will be needed to validate these findings in an independent cohort.

D. Research Implications:

Several of the signaling pathways altered in preterm placentas may be related to altered placental growth and invasion. We observed decreased expression of genes within the MAPK or JAK-STAT signaling, which are involved in the invasion and syncytialization of placental trophoblast cells,[70] We also observed decreased expression of VEGFA and HIF-1 signaling, which is essential for placental angiogenesis.[71,72] We also noted decreased expression of genes in endocrine pathways (including relaxin, estrogen, oxytocin and cortisol pathways) which are produced by the placenta that are involved in key endocrine

processes important to pregnancy.[16] Our research suggests that decreased expression of genes within these pathways may result in perturbed molecular mechanisms, and altered placental function and maternal-fetal signaling, ultimately culminating in spontaneous preterm birth. Both our gene and pathway level findings highlight the importance of immune signaling in the context of sPTB. We noted 17 different immune pathways with decreased expression in preterm vs term samples, as well as a number of individual genes, including decreased expression of *IL1B*, *CRLF1*, *CXCL8*, and *PTX3*. While there are numerous studies on the role of the fetal membranes and maternal blood in the context of premature birth, this study highlights the role of immune signaling within the placenta itself.

Our global findings also generate new hypothesis and both the gene and pathway level. For example, the top increased DEG across all analyses was *GABRP*, which encodes the inhibitory neurotransmitter Gaba A receptor π. This form of the GABA neurotransmitter has been found in a variety of reproductive tissues including the placenta, where it alters the sensitivity of GABA receptors to pregnanolone.[73] In extravillous trophoblast cells, GABRP promotes apoptosis, which ultimately resulted in decreased invasion.[74] Placental expression of GABRP was also positively associated with preeclampsia.[74] Our work suggests that placental expression of GABRP may be altered in the context of prematurity, and but more experimental work is needed to elucidate the biological basis behind changes in expression of these genes.

E. Strengths and Limitations:

Our results should be interpreted in light of inherent limitations in RNA sequencing analysis. We capture the placental transcriptome at birth, which is a snapshot of a highly regulated temporal process. We cannot collect samples matched for gestational age in preterm and term samples, so the differences we observe in preterm placentas may be reflective of gestational age-related differences instead of pathological changes related to prematurity. We address this in part using placental maturation signatures. Expression was quantified using bulk RNA sequencing data, so our findings may be confounded by different cell types collected within each sample and discrepancies in placental sampling protocols, which is a well-established challenge in this field,[75] although we address this in part by adjusting for cellular heterogeneity using surrogate variable analysis. This study did not deploy a spatial transcriptomics approach to investigate multiple sites within the same placenta, which is not feasible in such a large retrospective cohort study but is an important factor, as normal variation in the placental tissue has been noted.[76] We do not account for all risk factors and phenotypic subgroups of premature infants,[77] such as extra-uterine infections, cervical shortening or insufficiency, or maternal trauma, due to the low prevalence and inherent challenges in measuring these factors, although we accounted for as many as feasible in our study design. Our analysis did not include pregnancies from other pathologies (e.g., preeclampsia, gestational diabetes, etc.) as our sample collection strategy was not designed specifically for these complications. As this was a retrospective cohort study, we were unable to attain any histopathological data on these placental specimens, which limits our ability to understand molecular differences. Future analyses should consider integration of histopathology data with transcriptomic data, which was successfully used to provide molecular endotypes of preeclampsia[78] and fetal growth restriction[79] in other studies. Future

studies may also consider other subtypes of sPTB, due to factors such as chorioamnionitis, PPROM, or preeclampsia, which we were unable to fully profile with the clinical data available. Integration of other omics data including proteomics data from matched samples may also generate a more robust molecular signature.

Our findings stand apart as the largest transcriptome-wide assessment of sPTB-related differences in the placenta,[33] quantified using RNA sequencing. We collected detailed covariate data, which was harmonized across both cohorts, allowing us to exclude preterm births with other pathologies and adjust for potential confounding variables which were not addressed in our previous analyses of preterm birth, including key variables such as labor status.[23] Additionally, our study is more rigorous as we performed a stratified analysis utilizing gestational length subgroups as defined by ACOG. This is clinically significant as the majority of premature births are late preterm,[80] necessitating better understanding of molecular and pathological differences between these subgroups.

F. Conclusion:

Overall, this work supports the role of placental ‘omics analyses in understanding the relationship between placental physiology and premature birth. The differences in gene expression presented here can be used to understand underlying pathological changes in premature placentas, which can inform and improve clinical obstetrics practice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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GLOSSARY OF TERMS

American College of Obstetricians and Gynecologists (ACOG)
A professional organization of physicians specializing in gynecology and obstetrics

Benjamini-Hochberg *P* value
The Benjamini-Hochberg approach is applied to a list of *P* values to reduce the false discovery rate. The Benjamini-Hochberg method ranks the *P* values and calculates a new *P* value based on the gene’s rank divided by the total number of genes

Conditions Affecting Neurocognitive Development and Learning in Early childhood (CANDLE)

An observational, longitudinal cohort study which enrolled pregnant individuals from Shelby County Tennessee between 2006–2011

Confounding Variable
A variable that may influence both the dependent variable (In this analysis placental gene expression) and the independent variable (In this analysis preterm birth and preterm birth subgroups), causing spurious associations

Differentially Expressed Gene (DEG)
A gene whose expression levels are significantly different between 2 conditions, based on a predefined statistical cutoff. In this manuscript, we used a cutoff of FDR adjusted $P<0.05$

Early Preterm Birth
Birth occurring between 28–34 weeks of gestation

Early Term Birth
Birth occurring between 37–39 weeks of gestation, as defined by ACOG committee opinion 579[1]

Empirical Analysis of Digital Gene Expression Data in R (EdgeR)
This is a popular R package distributed through Bioconductor that contains functions to perform differential expression analysis of RNA sequencing data. This package implements methodologies based on negative binomial distributions of the data

False Discovery Rate (FDR)
The rate of rejected null hypothesis that are false. In studies that make a large number of multiple comparisons (including transcriptomics analyses), it is essential to control for the false discovery rate due to the increased chances of rejecting a null hypothesis due to the large number of tests

Full Term Birth
Birth occurring between 39–41 weeks of gestation, as defined by ACOG committee opinion 579[1]

Global Alliance to Prevent Prematurity and Stillbirth (GAPPS) biorepository
A placental biorepository which collected standardized, high-quality samples and questionnaire data from pregnant individuals. This study uses GAPPS biorepository samples collected from Seattle and Yakima Washington that are part of the ECHO PATHWAYS Consortium

Kallisto
a program to quantify transcript abundances from RNA sequencing files using pseudoaligments, which align the reads to a transcriptome index[2]

Kyoto Encyclopedia of Genes and Genomes (KEGG)
KEGG is a database for understanding high-level functions and utilities of biological systems molecular-level information, especially large-scale molecular datasets generated by

genome sequencing and other high-throughput experimental technologies. KEGG pathways are manually curated from existing biological literature and are continuously updated

Limma-Voom
The “limma voom” pipeline is a common statistical approach to analyze RNA sequencing data. VOOM (variance modeling at the observational level) calculates precision weights that estimate the mean-variance relationships of the log count data. This is then used in the LIMMA pipeline, which uses empirical bayes analysis to estimate the relationship between gene expression and covariate data

Late Preterm Birth
Birth occurring between 34–37 weeks of gestation

Late Term Birth
Birth occurring between 341–42 weeks of gestation, as defined by ACOG committee opinion 579[1]

Log Counts per million (LogCPM)
The log scaled counts that are scaled for the library count sum and multiplied by a million. This metric corrects for sequencing depth and is a popular unit among differential expression analysis methods

Placental Maturation Signatures
we created a curated list of 1420 gestational length signatures collected from maternal blood (N=522 genes)[3] cell free RNA across pregnancy (N=9 genes)[4] as well as another list of genes whose placental protein expression was significantly different between 2nd trimester and term gestations (N=954 genes)[5]

Premature Preterm Rupture of Membranes (PPROM)
Rupture of the amniotic sac prior to the onset of labor, occurring prior to 37 weeks of gestation. In this analysis, this was characterized via medical record abstraction

Precision Variable
A variable that may influence the dependent variable (In this analysis placental gene expression) but is not related to our independent variable, which can result in confounding

Rotational Gene Set Testing (roast)
A pathway analysis tool which compares the average t-statistic for each gene set to a null distribution estimated from random rotations of the residuals from the underlying model (10,000 rotations). We present results from the directional test, which first infers the direction of change for the gene set based on the preponderance of up or down-regulated genes, and then computes the average t-statistic based on the subset of genes in the set with the inferred direction of change using. This was implemented the roast function in the Bioconductor limma package[6]

Spontaneous Preterm Birth (SPTB)

Birth occurring before 37 weeks of gestation that is not medically indicated for conditions including preeclampsia, multi-fetal gestation, or placental abruption, and is unrelated to cervical insufficiency or chorioamnionitis

Surrogate Variable Analysis (SVA)

A computational approach to overcome problems in heterogeneity in data, including unmeasured confounding and cellular heterogeneity, by high dimensionality data reduction[7]

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CONDENSATION PAGE

1. **Condensation:** There are distinct differences in the placental transcriptome of early preterm (28–34 weeks) and late preterm infants (34–37 weeks) compared to full term infants (39–41 weeks).
2. **Short Title:** Placental Transcriptomic Signatures of Spontaneous Preterm Birth
3. **AJOG At a Glance.**
   - **A.** This Study aimed to identify differences in the placental transcriptome of placentas from preterm infants compared to term infants.
   - **B.** This study identified and characterized genes associated with prematurity that are involved in immune, endocrine, and signal transduction pathways. We noted a subset of pathways involved in metabolic processes containing genes with increased expression only in early preterm infants compared to full term infants.
   - **C.** This study contributes to the field by providing a catalogue of genes and pathways whose placental expression is associated with early and late spontaneous preterm birth (sPTB) based on investigations from a large retrospective cohort with nuanced covariate measures and robust adjustment for confounding. This study confirms gene expression changes identified in prior case-control investigations examining placental and chorioamniotic membrane physiology and preterm birth, and yielded novel insight into shared and distinct immune signaling mechanisms and metabolic perturbations related to early and late sPTB.

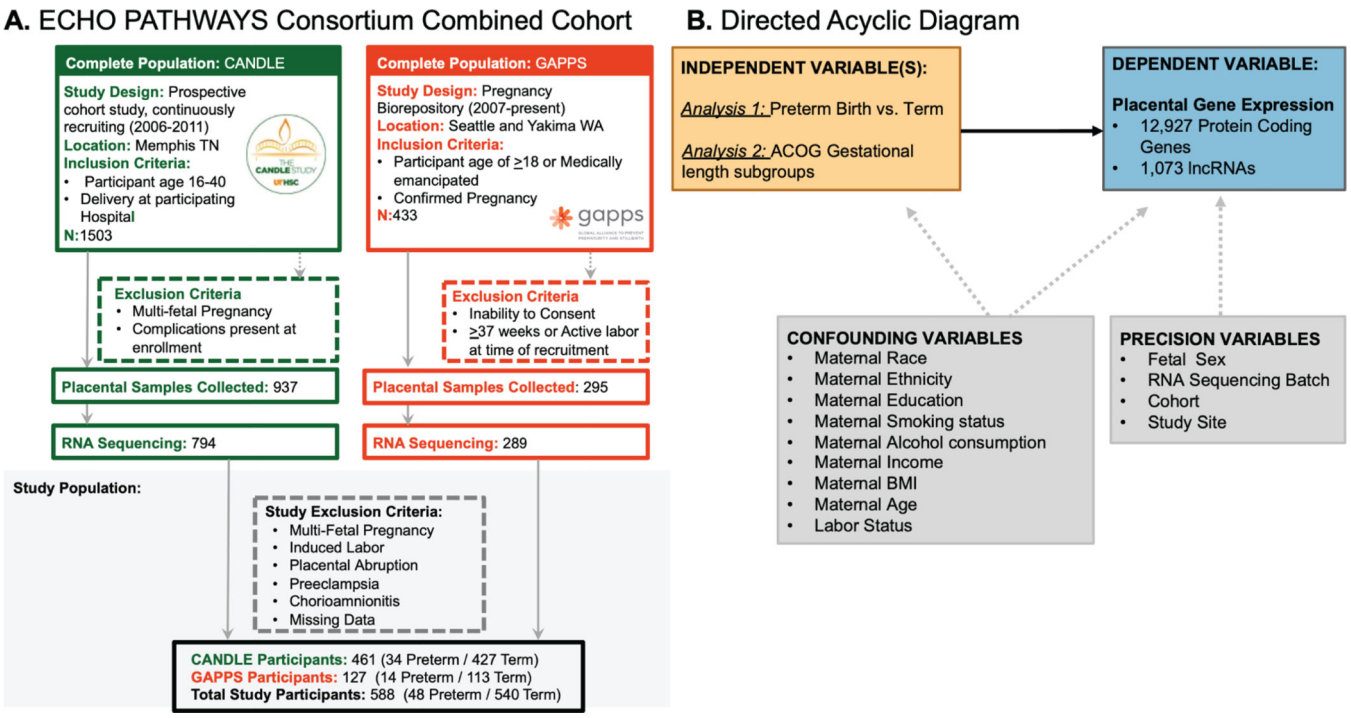

Figure 1:

(A) Summary of ECHO PATHWAYS Consortium Cohort and Study Specific Design. (B) Directed Acyclic Diagram of covariates used in Analysis.

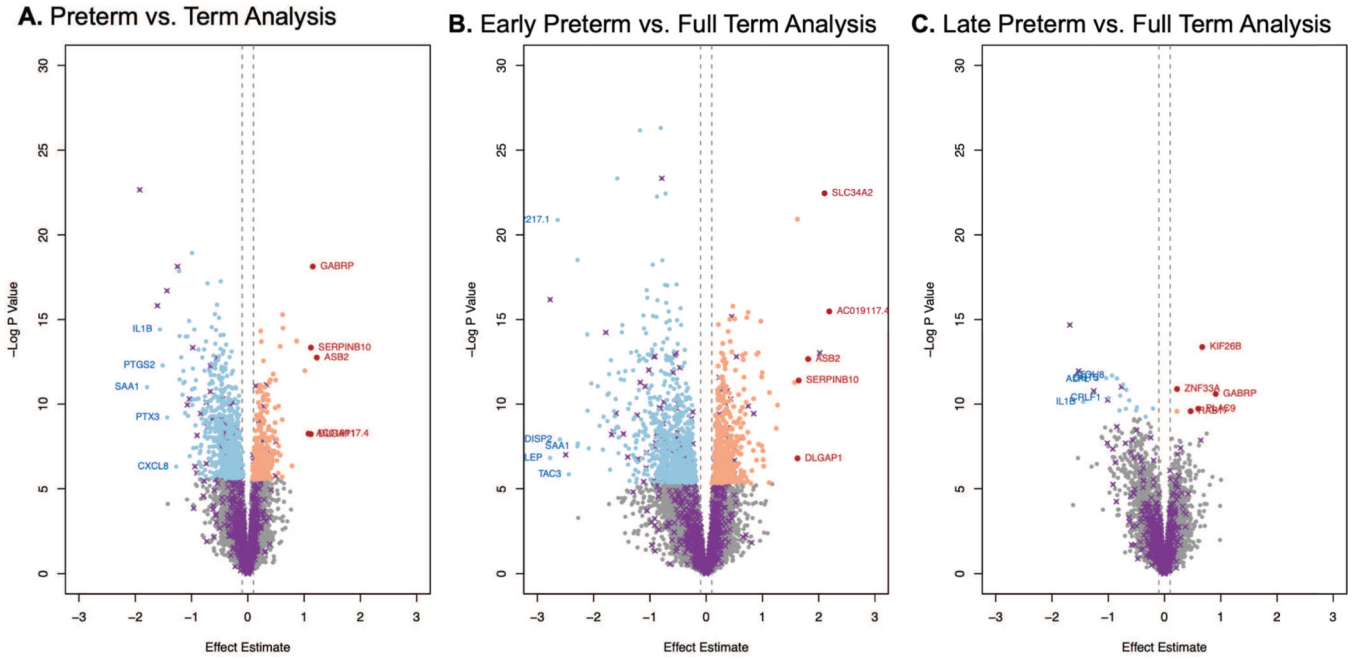

Figure 2:

Volcano plots depicting significance (-log10 p values, Y axis) and strength (LogFC, x axis) in our analyses of (A) Preterm vs.Term infants and (B) ACOG Gestational Length subgroups vs. full term infants. Purple Xs represent placental maturation signatures (from Supplemental table 1) not included in final analysis. The top 5 genes based on log fold change are labelled

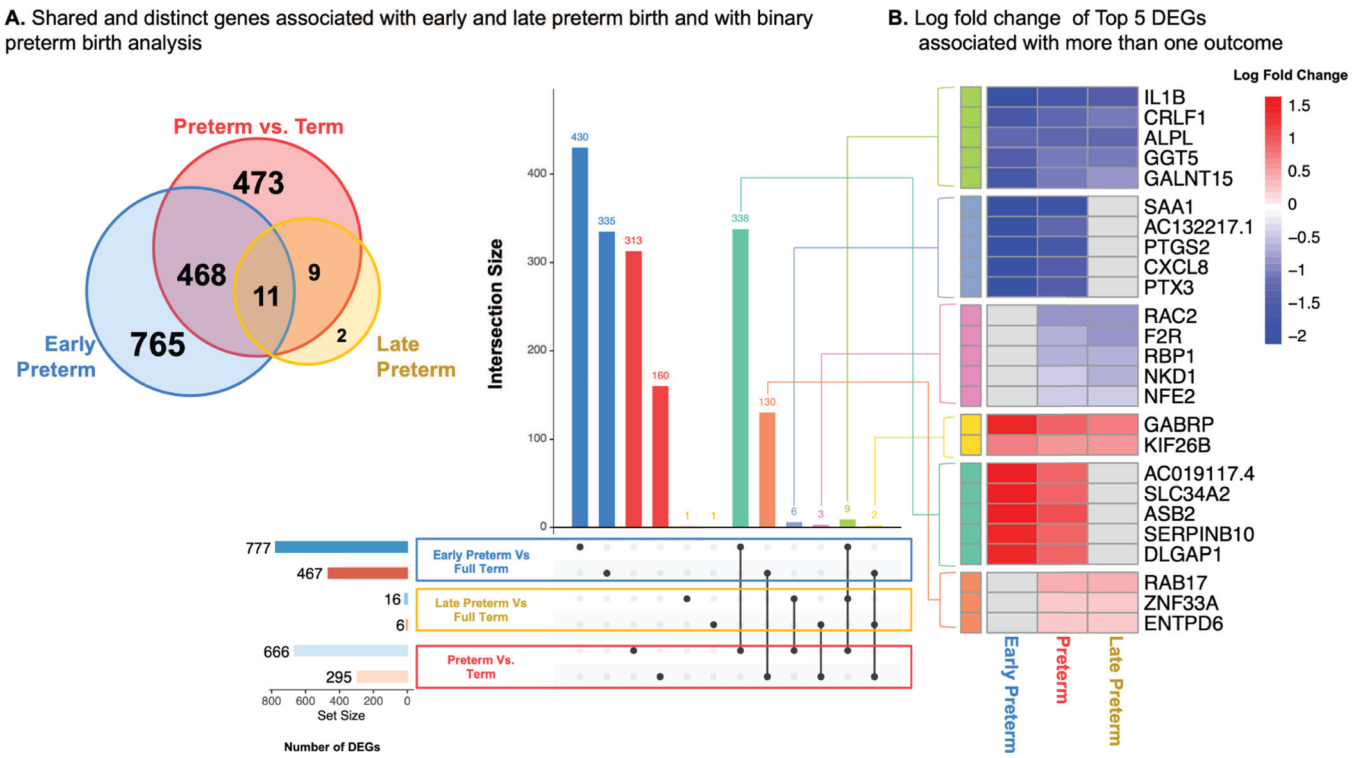

Figure 3:

Summary of Differentially Expressed Genes whose placental expression was associated with preterm birth and/or early and/or late preterm birth. (A) Venn Diagram and UpsetR plot depicting shared and distinct changes in placental gene expression. The horizontal bars indicate the total number of positive or negative DEGs in each subgroup, and the vertical bars represent the number of unique or overlapping DEG (B)The Log fold change of top 5 differentially expressed genes (based on combined FDR adjusted p values) for genes significantly associated with more than one outcome from the subgroups noted in the UpsetR plot, with subgroups highlighted by color in upsetR Plot.

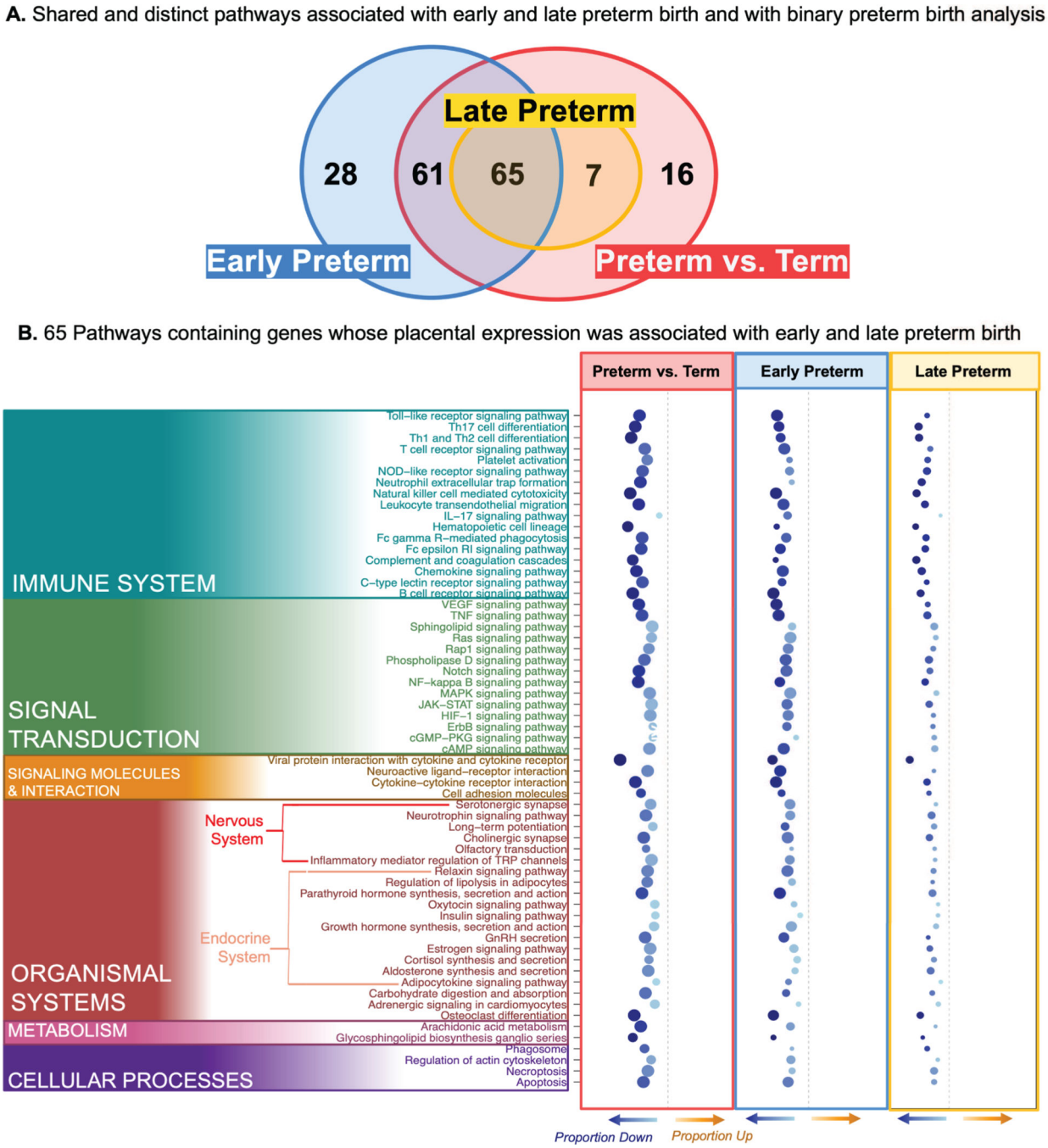

Figure 4:

Rotational gene set testing revealed shared and distinct pathways whose placental expression was associated with prematurity. (A) Venn diagram of shared and distinct pathways between analysis groups. (B) dotplot depicting results for 65 pathways associated with all three analyses. The size of the dots are scaled to the log adjusted p value, and ordered on the X axis based on the proportion of genes up or downregulated. Pathways are grouped into KEGG subgroups.

Table 1:

Participant Information

	Preterm (N= 48)	Term (N= 540)
Cohort		
CANDLE	34 (70.8%)	427 (79.1%)
GAPPS)	14 (29.2%)	113. (20.9%)
Gestational Length Subgroup		
Early Preterm (<34 Weeks)	11 (22.9%)	NA
Late Preterm (34-<37 weeks)	36 (75.0%)	NA
Early Term (37-<39 weeks)	NA	339 (62.8%)
Full Term (39-<41 weeks)	NA	181 (33.5%)
Late Term (41-<42 weeks)	NA	20 (3.7%)
Preterm Premature Rupture of Membranes		
No	27 (56.3%)	NA
Yes	21 (43.8%)	NA
Fetal Sex		
Female	22 (45.8%)	275 (50.9%)
Male	26 (54.2%)	265 (49.1%)
Labor Status		
Labor	44 (91.7%)	401 (74.3%)
No Labor	4 (8.3%)	139 (25.7%)
Delivery Method		
Caesarean Section	14 (29.2%)	214 (39.6%)
Vaginal	34 (70.8%)	326 (60.4%)
Maternal Ethnicity		
Non-Latino	44 (91.7%)	516 (95.6%)
Latino	4 (8.3%)	24 (4.4%)
Maternal Race		
Asian	4 (8.3%)	9 (1.7%)
Black	23 (47.9%)	228 (42.2%)
Multiple Race	4 (8.3%)	28 (5.2%)
American Indian/Alaska Native	0 (0.0%)	1 (0.2%)
Other	1 (2.1%)	4 (0.7%)
White	16 (33.3%)	270 (50.0%)
Maternal Ethnicity		

	Preterm (N= 48)	Term (N= 540)
High School or Less	27 (56.3%)	236 (43.7%)
College or Higher	21 (43.8%)	304 (56.3%)
Previous History of Preterm Birth		
No	38 (79.2%)	502 (93.0%)
Yes	10 (20.8%)	38 (7.0%)
Maternal Smoking Status During Pregnancy		
No	46 (95.8%)	500 (92.6%)
Yes	2 (4.2%)	40 (7.4%)
Maternal Alcohol Use During Pregnancy		
No	45 (93.8%)	474 (87.8%)
Yes	3 (6.3%)	66 (12.2%)