# Placental Neutrophil Infiltration Associated with Tobacco Exposure but Not Development of Bronchopulmonary Dysplasia

David M. Box [1], Abhishek Makkar [1], Zhongxin Yu [2], Hala Chaaban [1], Henry H. Tran [2], Kathryn Y. Burge [1] and Jeffrey V. Eckert [1,*]

[1] Neonatal-Perinatal Medicine, Department of Pediatrics, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA; davidbox22@gmail.com (D.M.B.); abhishek-makkar@ouhsc.edu (A.M.); hala-chaaban@ouhsc.edu (H.C.); kathryn-burge@ouhsc.edu (K.Y.B.)

[2] Pathology Department, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA; zhongxin-yu@ouhsc.edu (Z.Y.); hanh-tran@ouhsc.edu (H.H.T.)

[*] Correspondence: jeffrey-eckert@ouhsc.edu; Tel.: +1-(405)-271-4755; Fax: +1-(405)-271-1236

**Abstract:** Objective: In utero inflammation is associated with bronchopulmonary dysplasia (BPD) in preterm infants. We hypothesized that maternal tobacco exposure (TE) might induce placental neutrophil infiltration, increasing the risk for BPD. Study design: We compared the composite outcome of BPD and death in a prospective pilot study of TE and no-TE mothers and their infants born <32 weeks. Placental neutrophil infiltration was approximated by neutrophil gelatinase-associated lipocalin (NGAL) ELISA, and total RNA expression was analyzed via NanoString© (Seattle, WA, USA). Result: Of 39 enrolled patients, 44% were classified as tobacco exposure. No significant difference was noted in the infant's composite outcome of BPD or death based on maternal tobacco exposure. NGAL was higher in placentas of TE vs. non-TE mothers ($p < 0.05$). Placental RNA analysis identified the upregulation of key inflammatory genes associated with maternal tobacco exposure. Conclusion: Tobacco exposure during pregnancy was associated with increased placental neutrophil markers and upregulated inflammatory gene expression. These findings were not associated with BPD.

**Keywords:** bronchopulmonary dysplasia; preterm; maternal tobacco exposure; maternal smoking neutrophil; neutrophil gelatinase-associated lipocalin (NGAL)

## 1. Introduction

Tobacco exposure (TE) during pregnancy is highly prevalent in the United States. As reported by the Center for Disease Control and Prevention (CDC) in 2016, 7.2% of mothers smoked cigarettes during pregnancy [1]. It is well recognized that maternal tobacco use during pregnancy is linked to many negative outcomes for infants, including low birthweights, preterm birth, preterm prolonged rupture of membrane (PPROM), and other birth defects [2–5].

Recently, Antonucci et al. indicated that in utero exposure to smoking is an independent risk factor for the development of bronchopulmonary dysplasia (BPD) in premature infants born weighing less than 1500 g [6]. BPD is the most prevalent sequela of preterm birth, affecting 10,000–15,000 infants annually in the United States [7]. Known postnatal risk factors for the disease include hyperoxia, mechanical ventilation, patent ductus arteriosus (PDA), and sepsis; antenatal risk factors include chorioamnionitis, preeclampsia, and hypertension [8–12].

Neutrophil gelatinase-associated lipocalin (NGAL) is a glycoprotein found predominantly in neutrophil granules. NGAL is normally expressed at low levels but is often elevated in the blood, bronchoalveolar lavage (BAL) fluid, and sputum in adults with lung diseases, such as asthma and chronic obstructive pulmonary disease (COPD) [13]. Notably, serum levels of NGAL at birth are significantly higher in preterm infants who develop BPD

than in those who do not [14], suggesting a potential role for NGAL as a biomarker for BPD.

Little is known about the mechanism by which maternal tobacco exposure is associated with the development of BPD. A previous study demonstrated a higher number of neutrophils within the placentas of mothers who smoked during pregnancy; however, the incidence of bacterial infection in that group was higher, confounding the results [15]. Recent reviews have focused on injury and its contribution to fetal lung development, identifying inflammation and tobacco exposure as major contributors [16]. In addition, a meta-analysis of tobacco smoking during pregnancy showed significant association with BPD at a postmenstrual age of 36 weeks [17]. Finally, the adverse effects of maternal tobacco exposure are supported by epidemiological and animal studies, which demonstrate disrupted pulmonary development [18–20]. These observations taken together establish a link between maternal tobacco exposure and BPD and raise the possibility that neutrophils play a key role in the mechanism, with elevated levels in preterm infants who develop BDP.

To further understand the effect of antenatal tobacco exposure and its association with the development of BPD, we compared placental and infant characteristics of tobacco exposure and non-tobacco exposure mothers. Our hypothesis is two-fold; (1) we hypothesized that maternal tobacco exposure would result in increased inflammatory neutrophil infiltration of the placenta of preterm infants <32 weeks gestation, and (2) we hypothesize that infants <32 weeks gestation with tobacco exposure will subsequently be at increased risk for developing BPD. Therefore, we sought to achieve two aims/objectives in our study. The first was to identify increased neutrophil infiltration in the placenta of mothers with tobacco exposure (primary outcome). The second was to follow these infants for the composite outcome of BPD or death (secondary outcome).

## 2. Materials and Methods

Study design: This pilot prospective, observational study was conducted between October 2018 and December 2019 and was approved by the Institutional Review Board at the University of Oklahoma Health Sciences Center (OUHSC). Written informed consent was obtained for the mother and newborn either prior to delivery or within 24 h post-delivery. Following consent, a 9-item maternal questionnaire for self-identification of tobacco exposure during pregnancy was completed (Figure A1). Our maternal questionnaire on tobacco use was internally validated in a previous study, where cotinine levels (a nicotine metabolite) were detectable only in mothers who reported tobacco exposure [21]. Patients were stratified into two groups: TE mothers and non-TE mothers.

Study population: Participants included mothers and their preterm infants born at a gestational age of <32 weeks. Infants were excluded based on known major congenital anomalies, maternal concern for infection (e.g., clinical chorioamnionitis), maternal fever >38 °C 24 h before delivery, presence of meconium-stained fluid, maternal history of impaired immunity, or a concomitant medical condition impacting inflammatory response.

Data collection: Data were de-identified and prospectively collected and managed using a data collection sheet at OUHSC. Maternal and neonatal demographic characteristics were collected via chart review. The secondary outcome was a composite of BPD or death endpoints. BPD status was assessed at 36 weeks postmenstrual age (PMA) using the National Institutes of Health (NIH) workshop definition [22]. Mild BPD is defined as breathing room air at 36 weeks corrected or time of discharge, moderate BPD is defined as needing <30% oxygen at 36 weeks corrected/discharge, whereas severe BPD is defined as needing >30% O2 at 36 weeks corrected age/discharge. For the purpose of this study, infants were defined as having the presence or absence of BPD; absence of BPD was defined as no or mild BPD, and the presence of BPD was defined as moderate to severe BPD [22]. Additional outcomes included necrotizing enterocolitis (NEC), intraventricular hemorrhage (IVH), retinopathy of prematurity (ROP), PDA, and sepsis. A mother was considered to have received antenatal corticosteroids if she received a full or partial betamethasone or dexamethasone course. Intrauterine growth restriction (IUGR) was defined as intrauterine

estimated fetal weight less than the 10th percentile. PPROM was defined as having membranes ruptured for more than 18 h. Samples from the placenta from both groups were evaluated for histological chorioamnionitis by one of two pathologists blinded to maternal tobacco exposure status. Positive tobacco exposure was defined as maternal 'daily' to 'almost daily' active smoking or 'daily' to 'almost daily' secondhand smoke exposure, as reported on the maternal tobacco exposure questionnaire (Figure A1).

To determine the contribution of tobacco exposure to the development of BPD, the groups were further subdivided into (1) TE mothers with infants developing BPD (BPD TE group); (2) non-TE mothers with infants developing BPD (BPD No TE group); (3) TE mothers with infants not developing BPD (No BPD TE group); and (4) non-TE mothers with infants not developing BPD (No BPD No TE group).

Sample collection: Fresh placenta tissue samples were collected within 24 h of delivery. Three full-thickness sections of placenta parenchyma (including fetal and maternal surfaces), one section of extraplacental membrane roll, and two sections of the umbilical cord (proximal and distal) were collected and fixed in 10% formalin for routine histopathological examination and diagnosis. One full-thickness section was split and preserved for both RNA analysis (RNAlater™, Invitrogen, Carlsbad, CA, USA) and protein analysis (snap-frozen in liquid nitrogen). All samples were stored at −80 °C until further analysis.

Immunohistochemistry (IHC): IHC was performed according to the manufacturer's protocols using a Leica Bond-IIITM Polymer Refine Detection System (DS 9800). Formalin-fixed paraffin-embedded (FFPE) tissues were sectioned at the desired thickness (4 μm) and mounted on positively charged slides. The slides were dried overnight at room temperature and incubated at 60 °C for 45 min, followed by deparaffinization and rehydration in an automated multi-stainer (Leica ST5020). Subsequently, slides were transferred to the Leica Bond-IIITM and treated for antigen retrieval at 100 °C for 20 min in a retrieval solution, at either pH 6.0 or 9.0. Endogenous peroxidase was blocked using a peroxidase-blocking reagent, followed by 60 min of incubation with NGAL antibody (Catalog #711280, ThermoFisher Scientific, Waltham, MA, USA) diluted 1:100. Post-primary IgG-linker and/or poly-HRP IgG reagents were used as the secondary antibody. Detection was accomplished via the chromogen 3,3′-diaminobenzidine tetrahydrochloride (DAB), and counterstained with hematoxylin. Completed slides were dehydrated (Leica ST5020) and mounted (Leica MM24). The antibody-specific positive control and negative control (omission of primary antibody) were parallel stained. Additionally, two pathologists blinded to smoking and BPD status semi-quantitatively scored based on anatomical location, with scores from zero to four: score '0' signifying no staining; score '1' for 1–10 positive cells/per high power field (HPF); score '2' for 11–50 positive cells/HPF; score '3' for 51–75 positive cells/HPF; and score '4' for >75/HPF.

Protein analysis and enzyme-linked immunosorbent assay (ELISA): ELISA was used to quantify NGAL (Catalog #036RUO, BioPorto Diagnostics A/S, Hellerup, Denmark) following the manufacturer's instructions. Briefly, frozen placental tissue was mechanically homogenized using a BeadBeater (Next Advance Inc., Troy, NY, USA) in a buffer containing phosphatase, protease inhibitors (Catalog #524625 and #535140, Millipore, Burlington, MA, USA) and PMSF (Sigma-Aldrich, St. Louis, MO, USA). Results were normalized to total protein concentration determined by bicinchoninic acid (BCA) assay (Catalog #23227, Pierce Biotechnology, Rockford, IL, USA).

Total RNA analysis/NanoString©: A random subset of 12 patients from the four subgroups (n = 3/group): BPD, TE group; BPD, no TE group; no BPD, TE group; and no BPD, no TE group. A BeadBeater was used to homogenize placental tissue mechanically. Total RNA was extracted per the manufacturer's protocols using a Zymo Quick-RNA MidiPrep kit (Catalog #R1056, Zymo Research, Irvine, CA, USA). Total RNA, between 25 ng and 300 ng, was loaded onto a nCounter® Human Immunology v2 Panel (Catalog #XT-CSO-HIM2-12, NanoString, Seattle, WA, USA). This panel consisted of 594 genes of interest and 15 internal reference genes. Data were analyzed using nCounter Analysis and nCounter Advanced Analysis software. RCC output files were imported into NanoString

nSolver 4.0. Default quality control (QC) settings were used to verify the quality of all data (>95% of fields of view [FOV] and binding densities between 0.2 and 0.5). The background was corrected by subtracting the mean value of 8 engineered RNA negative control sequences from the raw counts of all genes. The geometric mean was calculated for the 15 housekeeping genes, and the nine genes with the lowest coefficient of variation were used to normalize the data. Genes with mean normalized counts of less than 50 were excluded from the analysis. The control group was defined as No TE or No BPD No TE for subgroup analysis. Gene expressions are estimated to have a log2-fold change, holding all other variables constant. The 95% confidence intervals (CI) for the log2-fold change and the *p* values are reported. A 1.2-fold change was selected as the differential threshold.

Given the unpredictable nature of preterm deliveries, we allowed up to 24 h for placenta collection. Once collected, the placenta was immediately placed at 4 °C. The pathologist then collected full-thickness sections and stored these at −80 °C or preserved with RNAlaterTM. Although we allowed up to 24 h for placenta collection in our protocol, the majority of samples were collected within 2–12 h. This methodology allows for collection of high-quality RNA from placentas stored at 4 °C or even room temperature for up to 48 h prior to being transferred to stabilizing solution, such as RNAlaterTM [23].

Statistical methods: Our study is a pilot/preliminary study on a topic where there is little known on the association between inflammation within the placenta and development of BPD in preterm neonates. While we have directional hypotheses, we felt it would be inappropriate to quantify an effect size given the paucity of research on the topic. Descriptive statistics were computed for demographic and clinical variables. Comparisons of categorical variables between patients developing BPD or death and those who did not were evaluated with Fisher's exact test. Continuous variables were assessed for normality, then compared between groups using a Kruskal–Wallis test or Student's *t*-test, as appropriate. Frequencies and percentages were reported for categorical variables across BPD status. Count means and standard deviations are reported for continuous variables. Statistical significance is defined, in all experiments, as $p < 0.05$.

## 3. Results

In total, 95 mothers were screened, and 49 mothers were approached for study enrollment based on the inclusion and exclusion criteria. Eight mothers declined and two approached mothers aged out of this study (delivered baby >32 weeks gestation). Demographic characteristics for the remaining 39 patients were stratified by the presence and absence of tobacco exposure (Table 1), as well as by the presence or absence of the composite outcome of BPD or death (Table A1). Of enrolled mothers, 43.6% reported tobacco exposure during pregnancy (Tables 1 and A2). Of these tobacco exposure mothers, two reported the exposure was via secondhand smoke.

No differences in birth weight, birth length, head circumference, gestational age, gender, maternal ethnicity, antenatal steroid, mode of delivery, intubation in delivery room, intubated in NICU, PDA medical or surgical treatment, IVH grade 3 or 4, ROP, IUGR <10th percentile, or death or BPD were noted with maternal tobacco exposure. There was an association with maternal age ($p = 0.048$), with tobacco exposure mothers being slightly older (Table 1). When comparing tobacco exposure mothers, no differences in diabetes status, maternal hypertension, prolong rupture of membranes, chorioamnionitis, antepartum hemorrhage, marijuana, or other illicit drug use were present (Table 2). No differences in the incidence of NEC, or sepsis based on maternal tobacco exposure were noted.

As expected, infants with the composite outcome of BPD or death had significantly lower ($p < 0.001$) birth weight, length, head circumference, and gestational age compared with the No BPD group. Additionally, more infants in the composite outcome required intubation in the delivery room ($p = 0.001$) or the NICU ($p < 0.001$), required medical management of PDA ($p = 0.01$), and developed threshold ROP ($p = 0.017$) compared to the No BPD group (Table A1). The remainder of the maternal and neonatal demographic characteristics did not differ between groups. From the maternal perspective, we found no

significant association between tobacco exposure status and maternal complications, with the exception of increased incidence of antepartum hemorrhage in the composite outcome group (*p* = 0.003) (Table A2).

**Table 1.** Maternal and Neonatal Demographic Data by Composite Outcome.

| | Tobacco Exposure | | | |
| | No (n = 22) | Yes (n = 17) | Total (n = 39) | *p* Value |
| --- | --- | --- | --- | --- |
| Birth weight, g (SD) | 1.141 (458) | 1.125 (464) | 1.134 (454) | >0.9 |
| Birth length, cm (SD) | 36.3 (4.9) | 36.6 (4.6) | 36.5 (4.7) | 0.9 |
| Head circumference, cm (SD) | 25.56 (2.87) | 25.48 (3.25) | 25.53 (3.00) | >0.9 |
| Gestational age, wk (SD) | 28.76 (2.64) | 28.40 (2.68) | 28.60 (2.63) | 0.5 |
| Maternal age, yr (SD) | 25.7 (4.9) | 29.8 (6.6) | 27.5 (6.0) | 0.048 |
| Sex | | | | |
|   F | 17 (77%) | 10 (59%) | 27 (69%) | 0.4 |
|   M | 5(23%) | 7(41%) | 12(31%) | |
| Maternal ethnicity | | | | |
|   Black | 5 (23%) | 3 (18%) | 8 (21%) | |
|   Black, Native American | 0 (0%) | 1 (5.9%) | 1 (2.6%) | |
|   Hispanic | 6 (27%) | 2 (12%) | 8 (21%) | |
|   Latino, White | 1 (4.5%) | 0 (0.0%) | 1 (2.6%) | 0.4 |
|   Native American | 1 (4.5%) | 3 (18%) | 4 (10%) | |
|   White | 9 (41%) | 7 (41%) | 16 (41%) | |
|   White, Native American | 0 (0%) | 1 (5.9%) | 1 (2.6%) | |
| Antenatal steroid, yes | 9 (41%) | 9 (53%) | 18 (46%) | 0.4 |
| Mode of delivery, C-Section | 11 (50%) | 10 (59%) | 21 (54%) | 0.8 |
| Intubated in delivery room, yes | 9 (41%) | 9 (53%) | 18 (46%) | 0.7 |
| Intubated in NICU, yes | 7 (32%) | 4 (24%) | 11 (28%) | 0.7 |
| PDA medical treatment, yes | 9 (41%) | 2 (12%) | 11 (28%) | 0.073 |
| PDA surgical treatment, yes | 1 (4.5%) | 0 (0%) | 1 (2.6%) | >0.9 |
| IVH grade 3 or 4, yes | 2 (9.1%) | 1 (5.9%) | 3 (7.7%) | >0.9 |
| Threshold ROP, yes | 3 (14%) | 1 (5.9%) | 4 (10%) | 0.6 |
| IUGR <10th percentile, yes | 3 (14%) | 0 (0%) | 3 (7.7%) | 0.2 |
| Death or BPD, yes | 15 (38%) | 9 (41%) | 6 (35%) | >0.9 |

All data are presented as the mean (standard deviation) or n (%). Statistical tests performed: Wilcoxon rank-sum test; chi-square test of independence; Fisher's exact test. *BPD*—bronchopulmonary dysplasia, *NICU*—neonatal intensive care unit, *PDA*—patent ductus arteriosus, *IVH*—intraventricular hemorrhage, *ROP*—retinopathy of prematurity, and *IUGR*—intrauterine growth restriction.

**Table 2.** Maternal Complications and Tobacco Exposure by Composite Outcome.

| | Tobacco Exposure | | | |
| | No (n = 22) | Yes (n = 17) | Total (n = 39) | *p* Value |
| --- | --- | --- | --- | --- |
| Maternal diabetes, yes | 3 (14%) | 2 (12%) | 5 (13%) | >0.9 |
| Maternal hypertension, yes | 3 (14%) | 2 (12%) | 5 (13%) | >0.9 |
| Prolonged rupture of membranes (>18h), yes | 4 (18%) | 3 (18%) | 7 (18%) | >0.9 |
| Chorioamnionitis, yes | 10 (45%) | 10 (59%) | 20 (51%) | 0.6 |
| Antepartum hemorrhage, yes | 4 (18%) | 4 (24%) | 8 (21%) | 0.7 |
| Marijuana use, yes | 1 (4.5%) | 1 (5.9%) | 2 (5.1%) | 0.4 |
| Illicit drugs, yes | 0 (0%) | 2 (12%) | 2 (5.1%) | 0.4 |

All data are presented as n (%). Statistical tests performed: Fisher's exact test; chi-square test of independence.

While there was no association between maternal tobacco exposure and an infant's risk for developing BPD, IHC of placental tissues showed a higher expression of NGAL in the fetal surfaces and upper portion of the placenta parenchyma of tobacco exposure mothers (Figure 1A,C) compared to those of No TE (Figure 1B,D) mothers. The IHC for the BPD TE group (Figure 1A) showed higher expression of NGAL as compared to the BPD
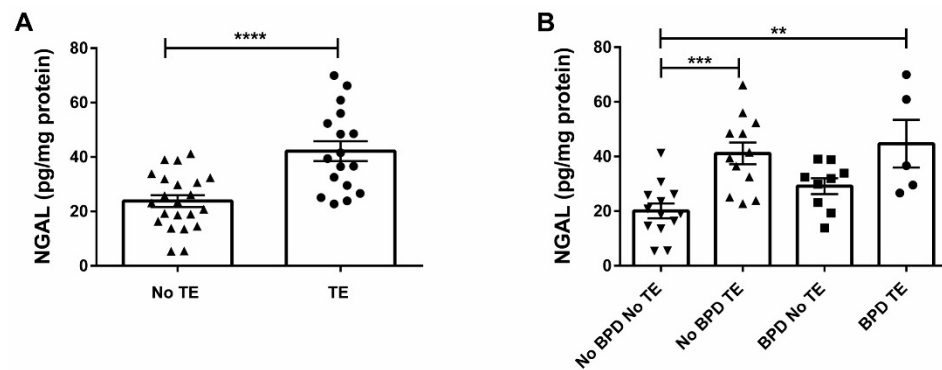
No TE group (Figure 1B). Regardless of BPD status, NGAL was highly expressed in the TE groups (BPD TE and No BPD TE) compared to the No TE group (BPD No TE and No BPD No TE). Additionally, NGAL intensity staining scores were higher in the chorionic plate and subchorionic space of placentas from tobacco exposure mothers, regardless of BPD status, though these differences did not reach statistical significance (Figure 1E,G; $p = 0.065$ and $p = 0.091$, respectively).



**Figure 1.** Representative diaminobenzidine (brown) and hematoxylin (blue) staining (**A**–**D**, 400X magnification), and staining quantification (**E**–**H**) for placental NGAL. (**A**) BPD, TE group (n = 5), with strong (3 to 4+) NGAL-positive staining in a chorionic plate and subchorionic space. (**B**) BPD, no TE group (n = 9), with mild (1–2+) NGAL-positive staining only in subchorionic space. (**C**) No BPD, TE group (n = 10), with strong (3–4+) NGAL-positive staining in the chorionic plate and subchorionic space. (**D**) No BPD, no TE group (n = 12), with rare (0–1+) NGAL-positive staining only in subchorionic space. (**E**,**F**) Quantification of chorionic plate staining stratified by maternal smoking status and subgroup analysis. (**G**,**H**) Quantification of subchorionic space staining stratified by maternal smoking status and subgroup analysis. NGAL—neutrophil gelatinase-associated lipocalin, BPD—bronchopulmonary dysplasia, and TE—tobacco exposure.

To confirm these histological findings, NGAL ELISA was performed in each of the four subgroups. As shown in Figure 2A, NGAL levels were significantly higher in the placentas of tobacco exposure compared to No TE mothers ($p < 0.0001$). Further subgroup analysis based on BPD outcomes showed that NGAL levels were significantly higher in infants of the BPD TE group compared to No BPD No TE infants (Figure 2B, $p < 0.01$). Notably, BPD No TE group also had significantly higher levels of NGAL as compared to No BPD No TE infants (Figure 2B, $p < 0.001$). Altogether, these data suggest that tobacco exposure during pregnancy is associated with increased neutrophil activation/infiltration in the placenta, and levels of neutrophil activation/infiltration are increased further still in the placentas of tobacco exposure infants developing BPD.

Next, the immune placental transcriptome from a subset of infants from all four subgroups was profiled using the NanoString nCounter™ Immunology Panel. Comparing BPD TE to No BPD No TE, 22 genes were significantly differentially expressed (Table 3) out of a total of 594 genes of potential interest (Table A3). Notably, transcript levels for the chemokines IL8 and CXCL10, the inflammatory molecules SA100A8/9, and the receptor CD44 were significantly upregulated in BPD TE compared to No BPD No TE infants (Table 3; $p < 0.05$), influencing cell signaling and inflammatory cytokine pathways (e.g., Figure A2). No other significant differences were found between the groups. We further compared the subgroups based on the neonatal outcome of BPD. Similarly, gene expression for CXCL8, CXCL10 were upregulated in the TE BPD group compared to no TE no BPD group.

**Figure 2.** ELISA for NGAL within placental tissue comparing (**A**) TE status and further comparing (**B**) TE and BPD status. (**A**) Compares TE group (n = 17) and No TE group (n = 22)—the NGAL is significantly higher in the TE group compared to No TE group (**** $p < 0.0001$). (**B**) Further subgroup analysis based on BPD status had significantly higher NGAL in the BPD TE group compared to No BPD No TE group (** $p < 0.01$). BPD No TE group also had significantly higher levels of NGAL as compared to No BPD No TE infants (*** $p < 0.001$). ELISA—enzyme-linked immunosorbent assay, NGAL—neutrophil gelatinase-associated lipocalin, BPD—bronchopulmonary dysplasia, and TE—tobacco exposure.

**Table 3.** Significantly Differential Gene Expressions (TE vs. No TE).

| Gene | Annotation | Log2-Fold Change | SE | *p* | Tentative Function |
|---|---|---|---|---|---|
| IL8 | Interleukin 8 | 4.77 | 0.898 | 0.00034 | Neutrophil Chemotaxis |
| S100A9 | S100 Calcium-Binding Protein A9 | 1.72 | 0.339 | 0.000477 | Leukocyte Activation |
| S100A8 | S100 Calcium-Binding Protein A8 | 3.33 | 0.912 | 0.00447 | Leukocyte Activation |
| IL1RL1 | Interleukin 1 Receptor Like 1 | −3.41 | 1.1 | 0.0115 | IL-33 Receptor/Inflammatory Signaling |
| CXCL10 | C-X-C Motif Chemokine Ligand 10 | 3.06 | 1.09 | 0.0187 | Peripheral Immune Cell Activation |
| CD44 | CD44 Molecule | 1.7 | 0.614 | 0.02 | Cell–Cell Signaling |
| TNFRSF10C | TNF Receptor Superfamily Member 10c | 1.53 | 0.562 | 0.0212 | Anti-Apoptosis |
| PLAUR | Plasminogen Activator, Urokinase Receptor | 1.78 | 0.676 | 0.0251 | Plasminogen Activation/Extracellular Matrix Degradation |
| IRF7 | Interferon Regulatory Factor 7 | 1.08 | 0.415 | 0.0261 | Anti-viral Immune Response |
| MALT1 | MALT1 Paracaspase | −0.572 | 0.22 | 0.0263 | NF-κB Activation |
| LILRB3 | Leukocyte Immunoglobulin-Like Receptor B3 | 2.04 | 0.795 | 0.0281 | Anti-B Cell Activation |

| Gene | Annotation | Log2-Fold Change | SE | *p* | Tentative Function |
|---|---|---|---|---|---|
| HLA-DRB1 | Major Histocompatibility Complex, Class II, DR Beta 1 | 2.74 | 1.14 | 0.0374 | Antigen Presentation |
| HLA-DRB3 | Major Histocompatibility Complex, Class II, DR Beta 3 | 1.78 | 0.746 | 0.0384 | Antigen Presentation |
| HFE | Homeostatic Iron Regulator | −1.25 | 0.529 | 0.0394 | Regulates Iron Absorption |
| TNFSF15 | TNF Superfamily Member 15 | −1.43 | 0.604 | 0.0397 | Endothelial Inflammatory Signaling |
| CD99 | CD99 Molecule | 1.12 | 0.475 | 0.0406 | Leukocyte Migration |
| PTPRC | Protein Tyrosine Phosphatase Receptor Type C | 1.94 | 0.847 | 0.045 | T Cell Activation |
| PTAFR | Platelet-Activating Factor Receptor | −2 | 0.881 | 0.0466 | Receptor for Inflammatory PAF |
| ZBTB16 | Zinc Finger- and BTB Domain-Containing 16 | −2.01 | 0.888 | 0.0469 | Transcription Repression/Myeloid Maturation |
| PLA2G2A | Phospholipase A2 Group IIA | −2.25 | 0.997 | 0.0479 | Phospholipid Metabolism |

*BPD*—bronchopulmonary dysplasia, *TE*—tobacco exposure, and *SE*—standard error.

## 4. Discussion

Bronchopulmonary dysplasia, a disease primarily affecting preterm infants, can be a challenge to manage both acutely and in the long term, as there are many persistent complications affecting patients and their families [24,25]. In this study, we sought to investigate whether tobacco exposure during pregnancy is a risk factor for developing BPD. Specifically, we questioned whether neutrophil activation/infiltration occurs in the placentas of tobacco exposure mothers and if this infiltration of neutrophils to the placenta is associated with the development of BPD or death, as a composite outcome, in preterm infants.

NGAL, neutrophil gelatinase-associated lipocalin, is a 25 kDa lipocalin originally purified from activated human neutrophils. This molecule is now known to be secreted by a variety of immune cells, hepatocytes, adipocytes, and renal tubular cells [26]. In the placenta, NGAL staining has been associated with inflammation and intra-amniotic infections [26]. NGAL levels in the plasma have also been associated with the development of BPD in preterm infants [14]. In this study, we showed for the first time that NGAL staining and NGAL protein levels are higher in the placentas of tobacco exposure mothers compared to those of No tobacco exposure mothers. Using IHC, NGAL staining was specifically high in the amniochorionic membrane and intervillous space, suggesting the presence of neutrophil activation on both the maternal and fetal surfaces. Levels of NGAL measured by ELISA in placenta homogenates were higher in BPD tabacco exposure infants compared to No BPD tobacco exposure infants. Notably, we found no difference in pathologically diagnosed chorioamnionitis or funisitis between the BPD and No BPD groups, suggesting that the observed elevated NGAL levels could be secondary to maternal tobacco exposure.

The potential physiological mechanisms associating maternal tobacco exposure with increased placental NGAL are currently unknown. However, it is reasonable to assume that tobacco exposure during pregnancy results in increased inflammation and immune

cell activation, both systemically and at the placenta [27]. Immune cell activation would result in the release of inflammatory cytokines and chemotactic factors [28], potentially affecting the maturation of the fetal lungs. Previous studies have confirmed an association of elevated levels of pro-inflammatory cytokines (interleukin 6 [IL-6], tumor necrosis factor-alpha [TNF-$\alpha$], IL-1$\beta$, and IL-8) in amniotic fluid 5 days preceding delivery with the development of BPD, suggesting that the mechanism responsible for BPD may begin before birth [29].

To determine if tobacco exposure is associated with increased inflammation in the placenta, we profiled the placental tissues as from tobacco exposure and no tobacco exposure mothers using the nCounter® Immunology NanoString Panel, which includes over 500 immunology genes involved with activation of the inflammatory cascade, including neutrophils, natural killer cell, B cell, and T cell activation, as well as various genes responsible for complement activation. Notably, IL8 and CXCL10 mRNA were significantly upregulated in tobacco exposure compared to no tobacco exposure placenta. Both genes encode chemokines known to recruit immune cells, including neutrophils, and are associated with inflammation in the placenta [28,30]. Additionally, the SA100A8 and SA100A9 genes, upregulated in tobacco exposure placentas, encode inflammatory proteins previously shown to play a role in pregnancy loss and other complications, such as preeclampsia [31]. These expression differences further support our suggestion that maternal tobacco exposure is associated with placental inflammation, at least at the transcript level.

Surprisingly, we found no association between maternal tobacco exposure and the incidence of BPD in preterm infants born <32 weeks gestation. This lack of association could be due to the small sample size, as well as a multitude of factors known to be involved in the pathogenesis of BPD [24]. Though a previous study showed a potential association of BPD with maternal tobacco exposure, the majority of the literature indicates that maternal smoking during pregnancy is not an independent risk factor for BPD development, after controlling for additional variables [6,8,32,33]. With the exception of antepartum hemorrhage incidence, which was significantly higher in the composite outcome group compared to the No BPD group (46.7% vs. 4.2%; $p = 0.003$), we found no difference in known risk factors for BPD, including maternal hypertension, PPROM, and chorioamnionitis [8–12]. In line with other studies [7], infants with the composite outcome of BPD or death had a lower gestational age and birth weight compared to infants in the No BPD group. Composite outcome infants also required more medical interventions, such as intubation after birth, medical management of PDA, and development of threshold ROP.

Our pilot study is subject to several limitations. First, maternal tobacco exposure status was based on a self-reported questionnaire rather than biochemical measurement, such as levels of cotinine, a nicotine metabolite. We previously showed that serum cotinine levels were significantly higher in cord blood of self-reported smokers than in cord blood of non-smokers, suggesting that self-reporting smoking status could be adequate in our patient population [21]. Secondly, we did not account for the amount of tobacco exposure (e.g., number of cigarettes smoked per day, or passive versus active smoking) in our results. It is possible that active smoking has a stronger association with placental pathology than passive tobacco exposure. Third, due to the small sample size, we focused on the clinically relevant outcome of moderate to severe BPD and did not adjust for the multiple confounding variables that contribute to the development of BPD. Lastly, our focus in this study was primarily on neutrophil activation. We did not evaluate the effect of tobacco exposure on activation or placental infiltration of other leukocytes.

Our studies provide direct evidence that maternal tobacco exposure leads to neutrophil infiltration into the placenta. One possible implication of this observation is an increased inflammatory environment which could amplify other risk factors, chorioamnionitis, preeclampsia, high oxygen or mechanical ventilation, resulting in the development of BPD [16]. Additional studies need to be carried out focusing on other leukocytes present in the placenta and the cytokines the neonate is exposed to that could contribute to inflammatory injury in the developing lungs. Further, an additional larger study should

be carried out to determine if an increase neutrophil infiltration into the placenta due to tobacco exposure is predictive of BPD.

## 5. Conclusions

In conclusion, our studies provide direct evidence that maternal tobacco exposure leads to neutrophil infiltration into the placenta. One possible implication of this observation is an increased inflammatory environment which could amplify other risk factors, chorioamnionitis, preeclampsia, high oxygen or mechanical ventilation, resulting in the development of BPD [16]. Additional studies need to be carried out focusing on other leukocytes present in the placenta and the cytokines the neonate is exposed to that could contribute to inflammatory injury in the developing lungs. Further, an additional larger study should be carried out to determine if an increase neutrophil infiltration into the placenta due to tobacco exposure is predictive of BPD.

Appendix A

---

# Maternal Tobacco Exposure Questionnaire

Thank you for choosing to participate in our research study.  The following questions ask about your exposure to tobacco products during your pregnancy, as well as some personal characteristics.  This is information will help to improve our understanding of how the body, specifically the placenta, responds to tobacco exposure.

1.  Have you **EVER** smoked or used tobacco/nicotine product (for example: cigarettes, cigar, pipe, chew/dip, nicotine gum or patch, e-cigarette/vape)? **\*\*if "NO,"** <u>skip to Question 5</u>**\*\***
    a)  No
    b)  Yes

2.  When was the last time you smoked or used tobacco/nicotine product?
    a)  Today or yesterday
    b)  2 days to 1 month ago
    c)  1 to 6 months ago
    d)  6 months to 1 year ago
    e)  Over 1 year ago
    **\*\*If answered (e) please SKIP to question #5\*\***

3.  On average, how often have you smoked or used tobacco/nicotine <u>during pregnancy</u>?
    a)  Daily to almost daily
    b)  3 to 5 days per week
    c)  1 to 2 days per week
    d)  Less than 1 day per week

4.  On average, how would you classify your smoking or nicotine habit <u>during pregnancy</u>?
    a)  20 cigarettes (1 pack) or more a day
    b)  10 to 19 cigarettes a day
    c)  5 to 9 cigarettes a day
    d)  Less than 5 cigarettes a day

5.  <u>Since becoming pregnant</u>, on average, how often are you exposed to secondhand smoke (at home, work, or in public places)?
    a)  I am not exposed to secondhand smoke
    b)  Daily to almost daily
    c)  3 to 5 days per week
    d)  1 to 2 days per week
    e)  Less than 1 day per week

6.  What is your marital status?
    a)  Single
    b)  Married
    c)  Long-term relationship (such as common law marriage)
    d)  Divorced or separated

---

**Figure A1.** *Cont.*

1.  What is your Race/Ethnicity? Please mark all that apply.
    a)  White
    b)  Black
    c)  Asian or Pacific Islander
    d)  Native American
    e)  Hispanic
    f)  Other (please list) _____

2.  What is the highest level of education completed?
    a)  Some high school or less
    b)  High school diploma or GED
    c)  Some college
    d)  College degree
    e)  Professional/post-grad-graduate degree

3.  How many adults (including you) live in your home?
    a)  1 to 2
    b)  3 to 4
    c)  5 or more

**Figure A1.** Following consent, a maternal tobacco exposure questionnaire was administered within 24 h of delivery to self-identify TE during pregnancy.
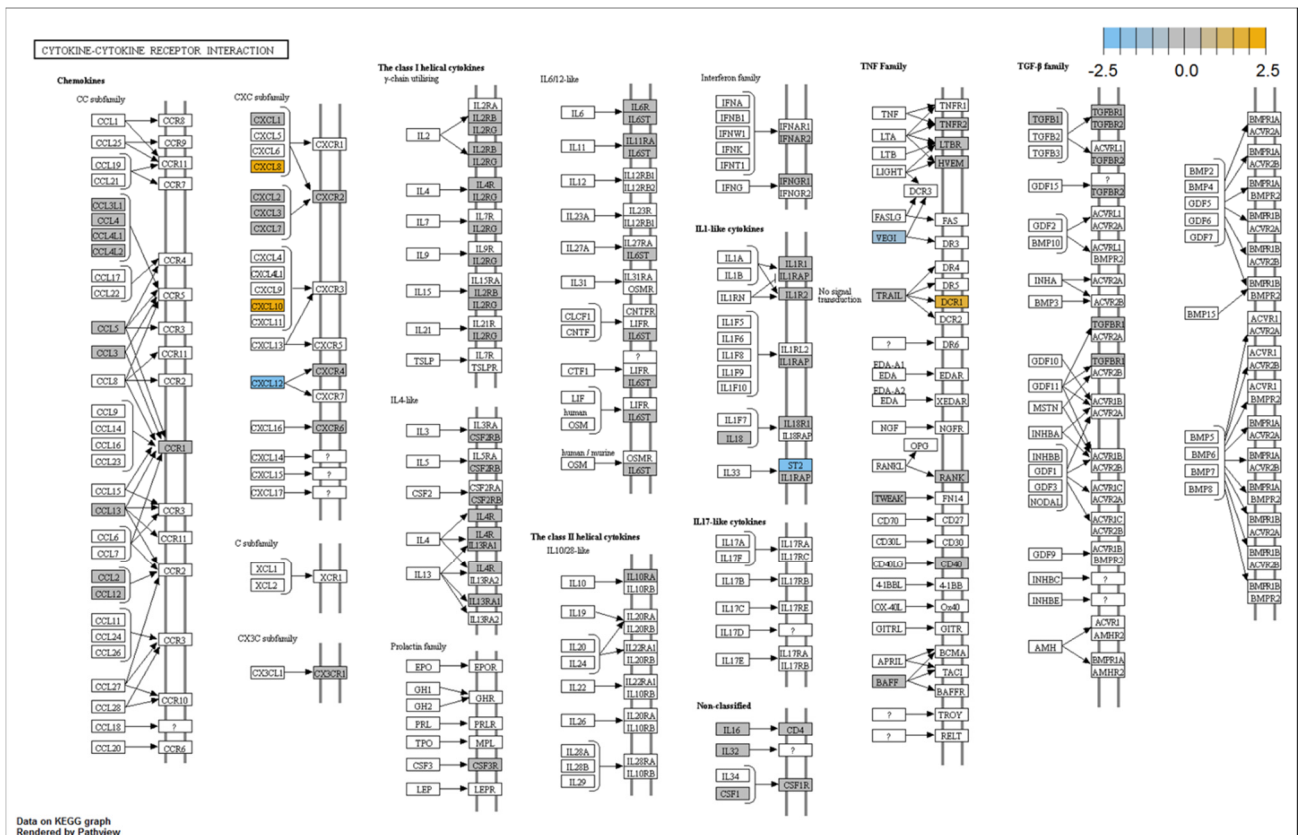


**Figure A2.** NanoString nSolver® pathway analysis indicating upregulation (yellow) and downregulation (blue) of genes encoding interactions between cytokines and cytokine receptors in BPD TE placentas compared to No BPD No TE.

**Table A1.** Maternal and Neonatal Demographic Data by Composite Outcome.

| Maternal and Neonatal Demographic Data | | | | |
|---|---|---|---|---|
| | **No BPD or Death** **(n = 22)** | **BPD or Death** **(n = 15)** | **Total (n = 39)** | ***p* Value** |
| Birth weight (g) | 1397.7 ± 355.1 | 711.7 ± 207.9 | 1133.8 ± 454.5 | <0.001 |
| Birth length (cm) | 39.4 ± 2.9 | 31.7 ± 2.6 | 36.5 ± 4.7 | <0.001 |
| Head circumference (cm) | 27.4 ± 1.8 | 22.5 ± 1.7 | 25.5 ± 3.0 | <0.001 |
| Gestational age (wk) | 29.8 ± 1.4 | 25.5 ± 1.9 | 28.2 ± 2.6 | <0.001 |
| Maternal age (yr) | 27.1 ± 5.6 | 28.2 ± 6.7 | 27.5 ± 6.0 | 0.643 |
| Sex | | | | |
| F | 14 (58.3) | 13 (86.7) | 27 (69.2) | 0.083 |
| M | 10 (41.7) | 2 (13.3) | 12 (30.8) | |
| Maternal ethnicity | | | | |
| Black | 3 (12.5%) | 5 (33.3%) | 8 (20.5%) | |
| Black, Native American | 0 (0%) | 1 (6.7%) | 1 (2.6%) | 0.233 |
| Hispanic | 7 (29.2%) | 1 (6.7%) | 8 (20.5%) | |
| Latino, White | 1 (4.2%) | 0 (0.0%) | 1 (2.6%) | |
| Native American | 3 (12.5%) | 1 (6.7%) | 4 (10.3%) | |
| White | 9 (37.5%) | 7 (46.7%) | 16(41.0%) | |
| White, Native American | 1 (4.2%) | 0 (0.0%) | 1 (2.6%) | |
| Antenatal steroid exposure | 23 (95.8) | 15 (100) | 38 (97.4) | 1.000 |
| Mode of delivery, C-Section | 14 (58.3) | 7 (46.7) | 21 (53.8) | 0.477 |
| Intubated in delivery room | 6 (25) | 12 (80) | 18 (46.2) | 0.001 |
| Intubated in NICU | 2 (8.3) | 9 (60.0) | 11 (28.2) | <0.001 |
| PDA medical treatment | 3 (12.5) | 8 (53.3) | 11 (28.2) | 0.010 |
| PDA surgical treatment | 0 (0) | 1 (6.7) | 1 (2.6) | 0.385 |
| IVH grade 3 or 4 | 1 (4.2) | 2 (13.3) | 3 (7.7) | 0.547 |
| Threshold ROP, yes | 0 (0) | 4 (26.7) | 4 (10.3) | 0.017 |
| IUGR <10th percentile, yes | 1 (4.2) | 2 (13.3) | 3 (7.7) | 0.547 |

All data are presented as the mean ± standard deviation or *n* (%). *BPD*—bronchopulmonary dysplasia, *NICU*—neonatal intensive care unit, *PDA*—patent ductus arteriosus, *IVH*—intraventricular hemorrhage, *ROP*—retinopathy of prematurity, and *IUGR*—intrauterine growth restriction.

**Table A2.** Maternal Complications and Tobacco Exposure by Composite Outcome.

| | No BPD or Death (n = 24) | BPD or Death (n = 15) | Total (n = 39) | *p* Value |
|---|---|---|---|---|
| Maternal diabetes | 4 (16.7) | 1 (6.7) | 5 (12.8) | 0.634 |
| Maternal hypertension | 3 (12.5) | 2 (13.3) | 5 (12.8) | 1.000 |
| Prolonged rupture of membranes (>18h) | 3 (12.5) | 4 (26.7) | 7 (17.9) | 0.396 |
| Histological chorioamnionitis | 12 (50) | 8 (53.3) | 20 (51.3) | 0.839 |
| Antepartum hemorrhage | 1 (4.2) | 7 (46.7) | 8 (20.5) | 0.003 |
| Maternal TE | 11 (45.8) | 6 (40) | 17 (43.6) | 0.721 |
| Maternal active smoking | 9 (37.5) | 3 (20) | 12 (30.8) | 0.305 |
| Maternal passive smoke exposure | 11 (45.8) | 7 (46.7) | 18 (46.2) | 0.959 |
| Illicit drugs, yes | 1 (4.21) | 1 (6.7) | 2 (5.1) | 0.765 |

All data are presented as *n* (%). *BPD*—bronchopulmonary dysplasia, *PPROM*—premature prolonged rupture of membranes, and *TE*—tobacco exposure.

**Table A3.** NanoString Gene Expressions (BPD TE vs. No BPD No TE).

| Gene | Log2-Fold Change | SE | Lower Confidence Limit (log2) | Upper Confidence Limit (log2) | *p* | Tentative Function |
|---|---|---|---|---|---|---|
| IL8 | 4.77 | 0.898 | 3.01 | 6.54 | 0.00034 | Cell Activation |
| S100A9 | 1.72 | 0.339 | 1.06 | 2.38 | 0.000477 | Cell–Cell Signaling |
| S100A8 | 3.33 | 0.912 | 1.54 | 5.12 | 0.00447 | Defense Response |
| IL1RL1 | −3.41 | 1.1 | −5.57 | −1.24 | 0.0115 | Receptor Signaling Protein Activity |
| CXCL10 | 3.06 | 1.09 | 0.922 | 5.21 | 0.0187 | Behavior |
| CD44 | 1.7 | 0.614 | 0.493 | 2.9 | 0.02 | Cell–Cell Signaling |
| TNFRSF10C | 1.53 | 0.562 | 0.433 | 2.64 | 0.0212 | Integral to Membrane |
| PLAUR | 1.78 | 0.676 | 0.454 | 3.11 | 0.0251 | Behavior |
| IRF7 | 1.08 | 0.415 | 0.269 | 1.9 | 0.0261 | Biopolymer Metabolic Process |
| MALT1 | −0.572 | 0.22 | −1 | −0.141 | 0.0263 | Cell Development |
| LILRB3 | 2.04 | 0.795 | 0.481 | 3.6 | 0.0281 | Cell Surface Receptor-Linked Signal Transduction |
| HLA-DRB1 | 2.74 | 1.14 | 0.502 | 4.99 | 0.0374 | Antigen Presentation |
| HLA-DRB3 | 1.78 | 0.746 | 0.316 | 3.24 | 0.0384 | Immune Response |
| HFE | −1.25 | 0.529 | −2.29 | −0.216 | 0.0394 | Cytoplasm |
| TNFSF15 | −1.43 | 0.604 | −2.61 | −0.244 | 0.0397 | Cell Development |
| CD99 | 1.12 | 0.475 | 0.186 | 2.05 | 0.0406 | Cytoplasm |
| PTPRC | 1.94 | 0.847 | 0.28 | 3.6 | 0.045 | Integral to Membrane |
| PTAFR | −2 | 0.881 | −3.73 | −0.273 | 0.0466 | Behavior |
| ZBTB16 | −2.01 | 0.888 | −3.75 | −0.272 | 0.0469 | Intracellular Organelle Part |
| PLA2G2A | −2.25 | 0.997 | −4.2 | −0.292 | 0.0479 | Cytoplasm |
| CXCL12 | −2.06 | 0.92 | −3.86 | −0.256 | 0.0492 | Behavior |
| HRAS | −1.39 | 0.621 | −2.6 | −0.169 | 0.0497 | Anatomical Structure Development |
| SELL | 1.66 | 0.757 | 0.177 | 3.15 | 0.053 | Integral to Membrane |
| PSMB9 | 1.13 | 0.578 | −0.00586 | 2.26 | 0.0798 | Antigen Presentation |
| NT5E | 0.917 | 0.482 | −0.0275 | 1.86 | 0.0862 | Biopolymer Metabolic Process |
| CCL3 | 1.66 | 0.885 | −0.0776 | 3.39 | 0.0907 | Cell Fraction |
| CD83 | 1.64 | 0.884 | −0.0944 | 3.37 | 0.0935 | Defense Response |
| NFKBIA | 0.72 | 0.398 | −0.0604 | 1.5 | 0.101 | Apoptosis |
| HLA-DRA | 1.16 | 0.648 | −0.114 | 2.43 | 0.105 | Cytoplasm |
| CLEC4A | 1.27 | 0.71 | −0.125 | 2.66 | 0.105 | Cell Surface Receptor-Linked Signal Transduction |

**Table A3.** *Cont.*

| Gene | Log2-Fold Change | SE | Lower Confidence Limit (log2) | Upper Confidence Limit (log2) | *p* | Tentative Function |
|------|------------------|-----|-------------------------------|-------------------------------|-----|--------------------|
| HLA-C | 1.22 | 0.686 | −0.124 | 2.57 | 0.106 | Antigen Presentation |
| CXCL1 | 1.66 | 0.942 | −0.184 | 3.51 | 0.108 | Behavior |
| BCL3 | 1.25 | 0.717 | −0.158 | 2.65 | 0.112 | Cytoplasm |
| ITGAX | 1.77 | 1.03 | −0.243 | 3.79 | 0.115 | Anatomical Structure Morphogenesis |
| HLA-DMA | 0.667 | 0.396 | −0.11 | 1.44 | 0.123 | Antigen Presentation |
| TRAF5 | −1.05 | 0.631 | −2.29 | 0.183 | 0.126 | IκB Kinase NFκB Cascade |
| HLA-A | 0.723 | 0.436 | −0.131 | 1.58 | 0.128 | Antigen Presentation |
| GATA3 | −1.13 | 0.679 | −2.46 | 0.204 | 0.128 | Anatomical Structure Morphogenesis |
| CD74 | 1.29 | 0.78 | −0.237 | 2.82 | 0.129 | Biosynthetic Process |
| LILRB2 | 1.22 | 0.737 | −0.228 | 2.66 | 0.13 | Cell–Cell Signaling |
| BST1 | 1.07 | 0.649 | −0.205 | 2.34 | 0.131 | Humoral Immune Response |
| LTB4R2 | −0.977 | 0.594 | −2.14 | 0.187 | 0.131 | Behavior |
| RARRES3 | −1.23 | 0.764 | −2.73 | 0.268 | 0.139 | Cell Proliferation |
| TNFSF13B | 0.903 | 0.566 | −0.207 | 2.01 | 0.142 | Cell Fraction |
| XBP1 | 1.34 | 0.84 | −0.307 | 2.99 | 0.142 | DNA Binding |
| CD24 | 1.22 | 0.778 | −0.299 | 2.75 | 0.146 | Cell Surface |
| NFKB2 | 0.877 | 0.56 | −0.221 | 1.98 | 0.148 | Biopolymer Metabolic Process |
| ITGAE | −0.775 | 0.505 | −1.77 | 0.215 | 0.156 | Integral to Membrane |
| VCAM1 | −1.04 | 0.69 | −2.39 | 0.312 | 0.162 | Leukocyte Adhesion |
| PSMD7 | 0.342 | 0.229 | −0.106 | 0.79 | 0.165 | Macromolecular Complex |
| ATG12 | −1.03 | 0.691 | −2.38 | 0.328 | 0.168 | Apoptosis |
| CXCL2 | 1.32 | 0.896 | −0.434 | 3.08 | 0.171 | Behavior |
| MAPK11 | 0.721 | 0.497 | −0.254 | 1.7 | 0.178 | Intracellular Signaling Cascade |
| IL11RA | −0.872 | 0.603 | −2.05 | 0.311 | 0.179 | Integral to Membrane |
| TAL1 | −1.01 | 0.702 | −2.39 | 0.364 | 0.18 | Cell Proliferation |
| PPBP | 1.06 | 0.736 | −0.382 | 2.5 | 0.18 | Establishment of Localization |
| TNFRSF14 | −0.854 | 0.601 | −2.03 | 0.324 | 0.186 | Cell Surface Receptor-Linked Signal Transduction |
| ITGAM | 0.932 | 0.659 | −0.359 | 2.22 | 0.187 | Integral to Membrane |
| C2 | −0.415 | 0.294 | −0.991 | 0.161 | 0.188 | Defense Response |
| CD59 | 0.626 | 0.446 | −0.247 | 1.5 | 0.19 | Cell Fraction |
| TGFB1 | 1.15 | 0.817 | −0.455 | 2.75 | 0.191 | DNA Metabolic Process |
| MIF | 0.686 | 0.489 | −0.272 | 1.65 | 0.191 | Biosynthetic Process |
| FCGR2A | 1.97 | 1.41 | −0.789 | 4.72 | 0.192 | Phagocytosis |
| BATF3 | −0.858 | 0.62 | −2.07 | 0.358 | 0.197 | Biopolymer Metabolic Process |
| CCL4 | 0.946 | 0.685 | −0.397 | 2.29 | 0.198 | Anatomical Structure Morphogenesis |
| IFI35 | −0.559 | 0.406 | −1.36 | 0.237 | 0.199 | Nucleus |
| HLA-DPB1 | 1.39 | 1.01 | −0.591 | 3.36 | 0.199 | Multi-Organism Process |
| FCER1G | 0.953 | 0.699 | −0.417 | 2.32 | 0.203 | Integral to Membrane |
| FCGR3A/B | 2.03 | 1.49 | −0.902 | 4.95 | 0.205 | Immune Response |
| HLA-DPA | 1.33 | 1 | −0.639 | 3.3 | 0.215 | Antigen Presentation |
| SRC | −0.655 | 0.497 | −1.63 | 0.32 | 0.217 | Cell Surface Receptor-Linked Signal Transduction |
| IL1R2 | 0.888 | 0.681 | −0.446 | 2.22 | 0.221 | Immune Response |
| CXCR2 | 0.877 | 0.675 | −0.447 | 2.2 | 0.223 | Receptor for IL-8 |
| ITGAL | 1.15 | 0.894 | −0.598 | 2.91 | 0.226 | Leukocyte Adhesion |
| CFD | −0.558 | 0.433 | −1.41 | 0.29 | 0.226 | Cellular Macromolecule Metabolic Process |
| SOCS3 | 0.87 | 0.681 | −0.464 | 2.2 | 0.23 | Cell Development |
| IL2RG | 0.973 | 0.763 | −0.522 | 2.47 | 0.231 | Cell Surface |
| PECAM1 | −0.491 | 0.386 | −1.25 | 0.265 | 0.232 | Membrane |
| TNFRSF1B | 1.34 | 1.06 | −0.729 | 3.41 | 0.233 | Receptor Activity |
| CASP8 | 1.27 | 1.02 | −0.725 | 3.27 | 0.241 | Cell Development |
| GBP1 | 1.41 | 1.13 | −0.81 | 3.63 | 0.242 | Cell Metabolism |
| TLR2 | 0.784 | 0.644 | −0.478 | 2.05 | 0.251 | Cell Development |

<div align="center"><b>Table A3.</b> <i>Cont.</i></div>

| Gene | Log2-Fold Change | SE | Lower Confidence Limit (log2) | Upper Confidence Limit (log2) | *p* | Tentative Function |
|---|---|---|---|---|---|---|
| CDKN1A | −0.618 | 0.51 | −1.62 | 0.381 | 0.253 | Cell Development |
| S1PR1 | 0.794 | 0.662 | −0.503 | 2.09 | 0.258 | S1P Receptor |
| IL18 | 0.562 | 0.475 | −0.369 | 1.49 | 0.264 | Anatomical Structure Morphogenesis |
| TFRC | −0.715 | 0.632 | −1.95 | 0.523 | 0.284 | Cytoplasm |
| VTN | −1.19 | 1.07 | −3.29 | 0.904 | 0.291 | Extracellular Region |
| GPI | 1.29 | 1.16 | −0.987 | 3.56 | 0.293 | Hemostasis |
| MR1 | −0.763 | 0.689 | −2.11 | 0.587 | 0.294 | Immune Response |
| PRKCD | 0.638 | 0.578 | −0.495 | 1.77 | 0.296 | Biopolymer Metabolic Process |
| BCL10 | 1.35 | 1.22 | −1.05 | 3.74 | 0.296 | Cytoplasm |
| MAPK14 | 1.55 | 1.4 | −1.21 | 4.3 | 0.297 | Behavior |
| ZEB1 | −0.667 | 0.607 | −1.86 | 0.521 | 0.297 | Biopolymer Metabolic Process |
| EBI3 | 0.721 | 0.661 | −0.574 | 2.02 | 0.301 | Biosynthetic Process |
| PTPN2 | 1.05 | 0.969 | −0.849 | 2.95 | 0.304 | Biopolymer Metabolic Process |
| TNFRSF11A | −0.464 | 0.43 | −1.31 | 0.379 | 0.306 | Cell–Cell Signaling |
| IL32 | 0.781 | 0.733 | −0.655 | 2.22 | 0.312 | Defense Response |
| C1QA | 1.34 | 1.27 | −1.15 | 3.84 | 0.317 | Cell–Cell Signaling |
| CHUK | 1.23 | 1.18 | −1.08 | 3.54 | 0.322 | Anatomical Structure Morphogenesis |
| AHR | −0.603 | 0.579 | −1.74 | 0.533 | 0.323 | Biopolymer Metabolic Process |
| TGFBR2 | −0.33 | 0.323 | −0.963 | 0.304 | 0.331 | Cell Proliferation |
| IL13RA1 | 0.217 | 0.214 | −0.202 | 0.637 | 0.334 | Cell Surface Receptor-Linked Signal Transduction |
| PDCD1LG2 | −0.741 | 0.732 | −2.18 | 0.694 | 0.335 | Antigen Presentation |
| ETS1 | −0.659 | 0.666 | −1.96 | 0.647 | 0.346 | Hemopoiesis |
| FADD | 0.531 | 0.538 | −0.524 | 1.58 | 0.347 | Cell Development |
| HLA-B | 1.45 | 1.47 | −1.43 | 4.33 | 0.348 | Cell Fraction |
| MYD88 | 1.15 | 1.17 | −1.15 | 3.45 | 0.352 | IκB Kinase NFκB Cascade |
| CR1 | 0.723 | 0.742 | −0.731 | 2.18 | 0.353 | Integral to Membrane |
| TGFBI | 1.51 | 1.55 | −1.53 | 4.54 | 0.353 | Cell Proliferation |
| TRAF6 | −0.132 | 0.137 | −0.401 | 0.136 | 0.358 | Biopolymer Metabolic Process |
| LTBR | 1.26 | 1.33 | −1.33 | 3.86 | 0.363 | IκB Kinase NFκB Cascade |
| TLR7 | −0.604 | 0.637 | −1.85 | 0.644 | 0.365 | Biosynthetic Process |
| BCAP31 | −0.968 | 1.02 | −2.97 | 1.03 | 0.366 | Integral to Membrane |
| CD45R0 | 0.666 | 0.707 | −0.719 | 2.05 | 0.368 | Integral to Membrane |
| PSMC2 | 1.35 | 1.45 | −1.5 | 4.19 | 0.375 | Cytoplasm |
| CUL9 | −0.547 | 0.596 | −1.72 | 0.622 | 0.381 | Microtubule Dynamics |
| MAP4K4 | 0.236 | 0.258 | −0.269 | 0.742 | 0.381 | Biopolymer Metabolic Process |
| TLR4 | 1.05 | 1.15 | −1.2 | 3.3 | 0.381 | Biosynthetic Process |
| STAT6 | 1.09 | 1.2 | −1.25 | 3.44 | 0.383 | DNA Binding |
| LTF | 1.28 | 1.4 | −1.47 | 4.02 | 0.384 | Endopeptidase Activity |
| STAT3 | 0.35 | 0.385 | −0.404 | 1.1 | 0.384 | Biopolymer Metabolic Process |
| BCL6 | 0.734 | 0.808 | −0.849 | 2.32 | 0.385 | Intracellular Non-Membrane-Bound Organelle |
| FYN | 0.626 | 0.695 | −0.735 | 1.99 | 0.389 | Behavior |
| IKBKAP | 0.885 | 0.986 | −1.05 | 2.82 | 0.39 | Cytoplasm |
| PPARG | 0.944 | 1.06 | −1.13 | 3.01 | 0.393 | Biopolymer Metabolic Process |
| IFITM1 | 1.26 | 1.42 | −1.52 | 4.05 | 0.395 | Cell Proliferation |
| CD40 | −0.655 | 0.741 | −2.11 | 0.797 | 0.398 | Defense Response |
| CASP3 | 1.03 | 1.17 | −1.26 | 3.33 | 0.398 | Cell Development |
| TAPBP | 0.332 | 0.383 | −0.42 | 1.08 | 0.407 | Cytoplasm |
| IFI16 | 1.09 | 1.27 | −1.4 | 3.58 | 0.411 | Cell Development |
| CD45RB | 0.708 | 0.825 | −0.91 | 2.33 | 0.411 | Integral to Membrane |
| GPR183 | −0.755 | 0.882 | −2.48 | 0.973 | 0.412 | Unknown |
| TAGAP | −0.671 | 0.79 | −2.22 | 0.877 | 0.416 | Rho GTPase Activation |
| ITGB2 | 0.619 | 0.732 | −0.815 | 2.05 | 0.417 | Behavior |
| NFATC2 | 0.97 | 1.15 | −1.28 | 3.22 | 0.418 | Biopolymer Metabolic Process |
| TAP2 | 0.575 | 0.682 | −0.761 | 1.91 | 0.419 | Cytoplasm |

**Table A3.** *Cont.*

| Gene | Log2-Fold Change | SE | Lower Confidence Limit (log2) | Upper Confidence Limit (log2) | *p* | Tentative Function |
|---|---|---|---|---|---|---|
| TBK1 | 1.12 | 1.33 | −1.49 | 3.74 | 0.42 | IκB Kinase NFκB Cascade |
| NCF4 | 0.51 | 0.607 | −0.681 | 1.7 | 0.421 | Cytoplasm |
| PTPN6 | 0.984 | 1.17 | −1.32 | 3.29 | 0.422 | Biopolymer Metabolic Process |
| ILF3 | 1.15 | 1.38 | −1.55 | 3.85 | 0.423 | Biopolymer Metabolic Process |
| CASP2 | 0.999 | 1.2 | −1.35 | 3.34 | 0.423 | Apoptosis |
| CD274 | 0.345 | 0.415 | −0.468 | 1.16 | 0.425 | Cell Proliferation |
| SPP1 | −0.645 | 0.779 | −2.17 | 0.882 | 0.427 | Osteoclast Attachment |
| FCGR1A/B | 0.507 | 0.613 | −0.695 | 1.71 | 0.428 | Establishment of Localization |
| RELA | 0.375 | 0.461 | −0.528 | 1.28 | 0.435 | Cell Development |
| SERPING1 | 1.26 | 1.55 | −1.78 | 4.3 | 0.436 | Regulation of Complement |
| TNFSF10 | 1.13 | 1.41 | −1.64 | 3.9 | 0.441 | Cell Development |
| CSF2RB | 0.579 | 0.732 | −0.856 | 2.01 | 0.447 | Integral to Membrane |
| NFKBIZ | 0.951 | 1.21 | −1.42 | 3.32 | 0.45 | Activation of IL-6 |
| PTK2 | 1.17 | 1.49 | −1.76 | 4.1 | 0.452 | Cell Surface Receptor-Linked Signal Transduction |
| RUNX1 | −0.403 | 0.52 | −1.42 | 0.617 | 0.456 | Biopolymer Metabolic Process |
| CTNNB1 | 1.44 | 1.86 | −2.2 | 5.08 | 0.457 | Cell Development |
| FCGR2B | −0.39 | 0.507 | −1.38 | 0.604 | 0.46 | Immune Response |
| TNFAIP3 | 0.511 | 0.665 | −0.792 | 1.81 | 0.46 | IκB Kinase NFκB Cascade |
| IL1R1 | −1 | 1.32 | −3.58 | 1.58 | 0.463 | Integral to Membrane |
| MAPKAPK2 | −0.436 | 0.573 | −1.56 | 0.687 | 0.464 | Biopolymer Metabolic Process |
| CD209 | 0.649 | 0.854 | −1.03 | 2.32 | 0.465 | Cell–Cell Adhesion |
| MX1 | −0.522 | 0.689 | −1.87 | 0.828 | 0.466 | Apoptosis |
| PSMB7 | 0.242 | 0.321 | −0.386 | 0.871 | 0.467 | Peptide Cleavage |
| ATG7 | 0.881 | 1.17 | −1.42 | 3.18 | 0.47 | Biopolymer Metabolic Process |
| CTSC | 1.31 | 1.74 | −2.11 | 4.72 | 0.47 | Cytoplasm |
| IL18R1 | −0.535 | 0.734 | −1.97 | 0.903 | 0.482 | Membrane |
| CCL5 | −0.467 | 0.648 | −1.74 | 0.803 | 0.487 | Behavior |
| LILRB4 | −0.632 | 0.877 | −2.35 | 1.09 | 0.488 | Antigen Binding |
| IL2RB | 0.594 | 0.83 | −1.03 | 2.22 | 0.491 | Cell Development |
| PSMB5 | −0.26 | 0.364 | −0.973 | 0.453 | 0.491 | Peptide Cleavage |
| CD46 | 1.35 | 1.9 | −2.37 | 5.08 | 0.492 | Integral to Membrane |
| C1QB | 1.2 | 1.7 | −2.12 | 4.53 | 0.494 | Extracellular Region |
| C14orf166 | 1.11 | 1.58 | −1.98 | 4.2 | 0.499 | Identical Protein Binding |
| FCGR2A/C | 1.08 | 1.59 | −2.03 | 4.19 | 0.513 | Phagocyte Cell Surface Receptor |
| LCP2 | 0.465 | 0.687 | −0.881 | 1.81 | 0.514 | Cell Surface Receptor-Linked Signal Transduction |
| EGR1 | −0.499 | 0.746 | −1.96 | 0.962 | 0.519 | Transcriptional Regulation |
| CD28 | −0.393 | 0.598 | −1.56 | 0.779 | 0.526 | Cell Development |
| TRAF2 | −0.126 | 0.194 | −0.506 | 0.253 | 0.528 | Macromolecular Complex Assembly |
| NOD2 | 0.524 | 0.806 | −1.06 | 2.1 | 0.531 | Cytoplasm |
| TLR1 | −0.302 | 0.468 | −1.22 | 0.616 | 0.534 | Biosynthetic Process |
| SMAD5 | 0.762 | 1.19 | −1.58 | 3.1 | 0.538 | Cell Surface Receptor-Linked Signal Transduction |
| PML | 0.387 | 0.607 | −0.803 | 1.58 | 0.538 | Cell Fraction |
| CXCR4 | 0.485 | 0.764 | −1.01 | 1.98 | 0.54 | Cell Development |
| IRF1 | −0.178 | 0.283 | −0.732 | 0.376 | 0.542 | Biopolymer Metabolic Process |
| LGALS3 | 1.02 | 1.63 | −2.17 | 4.2 | 0.545 | Carbohydrate Binding |
| ENTPD1 | −0.483 | 0.777 | −2.01 | 1.04 | 0.548 | Hemostasis |
| ICOSLG | −0.415 | 0.67 | −1.73 | 0.898 | 0.55 | Cell Activation |
| BCL2 | 0.496 | 0.801 | −1.07 | 2.06 | 0.55 | Cytoplasm |
| STAT2 | 0.804 | 1.3 | −1.74 | 3.35 | 0.55 | Biopolymer Metabolic Process |
| CD86 | −0.478 | 0.773 | −1.99 | 1.04 | 0.55 | Cell Proliferation |
| CD276 | 0.844 | 1.38 | −1.86 | 3.54 | 0.554 | Biosynthetic Process |
| MAF | 0.342 | 0.562 | −0.759 | 1.44 | 0.556 | Biopolymer Metabolic Process |

<div align="center">Table A3. *Cont.*</div>

| Gene | Log2-Fold Change | SE | Lower Confidence Limit (log2) | Upper Confidence Limit (log2) | *p* | Tentative Function |
|---|---|---|---|---|---|---|
| IFIT2 | −0.568 | 0.944 | −2.42 | 1.28 | 0.56 | Innate Immune |
| CD14 | 0.893 | 1.49 | −2.02 | 3.81 | 0.561 | Apoptosis |
| CD81 | 1.08 | 1.8 | −2.45 | 4.61 | 0.562 | Integral to Membrane |
| ITGA5 | −0.459 | 0.784 | −2 | 1.08 | 0.571 | Integral to Membrane |
| DPP4 | 0.884 | 1.51 | −2.08 | 3.84 | 0.571 | Immune Response |
| CSF1R | −0.326 | 0.564 | −1.43 | 0.78 | 0.577 | Cell Proliferation |
| IRF3 | 0.227 | 0.393 | −0.544 | 0.997 | 0.577 | Biopolymer Metabolic Process |
| JAK2 | 0.736 | 1.29 | −1.79 | 3.26 | 0.581 | Anatomical Structure Development |
| ICAM1 | 0.383 | 0.673 | −0.936 | 1.7 | 0.581 | Integral to Membrane |
| FKBP5 | −0.396 | 0.697 | −1.76 | 0.969 | 0.582 | Cellular Macromolecule Metabolic Process |
| TNFSF12 | 0.344 | 0.608 | −0.847 | 1.54 | 0.583 | Cell Development |
| MRC1 | 0.841 | 1.5 | −2.09 | 3.77 | 0.587 | Carbohydrate Binding |
| SYK | 0.343 | 0.612 | −0.856 | 1.54 | 0.587 | Anatomical Structure Morphogenesis |
| RELB | 0.316 | 0.564 | −0.79 | 1.42 | 0.588 | DNA Binding |
| IRF5 | 0.369 | 0.666 | −0.935 | 1.67 | 0.591 | Immune Transcription Factor |
| IRF8 | 0.368 | 0.681 | −0.967 | 1.7 | 0.601 | Biopolymer Metabolic Process |
| SKI | 0.684 | 1.27 | −1.8 | 3.17 | 0.602 | Repressor of TGF-β |
| C3 | 0.517 | 0.96 | −1.37 | 2.4 | 0.602 | Cell Surface Receptor-Linked Signal Transduction |
| PTGS2 | −0.508 | 0.945 | −2.36 | 1.34 | 0.603 | Cytoplasm |
| LITAF | 0.354 | 0.659 | −0.938 | 1.65 | 0.603 | Biopolymer Metabolic Process |
| BLNK | −0.312 | 0.586 | −1.46 | 0.836 | 0.606 | Anatomical Structure Development |
| IFNAR2 | 0.174 | 0.326 | −0.466 | 0.813 | 0.607 | Cell Surface Receptor-Linked Signal Transduction |
| MUC1 | 0.935 | 1.79 | −2.57 | 4.44 | 0.613 | Integral to Membrane |
| NOD1 | −0.62 | 1.2 | −2.97 | 1.73 | 0.616 | Biosynthetic Process |
| PDCD2 | 0.275 | 0.534 | −0.772 | 1.32 | 0.618 | Apoptosis |
| CD82 | −0.326 | 0.638 | −1.58 | 0.925 | 0.62 | Integral to Membrane |
| NFIL3 | 0.593 | 1.18 | −1.72 | 2.91 | 0.626 | Biopolymer Metabolic Process |
| TCF7 | 0.363 | 0.736 | −1.08 | 1.81 | 0.633 | Biopolymer Metabolic Process |
| ATG10 | −0.576 | 1.18 | −2.88 | 1.73 | 0.635 | Autophagocytosis |
| LILRB1 | 0.405 | 0.838 | −1.24 | 2.05 | 0.639 | Integral to Membrane |
| FCGRT | 0.757 | 1.58 | −2.33 | 3.85 | 0.641 | Immune Response |
| ICAM3 | −0.32 | 0.673 | −1.64 | 0.999 | 0.645 | Integral to Membrane |
| STAT1 | 0.33 | 0.695 | −1.03 | 1.69 | 0.645 | Biopolymer Metabolic Process |
| IL6ST | 0.905 | 1.91 | −2.84 | 4.65 | 0.645 | Cell Surface Receptor-Linked Signal Transduction |
| PDGFRB | −0.863 | 1.85 | −4.48 | 2.76 | 0.65 | Phosphotransferase Activity Alcohol Group as Acceptor |
| CSF1 | 0.335 | 0.722 | −1.08 | 1.75 | 0.653 | Cell Proliferation |
| IL1RAP | 0.358 | 0.782 | −1.17 | 1.89 | 0.657 | Cellular Component Assembly |
| MCL1 | 0.13 | 0.288 | −0.434 | 0.695 | 0.66 | Cell Development |
| NOTCH2 | 0.332 | 0.744 | −1.13 | 1.79 | 0.665 | Cell Development |
| IFNGR1 | 0.755 | 1.71 | −2.59 | 4.1 | 0.668 | Integral to Membrane |
| NFATC1 | −0.346 | 0.782 | −1.88 | 1.19 | 0.668 | Biopolymer Metabolic Process |
| SMAD3 | −0.262 | 0.594 | −1.43 | 0.902 | 0.668 | Macromolecular Complex |
| STAT4 | −0.358 | 0.812 | −1.95 | 1.23 | 0.669 | DNA Binding |
| C1S | −0.607 | 1.39 | −3.33 | 2.11 | 0.671 | Endopeptidase Activity |
| CX3CR1 | 0.364 | 0.859 | −1.32 | 2.05 | 0.681 | Behavior |
| LTB4R | 0.322 | 0.77 | −1.19 | 1.83 | 0.685 | Cell Surface Receptor-Linked Signal Transduction |
| ARHGDIB | 0.307 | 0.741 | −1.15 | 1.76 | 0.687 | Cytoplasm |
| JAK3 | 0.239 | 0.59 | −0.917 | 1.4 | 0.693 | Biopolymer Metabolic Process |
| TLR8 | 0.301 | 0.749 | −1.17 | 1.77 | 0.696 | Biosynthetic Process |
| RAF1 | 0.533 | 1.33 | −2.07 | 3.14 | 0.697 | Biopolymer Metabolic Process |
| CSF3R | 0.22 | 0.556 | −0.87 | 1.31 | 0.701 | Defense Response |
| SIGIRR | 0.272 | 0.693 | −1.09 | 1.63 | 0.703 | Membrane |

Table A3. *Cont.*

| Gene | Log2-Fold Change | SE | Lower Confidence Limit (log2) | Upper Confidence Limit (log2) | *p* | Tentative Function |
|------|------------------|-----|-------------------------------|-------------------------------|-----|--------------------|
| ATG16L1 | −0.436 | 1.13 | −2.66 | 1.79 | 0.709 | Autophagy |
| SOCS1 | −0.553 | 1.46 | −3.41 | 2.3 | 0.712 | Biopolymer Metabolic Process |
| POU2F2 | 0.26 | 0.694 | −1.1 | 1.62 | 0.715 | Biopolymer Metabolic Process |
| HLA-DMB | −0.311 | 0.839 | −1.96 | 1.33 | 0.719 | Antigen Presentation |
| MAP4K2 | −0.52 | 1.41 | −3.28 | 2.24 | 0.72 | Biopolymer Metabolic Process |
| IFIH1 | 0.186 | 0.509 | −0.812 | 1.18 | 0.722 | B-Cell Differentiation |
| TGFBR1 | 0.5 | 1.38 | −2.21 | 3.21 | 0.726 | Biopolymer Metabolic Process |
| B2M | −0.212 | 0.59 | −1.37 | 0.944 | 0.726 | Antimicrobial Protein |
| STAT5A | −0.286 | 0.804 | −1.86 | 1.29 | 0.729 | DNA Binding |
| IGF2R | 0.436 | 1.27 | −2.05 | 2.92 | 0.738 | Cytoplasm |
| CD34 | −0.175 | 0.516 | −1.19 | 0.836 | 0.741 | Carbohydrate Binding |
| ITGB1 | −0.191 | 0.563 | −1.29 | 0.912 | 0.741 | Cell–Cell Adhesion |
| TLR3 | −0.329 | 0.974 | −2.24 | 1.58 | 0.742 | Biosynthetic Process |
| CCL13 | −0.311 | 0.926 | −2.13 | 1.5 | 0.744 | Behavior |
| LAMP3 | 0.248 | 0.739 | −1.2 | 1.7 | 0.744 | Cell Proliferation |
| CCBP2 | −0.174 | 0.522 | −1.2 | 0.85 | 0.746 | Behavior |
| IDO1 | 0.261 | 0.787 | −1.28 | 1.8 | 0.747 | Tryptophan Catabolism |
| MME | 0.244 | 0.739 | −1.2 | 1.69 | 0.748 | Cell–Cell Signaling |
| MSR1 | 0.487 | 1.48 | −2.4 | 3.38 | 0.748 | Establishment of Localization |
| C7 | −0.292 | 0.908 | −2.07 | 1.49 | 0.754 | Integral to Membrane |
| CD36 | 0.603 | 1.88 | −3.09 | 4.3 | 0.755 | Cell Fraction |
| IL16 | −0.147 | 0.462 | −1.05 | 0.759 | 0.757 | Extracellular Region |
| CISH | −0.232 | 0.733 | −1.67 | 1.21 | 0.759 | Suppressor of Cytokine Signaling |
| CCL2 | 0.256 | 0.827 | −1.37 | 1.88 | 0.763 | Biopolymer Metabolic Process |
| CD163 | −0.265 | 0.886 | −2 | 1.47 | 0.771 | Integral to Membrane |
| STAT5B | 0.502 | 1.69 | −2.8 | 3.81 | 0.772 | DNA Binding |
| SLC2A1 | −0.123 | 0.419 | −0.944 | 0.698 | 0.776 | Cell Fraction |
| IRAK4 | −0.372 | 1.29 | −2.91 | 2.16 | 0.779 | Activates NFκB |
| DUSP4 | 0.203 | 0.708 | −1.18 | 1.59 | 0.78 | Biopolymer Metabolic Process |
| CEBPB | −0.158 | 0.553 | −1.24 | 0.925 | 0.78 | Biopolymer Metabolic Process |
| ITGA4 | 0.213 | 0.748 | −1.25 | 1.68 | 0.782 | Identical Protein Binding |
| CTSS | 0.22 | 0.819 | −1.39 | 1.83 | 0.794 | Cellular Macromolecule Metabolic Process |
| IKZF2 | −0.362 | 1.35 | −3.01 | 2.28 | 0.794 | Lymphocyte Development |
| LY96 | 0.189 | 0.711 | −1.2 | 1.58 | 0.796 | Cell Surface Receptor-Linked Signal Transduction |
| CLEC7A | 0.214 | 0.812 | −1.38 | 1.81 | 0.797 | Cell Activation |
| HAVCR2 | −0.207 | 0.787 | −1.75 | 1.34 | 0.798 | Th1 Surface Protein |
| ICAM2 | −0.388 | 1.48 | −3.29 | 2.51 | 0.798 | Integral to Membrane |
| PSMB8 | 0.354 | 1.35 | −2.3 | 3 | 0.799 | Antigen Presentation |
| C1R | −0.243 | 0.945 | −2.09 | 1.61 | 0.802 | Endopeptidase Activity |
| ABL1 | −0.375 | 1.48 | −3.27 | 2.52 | 0.805 | Biopolymer Metabolic Process |
| TLR5 | −0.117 | 0.466 | −1.03 | 0.796 | 0.807 | Innate Immunity |
| BST2 | −0.406 | 1.64 | −3.63 | 2.81 | 0.81 | IκB Kinase NFκB Cascade |
| IL6R | 0.159 | 0.653 | −1.12 | 1.44 | 0.813 | Cell Surface Receptor-Linked Signal Transduction |
| TMEM173 | 0.139 | 0.642 | −1.12 | 1.4 | 0.833 | Innate Immunity |
| IL10RA | −0.18 | 0.835 | −1.82 | 1.46 | 0.833 | Interleukin Binding |
| CDH5 | −0.401 | 1.9 | −4.13 | 3.33 | 0.837 | Cell–Cell Adhesion |
| PSMB10 | 0.273 | 1.31 | −2.3 | 2.85 | 0.839 | Humoral Immune Response |
| NFATC3 | 0.257 | 1.24 | −2.17 | 2.68 | 0.84 | Biopolymer Metabolic Process |
| BCL2L11 | 0.108 | 0.551 | −0.972 | 1.19 | 0.849 | Apoptosis |
| EDNRB | −0.308 | 1.62 | −3.49 | 2.87 | 0.853 | Integral to Membrane |
| TICAM1 | 0.119 | 0.632 | −1.12 | 1.36 | 0.854 | IκB Kinase NFκB Cascade |
| JAK1 | −0.286 | 1.56 | −3.35 | 2.77 | 0.858 | Interferon Signal Transduction |
| MAP4K1 | 0.115 | 0.632 | −1.12 | 1.35 | 0.859 | Biopolymer Metabolic Process |
| C1QBP | −0.0783 | 0.452 | −0.964 | 0.807 | 0.866 | Immune Response |
| CD97 | 0.302 | 1.76 | −3.15 | 3.75 | 0.867 | Cell–Cell Signaling |
| CMKLR1 | −0.271 | 1.6 | −3.41 | 2.86 | 0.869 | Behavior |

<div align="center">

**Table A3.** *Cont.*

</div>

| Gene | Log2-Fold Change | SE | Lower Confidence Limit (log2) | Upper Confidence Limit (log2) | *p* | Tentative Function |
|---|---|---|---|---|---|---|
| ABCB1 | −0.109 | 0.655 | −1.39 | 1.17 | 0.871 | Cell Fraction |
| ITGA6 | −0.0569 | 0.384 | −0.81 | 0.696 | 0.885 | Cellular Component Assembly |
| CFI | −0.123 | 0.832 | −1.75 | 1.51 | 0.885 | Endopeptidase Activity |
| TOLLIP | −0.227 | 1.55 | −3.27 | 2.81 | 0.886 | Cell–Cell Signaling |
| CFH | −0.144 | 1.02 | −2.14 | 1.85 | 0.89 | Extracellular Region |
| NFKB1 | −0.177 | 1.26 | −2.65 | 2.3 | 0.891 | Biopolymer Metabolic Process |
| FN1 | −0.0987 | 0.729 | −1.53 | 1.33 | 0.895 | Cytoplasm |
| IRAK3 | 0.114 | 0.846 | −1.54 | 1.77 | 0.896 | Biopolymer Metabolic Process |
| CXCR6 | 0.0995 | 0.75 | −1.37 | 1.57 | 0.897 | Cell Surface Receptor-Linked Signal Transduction |
| TCF4 | 0.0836 | 0.707 | −1.3 | 1.47 | 0.908 | Biopolymer Metabolic Process |
| LILRB5 | 0.0831 | 0.747 | −1.38 | 1.55 | 0.914 | Cell Surface Receptor-Linked Signal Transduction |
| IKBKB | 0.0595 | 0.55 | −1.02 | 1.14 | 0.916 | Activates NFκB |
| IKBKE | 0.0559 | 0.578 | −1.08 | 1.19 | 0.925 | Biopolymer Metabolic Process |
| CD19 | 0.126 | 1.36 | −2.54 | 2.79 | 0.928 | Cell Surface Receptor-Linked Signal Transduction |
| IRAK1 | −0.119 | 1.39 | −2.85 | 2.61 | 0.934 | Cellular Component Assembly |
| UBE2L3 | −0.112 | 1.49 | −3.03 | 2.8 | 0.942 | Biopolymer Metabolic Process |
| CD53 | 0.126 | 1.73 | −3.26 | 3.52 | 0.943 | Membrane |
| TRAF4 | −0.1 | 1.41 | −2.87 | 2.67 | 0.945 | DNA Binding |
| THY1 | −0.0637 | 0.979 | −1.98 | 1.86 | 0.949 | Cell Surface |
| ATG5 | −0.108 | 1.75 | −3.53 | 3.31 | 0.952 | Cytoplasm |
| CEACAM1 | 0.0489 | 0.824 | −1.57 | 1.66 | 0.954 | Cell Fraction |
| CCND3 | 0.0844 | 1.55 | −2.96 | 3.13 | 0.958 | Biopolymer Metabolic Process |
| MAPK1 | −0.0932 | 1.94 | −3.89 | 3.7 | 0.963 | Behavior |
| CD164 | −0.0906 | 1.98 | −3.96 | 3.78 | 0.964 | Cell–Cell Adhesion |
| NOTCH1 | 0.03 | 0.657 | −1.26 | 1.32 | 0.965 | Cell Development |
| CRADD | −0.0272 | 0.617 | −1.24 | 1.18 | 0.966 | Apoptosis |
| TP53 | −0.0682 | 1.76 | −3.52 | 3.38 | 0.97 | Cell Fraction |
| BAX | 0.0666 | 1.81 | −3.47 | 3.61 | 0.971 | Cytoplasm |
| CASP1 | 0.0524 | 1.43 | −2.75 | 2.86 | 0.971 | Cellular Protein Metabolic Process |
| IKBKG | −0.0416 | 1.19 | −2.37 | 2.28 | 0.973 | Cell Development |
| TYK2 | 0.0467 | 1.37 | −2.63 | 2.73 | 0.973 | Biopolymer Metabolic Process |
| CLEC4E | −0.0351 | 1.08 | −2.16 | 2.09 | 0.975 | Carbohydrate Binding |
| CFB | 0.0283 | 0.901 | −1.74 | 1.79 | 0.976 | Complement Activation |
| LAIR1 | −0.0191 | 0.739 | −1.47 | 1.43 | 0.98 | Inhibitory Receptor |
| CD58 | −0.0354 | 1.4 | −2.79 | 2.72 | 0.98 | T Cell Activation |
| KCNJ2 | 0.00799 | 0.384 | −0.744 | 0.76 | 0.984 | Establishment of Localization |
| PLAU | −0.016 | 0.788 | −1.56 | 1.53 | 0.984 | Behavior |
| TRAF3 | 0.0112 | 0.57 | −1.11 | 1.13 | 0.985 | Apoptosis |
| CCR1 | 0.0271 | 1.38 | −2.68 | 2.74 | 0.985 | Cell–Cell Signaling |
| CD9 | −0.0138 | 0.765 | −1.51 | 1.48 | 0.986 | Anatomical Structure Morphogenesis |
| APP | −0.00668 | 0.389 | −0.769 | 0.756 | 0.987 | Cell Surface |
| ARG2 | 0.0106 | 0.761 | −1.48 | 1.5 | 0.989 | Cytoplasm |
| IL4R | 0.0159 | 1.64 | −3.21 | 3.24 | 0.992 | Immune Response |
| CD4 | 0.0114 | 1.48 | −2.88 | 2.91 | 0.994 | Cell Activation |
| CYBB | 0.00269 | 0.554 | −1.08 | 1.09 | 0.996 | Defense Response |
| KIT | $-4.48 \times 10^{-16}$ | 0.576 | −1.13 | 1.13 | 1 | Phosphotransferase Activity Alcohol Group as Acceptor |

# References

1. Drake, P.; Driscoll, A.K.; Mathews, T.J. *Cigarette Smoking During Pregnancy*; NCHS Data Brief: Hyattsville, MD, USA, 2018; pp. 1–8.
2. Spencer, K.; Cowans, N.J. Accuracy of self-reported smoking status in first trimester aneuploidy screening. *Prenat. Diagn.* **2013**, *33*, 245–250. [CrossRef] [PubMed]
3. Kyrklund-Blomberg, N.B.; Cnattingius, S. Preterm birth and maternal smoking: Risks related to gestational age and onset of delivery. *J. Obstet. Gynaecol.* **1998**, *179*, 1051–1055. [CrossRef]
4. Nicoletti, D. Maternal smoking during pregnancy and birth defects in children: A systematic review with meta-analysis. *Cad. Saude Publica* **2014**, *30*, 2491–2529. [CrossRef] [PubMed]
5. Hackshaw, A.; Rodeck, C.; Boniface, S. Maternal smoking in pregnancy and birth defects: A systematic review based on 173 687 malformed cases and 11.7 million controls. *Hum. Reprod. Update* **2011**, *17*, 589–604. [CrossRef]
6. Antonucci, R.; Contu, P.; Porcella, A.; Atzeni, C.; Chiappe, S. Intrauterine smoke exposure: A new risk factor for bronchopulmonary dysplasia? *J. Perinat. Med.* **2004**, *32*, 272–277. [CrossRef]
7. Jensen, E.A.; Schmidt, B. Epidemiology of bronchopulmonary dysplasia. *Birth Defects Res. Part A Clin. Mol. Teratol.* **2014**, *100*, 145–157. [CrossRef]
8. Morrow, L.A.; Wagner, B.D.; Ingram, D.A.; Poindexter, B.B.; Schibler, K.; Cotten, C.M.; Dagle, J.; Sontag, M.K.; Mourani, P.M.; Abman, S.H. Antenatal Determinants of Bronchopulmonary Dysplasia and Late Respiratory Disease in Preterm Infants. *Am. J. Respir. Crit. Care Med.* **2017**, *196*, 364–374. [CrossRef]
9. Watterberg, K.L.; Demers, L.M.; Scott, S.M.; Murphy, S. Chorioamnionitis and Early Lung Inflammation in Infants in Whom Bronchopulmonary Dysplasia Develops. *Pediatrics* **1996**, *97*, 210–215. [CrossRef]
10. Hansen, A.R.; Barnés, C.M.; Folkman, J.; McElrath, T.F. Maternal Preeclampsia Predicts the Development of Bronchopulmonary Dysplasia. *J. Pediatrics* **2010**, *156*, 532–536. [CrossRef]
11. Eriksson, L.; Haglund, B.; Odlind, V.; Altman, M.; Ewald, U.; Kieler, H. Perinatal conditions related to growth restriction and inflammation are associated with an increased risk of bronchopulmonary dysplasia. *Acta Paediatr.* **2015**, *104*, 259–263. [CrossRef]
12. Gemmell, L.; Martin, L.; Murphy, K.E.; Modi, N.; Håkansson, S.; Reichman, B.; Lui, K.; Kusuda, S.; Sjörs, G.; Mirea, L.; et al. Hypertensive disorders of pregnancy and outcomes of preterm infants of 24 to 28 weeks' gestation. *J. Perinatol.* **2016**, *36*, 1067–1072. [CrossRef] [PubMed]
13. Xu, S.; Venge, P. Lipocalins as biochemical markers of disease. *Biochim. Biophys. Acta (BBA)-Protein Struct. Mol. Enzymol.* **2000**, *1482*, 298–307. [CrossRef]
14. Inoue, H.; Ohga, S.; Kusuda, T.; Kitajima, J.; Kinjo, T.; Ochiai, M.; Takahata, Y.; Honjo, S.; Hara, T. Serum neutrophil gelatinase-associated lipocalin as a predictor of the development of bronchopulmonary dysplasia in preterm infants. *Early Hum. Dev* **2013**, *89*, 425–429. [CrossRef] [PubMed]
15. Naeye, R.L. Effects of maternal cigarette smoking on the fetus and placenta. *BJOG. Int. J. Obstet.* **1978**, *85*, 732–737. [CrossRef]
16. Jobe, A.H. Mechanisms of lung injury and bronchopulmonary dysplasia. *Am. J. Perinatol.* **2016**, *33*, 1076–1078. [CrossRef]
17. Gonzalez-Luis, G.E.; Westering-Kroon, E.V.; Villamor-Martinez, E.; Huizing, M.J.; Kilani, M.A.; Kramer, B.W.; Villamor, E. Tobacco smoking during pregnancy is associated with increased risk of moderate/severe bronchopulmonary dysplasia: A systematic review and Meta-Analysis. *Front. Pediatrics* **2020**, *8*, 160. [CrossRef]
18. Gibbs, K.; Collaco, J.M.; McGrath-Morrow, S.A. Impact of tobacco smoke and nicotine exposure on lung development. *Chest* **2016**, *149*, 552–561. [CrossRef]
19. Been, J.V.; Millett, C. Reducing the global burden of preterm births. *Lancet Glob. Health* **2019**, *7*, 414. [CrossRef]
20. Wagijo, M.A.; Sheikh, A.; Duijts, L.; Been, J.V. Reducing tobacco smoking and smoke exposure to prevent preterm birth and its complications. *Paediatr. Respir.* **2017**, *22*, 3–10. [CrossRef]
21. Lauren Comarda, J.E. Maternal Tobacco Exposure Leads to Cytokine Dysregulation in Placental Membranes Stimulated with Lipopolysaccharide. In Proceedings of the Pediatric Academic Society, Toronto, ON, Canada, 18 September 2018; pp. 397–412.
22. Jobe, A.H.; Bancalari, E. Bronchopulmonary dysplasia. *Am. J. Respir. Crit. Care Med.* **2001**, *163*, 1723–1729. [CrossRef]
23. Fajardy, I.; Moitrot, E.; Vambergue, A.; Vandersippe-Millot, M.; Deruelle, P.; Rousseaux, J. Time course analysis of RNA stability in human placenta. *BMC Mol. Biol.* **2009**, *10*, 21. [CrossRef] [PubMed]
24. Davidson, L.M.; Berkelhamer, S.K. Bronchopulmonary Dysplasia: Chronic Lung Disease of Infancy and Long-Term Pulmonary Outcomes. *J. Clin. Med.* **2017**, *6*, 4. [CrossRef] [PubMed]
25. Cheong, J.L.Y.; Doyle, L.W. An update on pulmonary and neurodevelopmental outcomes of bronchopulmonary dysplasia. *Semin. Perinatol.* **2018**, *42*, 478–484. [CrossRef] [PubMed]
26. Tadesse, S.; Luo, G.; Park, J.S.; Kim, B.J.; Snegovskikh, V.V.; Zheng, T.; Hodgson, E.J.; Arcuri, F.; Toti, P.; Parikh, C.R.; et al. Intra-amniotic infection upregulates neutrophil gelatinase-associated lipocalin (NGAL) expression at the maternal-fetal interface at term: Implications for infection-related preterm birth. *Reprod. Sci.* **2011**, *18*, 713–722. [CrossRef]
27. Lee, J.; Taneja, V.; Vassallo, R. Cigarette smoking and inflammation: Cellular and molecular mechanisms. *J. Dent. Res.* **2012**, *91*, 142–149. [CrossRef]
28. Gonçalves, R.B.; Coletta, R.D.; Silvério, K.G.; Benevides, L.; Casati, M.Z.; da Silva, J.S.; Nociti, F.H. Impact of smoking on inflammation: Overview of molecular mechanisms. *Inflamm. Res.* **2011**, *60*, 409–424. [CrossRef]

29. Yoon, B.H.; Romero, R.; Jun, J.K.; Park, K.H.; Park, J.D.; Ghezzi, F.; Kim, B.I. Amniotic fluid cytokines (interleukin-6, tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8) and the risk for the development of bronchopulmonary dysplasia. *Am. J. Obstet. Gynecol.* **1997**, *177*, 825–830. [CrossRef]

30. Shimoya, K.; Moriyama, A.; Matsuzaki, N.; Ogata, I.; Koyama, M.; Azuma, C.; Saji, F.; Murata, Y. Human placental cells show enhanced production of interleukin (IL)-8 in response to lipopolysaccharide (LPS), IL-1 and tumour necrosis factor (TNF)-alpha, but not to IL-6. *Mol. Hum. Reprod.* **1999**, *5*, 885. [CrossRef]

31. Nair, R.R.; Khanna, A.; Singh, K. Role of inflammatory proteins S100A8 and S100A9 in pathophysiology of recurrent early pregnancy loss. *Placenta* **2013**, *34*, 824–827. [CrossRef]

32. Isayama, T.; Shah, P.S.; Ye, X.Y.; Dunn, M.; Da Silva, O.; Alvaro, R.; Lee, S.K. Adverse Impact of Maternal Cigarette Smoking on Preterm Infants: A Population-Based Cohort Study. *Am. J. Perinatol.* **2015**, *32*, 1105–1111. [CrossRef]

33. Spinillo, A.; Ometto, A.; Stronati, M.; Piazzi, G.; Iasci, A.; Rondini, G. Epidemiologic association between maternal smoking during pregnancy and intracranial hemorrhage in preterm infants. *J. Pediatrics* **1995**, *127*, 472–478. [CrossRef]