

**THE AMME STUDY:
AMNIOTIC FLUID AS MEDIATOR OF THE
MATERNAL ENVIRONMENT**

BY

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Abstract

The development of chronic lung disease is partly due to fetal programming during gestation. *In utero* cigarette smoke exposure has been linked to adverse neonatal outcomes as well as detrimental effects to the lungs. The mechanism by which *in utero* cigarette smoke exposure is translated to negative effects to the neonate is not elucidated. Fetal breathing movements allow for the exchange of amniotic fluid and fetal lung liquid, a liquid secreted by the pulmonary epithelium. These movements are vital for optimal fetal lung development, growth, and pulmonary cell differentiation. Elevated levels of IL-5, TNF α , IL-1 α , and IL-1 β in the amniotic fluid promote airway hyperresponsiveness and lung inflammation in the offspring, both are hallmarks of chronic lung disease. However, the non-pathologic cytokine/chemokine profile and whether environmental exposures alter it is unknown. Therefore, I hypothesize that *in utero* cigarette smoke exposure alters the cytokine/chemokine profile of the amniotic fluid, and that amniotic fluid can alter airway epithelial barrier function.

Matched amniotic fluid (n = 32), umbilical cord serum (n = 33), maternal serum (n = 33), and placenta tissue (n = 36) were collected from singleton, uncomplicated, term, and scheduled caesarean delivery. Cigarette smoke exposure was collected by questionnaire and confirmed by measuring cotinine, a nicotine metabolite, by direct ELISA. All samples were sent for multiplex cytokine analysis by Eve Technologies (Calgary, AB) to assay for 99 cytokines/chemokines. In a transwell culture using Calu-3 airway epithelial cells, epithelial barrier function and wound recovery was assessed by exposing cells to individual amniotic fluid samples and measuring transepithelial electrical resistance.

Cotinine bioaccumulates in amniotic fluid compared to umbilical cord serum and maternal serum. Cotinine was not detected in placenta samples. Cotinine-positive amniotic fluid samples

have a different cytokine/chemokine profile characterized by higher levels of MIG/CXCL9, IP-10, and IL-6 relative to cotinine-negative samples. Barrier function was significantly negatively correlated to abundances of such as IL-13 and IL-9. Wound healing capacity was significantly positively correlated to abundances of EGF and CXCL16.

In utero exposure to cigarette smoke alters the amniotic fluid cytokine/chemokine profile thereby increasing the abundances of a number of cytokines, such as IL-6. Additionally, cotinine, the major metabolite of nicotine, bioaccumulates within the amniotic fluid. The barrier function and wound healing capacity of airway epithelial cells are altered by amniotic fluid and is associated with its cytokine/chemokine profile. These results provide evidence that the amniotic fluid composition, including its cytokine/chemokine profile, can be influenced by the maternal environment and may be an important communication pathway between *in utero* exposures and the fetal lungs.

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Dedication

Behind every scientist is a group chat of family or friends pretending to know what you're talking about. For that, I would like to dedicate this thesis to my family, especially to my grandparents who have instilled in me the importance of education. Without my family, I would not have been able to attain higher education. I would also like to dedicate this thesis to my girlies for supporting me as I went through the highs, the lows, and the extreme lows of research: Patricia, Chester, and Bryan. Finally, to Riche Ann Valencia and Manuel Siguenza, thank you for being my best friends and for *always* being there for me.

Table of Contents

Abstract.....	i
Acknowledgements	iii
Dedication	v
List of Figures.....	ix
List of Tables.....	x
List of Appendices	xi
List of Abbreviations.....	xii
Chapter 1: Introduction	1
1.1 Developmental Origins of Health and Disease	2
1.1.1 Overview of Developmental Origins of Health and Disease (DOHaD).....	2
1.1.2 Developmental Origins of Lung Health and Disease	2
1.2 Amniotic Fluid	5
1.2.1 Amniotic Fluid Composition	5
1.2.2 Fluid Exchange Between Amniotic Fluid and Fetal Lung	6
1.2.3 Amniotic Fluid Role in Fetal Development.....	7
1.3 Inflammation in Pregnancy	8
1.3.1 Introduction.....	8
1.3.2 Intra-amniotic Inflammation and Pulmonary Outcomes: Exogenous Sources of Inflammation	9
1.3.3 Intra-amniotic Inflammation and Pulmonary Outcomes: Endogenous Sources of Inflammation	12
1.4 Amniotic Fluid Inflammatory Profiles and Fetal Lung Development	12
1.4.1 Inflammatory Biomarkers in the Amniotic Fluid	12
1.4.2 Umbilical Cord as a Communication Pathway	14
1.5 Airway Epithelium and Epithelial Cells	15
1.5.1 Airway Epithelium Introduction	15
1.5.2 Airway Epithelial Remodeling and Dysfunction	16
1.5.3 Nicotine Disrupts the Epithelial Barrier.....	17
1.5.4 Airway Epithelial Cell Cytokines	18
1.6 Knowledge Gaps and Hypothesis	21
1.6.1 Summary of Knowledge Gaps	21
1.6.2 Hypotheses	22
1.7 Project Objectives	22

Chapter 2: Methods	23
2.1 Patient Recruitment and Sample Collection	24
2.1.1 Patient Recruitment Protocol and Questionnaire.....	24
2.1.2 Sample Collection.....	25
2.1.3 Cigarette Smoke Exposure Confirmation.....	26
2.1.4 Processing of Placenta Tissue.....	26
2.2 Cytokine Analysis.....	27
2.2.1 Amniotic Fluid and Serum Sample Preparation	28
2.2.2 Cytokine Profile Data Pre-Processing.....	28
2.3 Cell Experiments	29
2.3.1 Cell Culture and Barrier Function Measurement	29
2.3.2 Amniotic Fluid Effect on Epithelial Barrier Function.....	30
2.3.3 Amniotic Fluid Effect on Epithelial Wound Healing (Barrier Recovery).....	31
2.4 Data Analysis	32
2.4.1 Cytokine Profile Data Analysis	32
2.4.2 Cotinine ELISA Analysis	33
2.4.3 Statistical Analysis	33
Chapter 3: Results.....	34
3.1 Objective 1 Results.....	35
3.1.1 Participant Characteristics	35
3.1.2 The Amniotic Fluid has a Unique Inflammatory Profile.....	37
3.1.3 The Amniotic Fluid Inflammatory Profile is a Network	42
3.1.4 The Amniotic Fluid Inflammatory Profile is Altered by Cigarette Smoke Exposure	50
3.2 Objective 2 Results.....	55
3.2.1 Amniotic Fluid Alters Calu-3 Barrier Function at 24 Hours of Exposure	55
3.2.2 Amniotic Fluid Augments Calu-3 Wound Healing	59
Chapter 4: Discussion	64
4.1 Summary of Study Findings	65
4.2 Relevance to Developmental Origins of Health and Disease	66
4.3 The Amniotic Fluid as a Medium of Communication	70
4.4 Amniotic Fluid is Influenced by the Maternal Environment.....	72
4.5 Amniotic Fluid Affects Airway Epithelial Barrier Function.....	74
4.6 Amniotic Fluid Augments Airway Epithelial Barrier Recovery After Wounding	75
4.7 Significance.....	77

4.8 Limitations.....	78
4.8 Future Directions	80
Appendices.....	83
Appendix A	84
Appendix B	85
Appendix C	88
Appendix D.....	92
References	95

List of Figures

Figure 1. Workflow of cytokine/chemokine profile identification and quantification.	29
Figure 2. Schematic of experiments measuring Calu-3 epithelial barrier function in response to amniotic fluid.	30
Figure 3. Schematic of wound healing experiments measuring the effect of amniotic fluid on barrier function recovery of Calu-3 cells after wounding.....	31
Figure 4. Principal component analysis (PCA) reveals unique cytokine/chemokine profiles for tissues present in pregnancy	39
Figure 5. Umbilical cord serum and maternal serum are characterized by abundances of some cytokines.....	40
Figure 6. Amniotic fluid and placenta tissue are characterized by abundances of some cytokines.....	41
Figure 7. Cytokines and chemokines in the amniotic fluid show positive correlation.....	43
Figure 8. Amniotic fluid cytokine/chemokine profile is characterized by groups with correlated abundances.....	44
Figure 9. Some amniotic fluid cytokine/chemokine clusters correlate negatively with each other	45
Figure 10. Functional enrichment analyses of cytokine/chemokine clusters predict protein-protein interactions	47
Figure 11. Cotinine bioaccumulates in amniotic fluid	51
Figure 12. The cytokine/chemokine profile of amniotic fluid is altered by cigarette smoke exposure	52
Figure 13. Amniotic fluid exposed to cigarette smoke is characterized by higher abundances of some cytokines.....	53
Figure 14. Differential abundance analysis shows some cytokines are significantly elevated in cotinine-positive amniotic fluid	54
Figure 15. Calu-3 barrier function increases at 24 hours after exposure	56
Figure 16. Calu-3 barrier function is altered by exposure to amniotic fluid.....	57
Figure 17. Fractalkine, IFN α 2, IL-9, and IL-13 abundances in amniotic fluid are significantly negatively correlated to Calu-3 barrier function	58
Figure 18. Amniotic fluid augments the recovery of barrier function of Calu-3 cells after wounding.....	59
Figure 19. Abundances of certain cytokines and chemokines in amniotic fluid are significantly correlated to Calu-3 barrier function.....	61
Figure 20. Amniotic fluid augments the wound healing capacity of Calu-3 cells	62
Figure 21. Abundances of cytokines and chemokines are positively and negatively correlated with wound healing capacity	63
Figure 22. Experimental design for the <i>in utero</i> cigarette smoke exposure in a rhesus macaque model to assess the influence of the amniotic fluid milieu on offspring airway epithelial barrier function.	82

List of Tables

Table 1: Inclusion criteria for study groups healthy control and cigarette smoke cohort. ..	24
Table 2: Exclusion criteria for study participants.	25
Table 3. Demographic and clinical characteristics of study participants with the comparison of cotinine-negative and cotinine-positive groups.	36
Table 4. Cluster groupings of cytokines within the amniotic fluid.	46
Table 5. Predicted protein-protein interactions in cytokine/chemokine clusters are involved in various pathways.	49
Table 6. Abundances and unadjusted <i>p</i> values of significantly different cytokines between cotinine-positive and cotinine-negative amniotic fluid samples.....	55

List of Appendices

Table A 1. Patient demographic and clinical measures data sheet.	84
Table A 2. List of cytokines and chemokines analyzed in multiplex cytokine analyses.	85
Table A 3. Average abundances of cytokines and chemokines for all samples collected	88
Table A 4. Abundances of cytokines and chemokines comparing cotinine-positive and cotinine-negative samples.	92

List of Abbreviations

Abbreviation	Name
AF	Amniotic Fluid
ATI	Alveolar Type 1 Cell
ATII	Alveolar Type 2 Cell
CB	Cord Blood (Umbilical Cord Serum)
FBS	Fetal Bovine Serum
FEF ₂₅₋₇₅	Forced Expiratory Flow At 25-75% Of Vital Capacity
FEV ₁	Forced Expiratory Volume At 1 Second
FRC	Functional Residual Capacity
ITS	Insulin-Transferrin-Selenium
LPS	Lipopolysaccharide
MB	Maternal Blood (Maternal Serum)
PBS	Phosphate Buffered Saline
PCA	Principal Component Analysis
PCLS	Precision Cut Lung Slices
PL	Placenta Tissue
PLS-DA	Partial Least Squares-Discriminant Analysis
PNS	Penicillin-Streptomycin
RIPA	Radioimmunoprecipitation Assay Buffer
TEER	Transepithelial Electrical Resistance
VFRC	Flow At Functional Residual Capacity
$\alpha 7$ nAChR	Alpha 7 Nicotinic Acetylcholine Receptor

Chapter 1: Introduction

1.1 Developmental Origins of Health and Disease

1.1.1 Overview of Developmental Origins of Health and Disease (DOHaD)

The series of work by Barker in the 80s laid the groundwork for the current hypothesis of developmental origins of health and disease (DOHaD). His findings highlight the relationship of prenatal conditions as an important determinant of prenatal and postnatal growth and risk for chronic diseases in later life (1,2). Many prenatal factors have been studied within the context of DOHaD. For example, undernutrition during pregnancy is associated with adult obesity and cardiovascular disease in the offspring. As well, low birthweight has been associated with increased risk for diseases such as type 2 diabetes. While many more factors including epigenetics have been studied in DOHaD, this thesis will specifically focus on prenatal cigarette smoke exposure (3). Cigarette smoking during pregnancy has been well established to be associated with numerous adverse neonatal outcomes such as preterm birth and respiratory diseases. Regardless, cigarette smoking during pregnancy remains prevalent especially in Manitoba where about 1,800 babies are born each year who were exposed to cigarette smoke *in utero* (4,5).

1.1.2 Developmental Origins of Lung Health and Disease

Although the lung is fully functional at birth, lung growth and development continue from early childhood to early adulthood, termed “lung function trajectory,” reaching a theoretical maximal growth and function at about age 20 and remains stable before experiencing a gradual decline thereafter (6,7). The rate of decline in lung function can be influenced by many factors, most especially environmental exposures to the lungs, which could lead to an atypical steep decline in lung function. These factors could be cigarette smoke exposure, family history of asthma, respiratory infections, low birth weight, and social determinants of health (8,9). Low lung function trajectory, deficits in lung function, and an early decline in lung function are all associated with

increased risk for chronic lung diseases such as asthma and chronic obstructive pulmonary disease (7,10). Postnatal cigarette smoke exposure, both during childhood and in adulthood, has been well established to be linked to deficits in lung function and increased risk for chronic lung diseases such as asthma (8,9,11–16). However, it is important to consider that since the lung continues to grow and develop after birth, prenatal exposures that affect fetal lung development can also influence lung function trajectories and can predispose the neonate to chronic lung diseases.

Environmental exposures during pregnancy, specifically air pollution (particulate matter) and cigarette smoke have been linked to increased risk of asthma in the offspring. Air pollution can indirectly affect fetal lung development by causing fetal growth restriction which can lead to developmental defects (17). Furthermore, *in utero* exposure to ambient particulate matter, especially during mid gestation (weeks 15-25), is associated with an increased risk for asthma by age 6. This association is more pronounced in males compared to females (18).

In utero (or prenatal) cigarette smoke exposure and its effect on offspring lung function is well studied (19–23). Lung function measurements of infants 2 weeks up to 18 months of age who were exposed to cigarette smoke *in utero* reveal reduced metrics of lung function compared to neonates who are unexposed. Of note, there is a reduction in functional residual capacity (FRC) and airflow at functional residual capacity (V_{FRC}). Additionally, these impairments were observed in term infants, none were pre-term (19,20). Furthermore, impairments in lung mechanics such as tidal breathing and lung compliance can be observed in neonates exposed to cigarette smoke *in utero* (22). These detrimental effects persist into adulthood whereby at age 21, young adults who were exposed were observed to have impairments in lung function measurements such as forced

expiratory flow at 25-75% of vital capacity (FEF₂₅₋₇₅) and forced expiratory volume at 1 second (FEV₁) (21). In regard to lung development, *in utero* exposure to cigarette smoke (nicotine) leads to increased alveolar spaces, decreased total lung surface area, and an altered lung branching morphogenesis (24,25). *In utero* exposure to nicotine and fetal lung development is further discussed in Section “1.5.3 Nicotine Disrupts the Epithelial Barrier.”

Furthermore, *in utero* exposure to cigarette smoke has been linked to youth- and adult-onset asthma. A dose-response relationship with level of cigarette smoke exposure and risk for asthma and asthma exacerbations can be observed. Children of mothers who smoked more than 20 cigarettes daily during pregnancy have a 25% higher risk of persistent asthma exacerbations than those whose mothers smoked fewer than 20 daily, who themselves face a 36% higher risk compared to those who are unexposed (26). The effects of *in utero* cigarette smoke exposure are also intergenerational. Maternal grandmaternal cigarette smoking during the mother’s fetal development is associated with an increased risk of childhood asthma for the offspring (27–29). In contrast, paternal grandmaternal smoking is not associated with increased risk for asthma in the third generation. However, parental smoking before conception has been linked to early-onset asthma (30). These observations provide evidence that environmental exposures *in utero* contribute to the pathogenesis of chronic lung diseases. Therefore, chronic lung diseases have developmental origins whose mechanism is yet to be elucidated.

Intra-amniotic inflammation, such as chorioamnionitis, and cytokines associated with such inflammation have been linked to adverse pulmonary outcomes such as fetal lung inflammation, lung injury, and bronchopulmonary dysplasia (31–34). Previous works have identified the critical

role of amniotic fluid (AF) in fetal lung development, and the inhalation of which through breathing movements is important for lung organogenesis and alveolar epithelial cell differentiation (35–37). In addition, attempts to perform *in utero* gene editing of the airway epithelium has proven successful through intra-amniotic injection of CRISPR-Cas9. Fetal breathing movements then move the CRISPR-Cas9 into the fetal lungs leading to targeted editing of both airway and alveolar epithelial cells (38). Therefore, through fetal breathing movements, the amniotic fluid components can be exposed to the fetal lungs and can influence the airway epithelium. A dysfunctional epithelial barrier is associated with the pathogenesis and severity of asthma, a chronic lung disease (39). These observations suggest that fetal lungs can be exposed to the amniotic fluid milieu and potentially influence the airway epithelial layer.

1.2 Amniotic Fluid

1.2.1 Amniotic Fluid Composition

Amniotic fluid is a rich source of biomolecules that provide the developing fetus nutrients and growth factors. Amniotic fluid also provides antimicrobial protection, mechanical protection against blunt trauma, and fosters typical fetal development (40–42). The composition and volume of amniotic fluid changes throughout gestation starting at about 25 mL at week 10 to a maximum of about 800 mL by week 28 followed by a decrease to about 400 mL thereafter by term (40). The source of amniotic fluid in early gestation is through passive diffusion from maternal serum across the uterine decidua and the amniochorionic membrane and thus its composition is isotonic with maternal serum. During the second half of gestation, fetal urine and lung liquid production are the main contributors to the volume and composition of amniotic fluid thereby altering its tonicity in comparison to maternal serum (40,41). Amniotic fluid volume is tightly regulated by its reabsorption through fetal swallowing and intramembranous flow from the amniotic fluid itself

into fetal vessels through the amniochorionic membrane. Perturbations in typical amniotic fluid volume can lead to pathologies such as oligohydramnios (decreased volume) or polyhydramnios (increased volume) (41).

The biochemical composition of amniotic fluid has been widely studied. Amniotic fluid contains many solutes such as sodium, potassium, chloride, glucose, phosphate, among many others. It also contains macromolecules such as amino acids, cytokines, lipids, and proteins, many of which have been explored as possible biomarkers for fetal pathologies (42–46). In early gestation, the source of these biomolecules can come from maternal plasma through transplacental transport. In later gestation, the biggest contributor to amniotic fluid is fetal urine and lung liquid efflux. So, the source of the biomolecules in the amniotic fluid is mostly from the fetus itself (40,41). The amniotic fluid milieu changes over gestation and is altered by intra-amniotic inflammation and fetal well-being (44,47,48). Interestingly, some parental factors have been linked to influence the amniotic fluid. Maternal diet has been found to alter the amino acid composition of amniotic fluid (49). Furthermore, air pollutants like microplastics have been detected in amniotic fluid indicating that particulate matter could indeed enter the amniotic space (50). However, not much is known on the influence of environmental exposures on the amniotic fluid composition.

1.2.2 Fluid Exchange Between Amniotic Fluid and Fetal Lung

During development, the fetal lung is filled with fluid that is mainly produced by the pulmonary epithelia of the fetal lung, termed fetal lung liquid (51). An optimal level of distention is required for normal lung growth and development, and distention is mainly controlled by fetal breathing movements (36,37). Early studies of fetal breathing movements suggest that its main

purpose is to reduce the efflux of fluid from the lungs into the amniotic sac by preventing the elastic recoil of the lungs thereby preventing the expelling of fetal lung liquid (37). However, visualization of fetal breathing movements using Doppler has revealed fluid flow both into and out of the lungs during fetal inspiratory and expiratory movements. This provides evidence that amniotic fluid and fetal lung liquid intermix during fetal breathing movements. In a cohort of fetuses with gestational ages ranging 28 weeks to 38 weeks, the average volume influx and efflux during inspiratory and expiratory movements are 1.36 ± 0.67 mL and 1.32 ± 0.56 mL, respectively (52). For each breath, fetuses exchange about 2.5% at week 28 and about 5.6% at week 38 of total lung volume in amniotic fluid. In the adult lung, each breath (tidal volume) exchanges about 8% of total lung volume in air, which is similar to fetal breathing. Since fetal breathing begins at week 10 and continues until term, the amount of inspired amniotic fluid is physiologically relevant (52–55).

Furthermore, during lung development, the differentiation of alveolar cells is dependent on the influx of amniotic fluid and the mechanical stretch that it provides during inspiratory movement (35). The therapeutic utility of the inhalation of amniotic fluid during inspiratory movements has also been explored. Successful gene editing of the airway and alveolar epithelium through intra-amniotic injection of CRISPR-Cas9 with subsequent delivery to the fetal lungs via breathing movements have proven that amniotic fluid does substantially enter and interact with the fetal lungs (38). Therefore, it is possible for the amniotic fluid milieu, especially cytokines and chemokines, to directly interact with and influence the fetal lungs.

1.2.3 Amniotic Fluid Role in Fetal Development

In addition to its utility in delivering nutrients such as carbohydrates, lipids, and proteins to the developing fetus, amniotic fluid is also crucial for the morphological development of the

lungs (40). As alluded to earlier, the fetal lung liquid is mainly produced by the pulmonary epithelial and fills the lung during fetal development (36,51). Keeping the developing lungs at an optimal distention is vital for proper lung development, and its distention is controlled both by lung liquid production and fetal breathing movements that allow for the mixing and exchange of amniotic fluid and the fluid produced by the pulmonary epithelia (35–37,56). A reduction or absence of fetal breathing movements and/or oligohydramnios impairs lung growth and development and could lead to pulmonary hypoplasia which is detrimental for neonatal health. In extreme cases, oligohydramnios could lead to pregnancy loss (36,56,57). It is important to note, however, that in the absence of oligohydramnios, a reduction in amniotic fluid pressure through prolapsed (amniochorionic) membranes could also detrimentally affect lung development and maturity resulting in immature pulmonary structures such as the airways and alveoli (58). Furthermore, the mechanical stretch of the fetal lungs that is provided by the influx of amniotic fluid during fetal breathing movements is essential for successful alveolarization. The differentiation of alveolar progenitor cells into mature and functional alveolar type I (ATI) and type II (ATII) cells is dose-dependent on mechanical stretch (35).

1.3 Inflammation in Pregnancy

1.3.1 Introduction

Inflammation during pregnancy or *in utero* exposure to inflammation is an important risk factor for the pathogenesis of many diseases, is detrimental to the fetus, and is also linked with adverse birth outcomes (20,33,59,60). However, inflammation is not inherently negative. The maternal immune system undergoes several changes throughout pregnancy to accommodate and tolerate the fetus, which is immunologically foreign to the maternal system (61). In uncomplicated pregnancies, a pro-inflammatory milieu is needed for successful implantation and placentation of

the blastocyst into the uterine decidua followed by an anti-inflammatory milieu to support the growth and development of the fetus. Finally, the onset of parturition and the induction of labour at term is characterized by a shift to a pro-inflammatory milieu in the maternal system. The three characteristic shifts in inflammatory profiles are widely thought as the three immunological phases of pregnancy (61–64). Non-typical production of cytokines or chemokines due to both endogenous (e.g. autoimmune disease) and exogenous sources (e.g. viral/bacterial infection) could disrupt the immunological phases and have detrimental effects such as pregnancy loss and preterm labour (63,64). What remains to be elucidated is the normal cytokine/chemokine profile of the amniotic fluid and whether it is altered by maternal exposures such as cigarette smoke and air pollution. Fetal breathing movements are an important mechanism at which the amniotic fluid milieu communicates with the fetal lung, more specifically its epithelia. As earlier discussed, the interaction between fetal breathing movements and the pulmonary epithelia is substantial that even intra-amniotic gene editing is possible (38). The focus of this thesis is to characterize the amniotic fluid cytokine and chemokine profile because *in utero* exposure to inflammation is linked to adverse outcomes. Since the amniotic fluid milieu can interact with and influence the fetal lungs, this thesis also aims to determine whether environmental exposures, specifically cigarette smoke, can alter the cytokine and chemokine profile.

1.3.2 Intra-amniotic Inflammation and Pulmonary Outcomes: Exogenous Sources of Inflammation

Exogenous sources of intra-amniotic inflammation could be from pathogenic agents, such as viruses and bacteria, or sterile agents, such as experimental induction of inflammation. Intra-amniotic inflammation is clinically termed chorioamnionitis and could lead to adverse outcomes for the neonate (33,65–70). Consequently, intra-amniotic inflammation and/or chorioamnionitis

has been linked to fetal lung changes such as bronchopulmonary dysplasia, lung injury, and altered lung development (31,67,69,71). Although intra-amniotic inflammation has been broadly linked to detrimental fetal lung effects, the knowledge on the inflammatory profiles that lead to such detrimental effects are little. Furthermore, we do not know whether maternal exposure to cigarette smoke alters the amniotic fluid cytokine and chemokine profile such that the fetal lungs are negatively affected. This thesis will focus on whether the amniotic fluid cytokine and chemokines affect the airway epithelium.

Experimental models of chorioamnionitis have linked that cytokines in the amniotic fluid interact with the developing lung. The role of Interleukin (IL)-1 in mediating fetal lung inflammation has been elucidated through a lipopolysaccharide (LPS) challenge of an ovine model of chorioamnionitis. Intra-amniotic injection of LPS induces fetal lung inflammation characterized by lung infiltration of neutrophils and monocytes and by increased production of cytokines such as IL-6 and IL-8 (32). An elevation of both IL-6 and IL-8 in amniotic fluid has been associated with fetal lung injury and inflammation (72). Interestingly, inhibition of IL-1 signalling via intra-amniotic injection of an IL-1 receptor antagonist, which blocks both IL-1 α and IL-1 β , successfully reduces fetal lung infiltration of neutrophils and monocytes and reduces production of IL-6 and IL-8 in the fetal lung signifying an amelioration of fetal lung inflammation (32). Furthermore, LPS challenge of a non-human primate model of chorioamnionitis has associated intra-amniotic TNF α signalling as a contributor to fetal lung inflammation and injury. The inflammatory response to intra-amniotic LPS injection impairs the close association between ATI and the pulmonary capillaries. Furthermore, in severely injured fetal lungs, loss of mature differentiated alveolar epithelial (ATI and ATII) cells is observed, leading to disrupted alveolarization (31). However,

combined inhibition of IL-1 (both IL-1 α and IL-1 β) and TNF α signalling by intra-amniotic injection of an IL-1 receptor antagonist and an anti-TNF α antibody reduced levels of inflammatory cytokines in the fetal airways and alveoli. Histologic imaging revealed that inhibiting IL-1 and TNF α signaling in fetal lungs prevented lung injury with pulmonary structures indistinguishable from lungs unaffected by inflammation (31,73). In the absence of an inflammatory challenge, transgenic overexpression of IL-5 in mice dams exposes fetuses to IL-5 *in utero* via the amniotic fluid. This leads to airway hyperreactivity in the offspring secondary to increased airway innervation that can respond to noxious stimuli. Airway hyperreactivity is a hallmark of asthma (74). These studies provide evidence and emphasize that the amniotic fluid milieu, including cytokines and chemokines, interact with the fetal lungs.

Environmental exposures are also linked to chorioamnionitis. Exposure to ambient particulate matter (PM_{2.5}) during pregnancy is significantly associated with chorioamnionitis as determined through histopathologic examination of placentae. This holds true even when stratifying for the three trimesters of pregnancy (75). Consequently, prenatal exposure to inflammation, including chorioamnionitis, is associated with slower/decreased postnatal growth which could negatively affect postnatal lung growth trajectory, thereby increasing risk for chronic lung disease (76). *In utero* cigarette smoke exposure is known to disrupt amino acid metabolism in the amniotic fluid, but no data is present for the production and degradation of proteins, including cytokines and chemokines. Furthermore, maternal smoking alters the neonatal immune response and cytokine production as studied in the umbilical cord blood. So, whether cigarette smoke exposure alters the amniotic fluid cytokine and chemokine profile remains unknown (77–79).

Chorioamnionitis typically arises secondary to infection as a result of ruptured fetal membranes (which includes both the chorionic and the amniotic membranes). Pathogens such as bacteria found in the vaginal microflora or viruses like herpes simplex virus are common infectious agents leading to congenital disease. In these instances of chorioamnionitis, an acute and high innate inflammatory response occurs as a result (66). However, *in utero* cigarette smoke exposure is unlikely to cause the same response as infectious chorioamnionitis that induces an active immune response.

1.3.3 Intra-amniotic Inflammation and Pulmonary Outcomes: Endogenous Sources of Inflammation

Endogenous sources of inflammation are those that incite an inflammatory response in the absence of pathogens, commonly termed “sterile inflammation.” Causes of sterile inflammation include, but are not limited to, autoimmune disease, necrotic cell death, and cell stress (80). In pregnancy, sterile inflammation can lead to chorioamnionitis and intra-amniotic inflammation. Although these are associated with an increase in fetal (both placental and amniotic fluid) pro-inflammatory cytokines and adverse neonatal outcomes such as pre-term birth and bronchopulmonary dysplasia, the focus of this thesis will be exogenous, namely cigarette smoke, as an inflammatory agent (34,81).

1.4 Amniotic Fluid Inflammatory Profiles and Fetal Lung Development

1.4.1 Inflammatory Biomarkers in the Amniotic Fluid

The study of inflammatory biomarkers, specifically cytokines, in the amniotic fluid has historically been focused on clinical associations with adverse fetal and neonatal outcomes such as preterm birth, intrauterine growth restriction, bronchopulmonary dysplasia, and neurological

pathologies (48,82–86). However, not much is known on the cytokine and chemokine profile and abundances in the amniotic fluid of uncomplicated pregnancies.

There are two possible pathways of communication between the external maternal environment and the fetus: the umbilical cord and the amniotic fluid. Although this thesis will focus on amniotic fluid and the intra-amniotic pathway of communication, a brief discussion on the umbilical cord pathway is given below. Experimental models studying the effect of the interaction between amniotic fluid cytokines and the developing lung has yielded substantial evidence that lung development is altered by its intra-amniotic exposure to cytokines (31,74). Intra-tracheal delivery of IL-1 α into fetal lamb lungs induces lung inflammation characterized by infiltration of neutrophils and monocytes and production of pro-inflammatory cytokines. This is evidence that with direct delivery of a single cytokine, the lungs react with an acute inflammatory response that could affect further lung development (87). Interestingly, and as previously highlighted, intra-amniotic inflammation results in lung inflammation and lung injury, and intra-amniotic administration of inhibitors of cytokine signalling, specifically (IL-1 α , IL-1 β , and TNF α) prevents or ameliorates lung inflammation and damage to pulmonary structures (31,32). Intra-amniotic administration of IL-1 β , as opposed to inducing intra-amniotic inflammation using LPS, also results in lung inflammation characterized by a 6-fold increase in neutrophil population in the fetal airways and abundance in activated macrophages. Moreover, IL-1 β also causes a shift in the ratio of immune cells in lymphatic organs, especially the mediastinal lymph nodes (69). These provide evidence that cytokines and chemokines in amniotic fluid can affect the fetal lungs.

1.4.2 Umbilical Cord as a Communication Pathway

The placenta and umbilical cord are vital for a successful pregnancy. Through these structures, the fetus is supplied with oxygen and allows for the exchange of gases, byproducts, nutrients, hormones, and antibodies (88). The umbilical cord is a well-studied communication pathway between the maternal environment and the fetus. Maternal infection of respiratory syncytial virus during pregnancy allows for vertical transmission of the virus to the fetus, and this is associated with detrimental outcomes for the fetus such as weak immunity to later reinfection, alteration of pulmonary immune response, and airway hyperreactivity secondary to reinfection (89–91). Furthermore, maternal exposure to air pollutants such as nanoparticles is also known to be vertically transmitted via the umbilical cord to the fetus. Although transplacental transport of particles is size-exclusionary, nanoparticles that are small enough can nonetheless accumulate in the fetal environment and can lead to altered abundances of amniotic fluid and fetal lung cytokines. In addition, both pre- and postnatal lung development is irreversibly altered resulting in enlarged alveolar spaces even at lung maturity thereby reducing ventilatory efficiency (92). In the context of maternal cigarette smoke exposure, the response of umbilical cord immune cells to allergens is altered characterized by increased activity of T helper type 2 associated responses and attenuated responses to toll-like receptor ligands (78,79). It is important to note that only 11% of the total fetal cardiac output flows to the lung. This alludes to an alternate pathway of communication with fetal lungs, this is the amniotic fluid through fetal breathing movements (93).

1.5 Airway Epithelium and Epithelial Cells

1.5.1 Airway Epithelium Introduction

The airway epithelium is comprised of a myriad of epithelial cell types such as ciliated epithelial cells, goblet cells, basal cells, and club cells. The main function of the airway epithelium is to provide a physical barrier between the internal and external environments and to provide innate defense against pathogens (94). Ciliated cells make up over 50% of all airway epithelial cells and function in the mucociliary clearance of debris, pathogens, and allergens trapped within the mucus that is produced by goblet cells (94,95). Epithelial cells are connected to each other via intercellular cell-cell adhesion complexes called tight junctions, that are found at the apical surface of the airway epithelium. Tight junctions control the paracellular permeability of the airway epithelium providing size- and ion-selectivity. This provides a substantial control over what can paracellularly cross the airway epithelium. Therefore, insults that can disrupt the tight junctions could be associated with disease pathophysiology (94). Indeed, epithelial barrier dysfunction is characteristic of chronic lung diseases such as asthma. The barrier dysfunction is characterized by increased permeability due to loss of tight junctions and epithelial cell detachment. Barrier dysfunction is also proportional to severity of disease (39).

Environmental toxicants such as noxious chemicals and cigarette smoke affect the airway epithelium by disrupting mucociliary clearance and affecting the balance of epithelial cell types (96). Particulate matter (PM_{2.5}) disrupts the nasal epithelial barrier as measured by a reduction in transepithelial electrical resistance (TEER) compared to levels prior to PM_{2.5} exposure. TEER measures paracellular ion flux and can be used as a surrogate marker of epithelial barrier function (97,98). Disruption in the integrity of the epithelial barrier allow environmental toxicants to easily translocate from the airway lumen and into the lung tissue where resident immune cells can be

activated, leading to an immune response (98). The morphology and physiology of the airway epithelium changes in disease. In people with asthma, chronic lung inflammation and atypical immune reaction to environmental toxicants and allergens (called airway hyperresponsiveness) result in structural changes to the airways, collectively termed “airway remodelling” (99).

1.5.2 Airway Epithelial Remodeling and Dysfunction

Airway remodelling is one of the hallmarks of asthma and is characteristic of other chronic lung diseases. Airway remodelling is not secondary to asthma, it may occur prior to clinical manifestation of the disease and is characterized by airway smooth muscle hypertrophy and hyperplasia, goblet cell hyperplasia, increased mucus production, and narrowing of the airway lumen. This ultimately leads to an imbalance of epithelial cell types, fibrosis of the airways, and airway hyperresponsiveness (99,100). Dysfunction of the airway epithelium is a key driver in the pathogenesis of asthma. In asthma, the airway epithelium barrier function is compromised due to the reduced expression of tight junction proteins resulting in increased permeability of the airway epithelium to environmental insults, air pollution, pathogens, and allergens (98,101). Airway epithelial cells also play a role in regulating innate defense by using pattern-recognition receptors to detect immunogenic macromolecules, such as surface proteins of pathogens and allergens. Once activated, airway epithelial cells release cytokines and chemokines to trigger an immune response. Abnormal or heightened immune response, termed airway hyperresponsiveness, to various triggers is observed in people with asthma, characterized by an increased release of cytokines and chemokines (39). Furthermore, the tissue repair capacity of the airway epithelium is compromised in asthma due to the impairment of epithelial cell proliferation and differentiation (39,94). Persistent activation of an immune response in the airways due to environmental insults, coupled

with impaired tissue repair and loss of tight junctions, can result in chronic inflammation, aberrant airway epithelial cell proliferation, and a dysfunctional epithelial barrier (39,99).

1.5.3 Nicotine Disrupts the Epithelial Barrier

Nicotine has been linked to epithelial barrier dysfunction and lung function deficits through activation of the alpha 7 nicotinic acetylcholine receptor ($\alpha 7$ nAChR) expressed by airway epithelial cells. *In utero* exposure to nicotine leads to an altered fetal lung development characterized by enlarged alveolar spaces with a concomitant decrease in lung internal surface area. Exposure to nicotine also results in an increased expression of $\alpha 7$ nAChR (24,102). Furthermore, the activation of $\alpha 7$ nAChR by nicotine in fetal lungs stimulates atypical branching morphogenesis leading to an increase in the total length and number of airways. This permanent alteration in the lung architecture translates to a deficit in lung function such as increased airway resistance and reduced forced expiratory flow (25,103). Interestingly, in a murine model, *in utero* exposure to nicotine only between gestational day 14 to postnatal day 7 is associated with the lung function deficits described above. This strongly suggests a critical “window of exposure” wherein alterations in lung development could be permanent and lead to lung function deficits (103). At the cellular level, $\alpha 7$ nAChR seems to play a major role in the airway epithelial morphology. Whereas both absence of the $\alpha 7$ nAChR and its pharmacological antagonism results in epithelial basal cell hyperplasia (a pathological phenotype), its activation implies that it regulates proliferation and differentiation of the airway epithelium (104,105). This initially suggests that nicotine can activate $\alpha 7$ nAChR and have beneficial effects on the airway epithelial structure. However, it is now known that chronic nicotine exposure leads to desensitization of the $\alpha 7$ nAChR, thereby leading to the pathological alterations in the lungs as described above (104). Nicotine, the bioactive component of cigarette smoke, is metabolized to cotinine, its major metabolite in humans.

Cotinine has a longer half-life and persists in circulation much longer than nicotine. Therefore, the levels of cotinine is inherently dependent on levels of nicotine exposure (106). Both nicotine and cotinine are found in the amniotic fluid with cotinine at higher concentrations than nicotine, presumably due to the metabolism of nicotine and its longer half-life (107). Although cotinine binds to $\alpha 7$ nAChR at a lower potency than nicotine, both compounds enter and persist in the amniotic fluid which can then interact with the fetal lungs leading to permanent alterations in lung epithelial morphology (103,106). Therefore, there is a possibility that exposure to nicotine and cotinine can directly alter the airway epithelial structure and lead to barrier dysfunction that could start a positive feedback loop of airway inflammation and remodelling which is related to chronic lung diseases like asthma (108).

1.5.4 Airway Epithelial Cell Cytokines

Airway epithelial cells release cytokines in response to immunogenic macromolecules trapped within the mucus layer in the airway lumen. Epithelial cytokines include: TSLP, IL-33, and IL-25. These cytokines mediate immune response in the airways and are basally elevated in people with asthma (99,101). Once released, neighbouring cells with receptors for such cytokines are then activated and thus begins a series of downstream intra- and intercellular signalling that can result in the proliferation of lung fibroblasts, the release of other cytokines such as vascular endothelial growth factor-A (VEGF-A), and the production of extracellular matrix molecules such as collagen. Furthermore, these cytokines induce lung resident immune cells to produce pro-inflammatory cytokines such as IL-5 and IL-13 that further recruit and activate other immune cells ultimately resulting in bronchoconstriction, airway hyperresponsiveness, and airway smooth muscle proliferation (99,101).

In asthma, increased IL-13 production is associated with the reduced expression of critical tight junction proteins that maintain the epithelial barrier. Cell detachment due to loss of tight junction proteins leads to the release of pro-inflammatory cytokines with the subsequent recruitment of immune cells to the epithelium as well as cytokines that promote remodelling such as epidermal growth factor (EGF) and transforming growth factor (TGF)- β (39,105). These growth factors then lead to airway epithelial dysfunction by stimulating the hypertrophy of airway smooth muscles and by skewing the balance of epithelial cell types (39). These observations provide evidence that airway epithelial cell autocrine and paracrine signaling through the release of cytokines affect the epithelial barrier itself.

A recently published study explored a panel of 71 cytokines and chemokines in amniotic fluid from preterm and term pregnancies and its effect on esophageal epithelial organoids (109). The authors observed correlations in the expression profiles of certain cytokines across the gestational ages of their study cohort. Whereas 17 cytokines were highly expressed, 5 cytokines were undetected, and 49 cytokines were classified as having variable expression (high or low) among their participants. However, no comparison was made between the cytokines and chemokines in the amniotic fluid and other tissues present in pregnancy (109). Furthermore, in a 3D spheroid model of esophageal epithelium, exposure of epithelial cells to amniotic fluid alters the transcriptome during the *in vitro* differentiation process which could alter their resulting cell fates (109). Interestingly, exposure to amniotic fluid alters esophageal epithelial response to IL-13 characterized by differential transcriptomic production of IL-13 target genes which are linked to a chronic inflammatory disease, eosinophilic esophagitis (109). Although the influence of gestational age on the abundances of cytokines and chemokines in the amniotic fluid was assessed,

this study did not evaluate other environmental factors. The effect of amniotic fluid exposure, with respect to its cytokine and chemokine profiles, on epithelial barrier function was also not assessed in this study. Nevertheless, this provides evidence that the amniotic fluid influences epithelial cells. Therefore, whether amniotic fluid alters the airway epithelial barrier function and whether barrier function is associated with the amniotic fluid cytokine and chemokine profile remains an enigma.

1.6 Knowledge Gaps and Hypothesis

1.6.1 Summary of Knowledge Gaps

In gist, *in utero* and intra-amniotic exposure of fetal lungs to cytokines such as IL-5, IL-1, and TNF α have been linked to detrimental effects such as airway hyperreactivity, lung inflammation, and lung injury, all of which are related to chronic lung diseases such as asthma (31,39,74,108). Although a part of the amniotic fluid cytokine and chemokine profile has been characterized in relation to gestational age, the basal amniotic fluid cytokine/chemokine profile and whether environmental factors influence it remains unknown (109). Furthermore, *in utero* exposure to cigarette smoke is linked to adverse neonatal and pulmonary outcomes such as lung function deficits which are associated with a low lung function trajectory and increased risk for chronic lung diseases (7,10,19–23,110). However, it is unknown how the negative effects of *in utero* cigarette smoke exposure are communicated with the fetal lungs. Since only a fraction of fetal circulation reaches the fetal lungs, the alternate communication medium could be the amniotic fluid through fetal breathing movements (93).

1.6.2 Hypotheses

- Hypothesis 1: *In utero* cigarette smoke exposure alters the cytokine/chemokine profile of amniotic fluid in uncomplicated pregnancy.
- Hypothesis 2: Amniotic fluid alters the airway epithelial barrier function, and the altered function is associated with the cytokine/chemokine profile of amniotic fluid.

1.7 Project Objectives

To test my hypotheses, the amniotic fluid cytokine and chemokine profile must first be fully characterized and then exposed to airway epithelial cells to assess its effect on barrier function.

Objective 1: Characterize the amniotic fluid cytokine and chemokine profiles.

- Aim 1: To define the amniotic fluid cytokine/chemokine profiles and abundances in term pregnancies.
- Aim 2: To characterize the amniotic fluid cytokine/chemokine profiles in reference to other tissues present in pregnancy: umbilical cord serum, maternal serum, and placenta.
- Aim 3: To assess whether the amniotic fluid cytokine/chemokine profiles are altered by cigarette smoke exposure.

Objective 2: Assess the influence of amniotic fluid on airway epithelial barrier function

- Aim 1: To measure the impact of amniotic fluid on airway epithelial barrier function.
- Aim 2: To measure the impact of amniotic fluid on airway epithelial barrier recovery after wounding

Chapter 2: Methods

2.1 Patient Recruitment and Sample Collection

2.1.1 Patient Recruitment Protocol and Questionnaire

Recruitment of participants for this study was done in collaboration with Dr. Meghan Riddell's group in the Women & Children's Health Research Institute (WCHRI) and the University of Alberta in Edmonton, AB. In brief, the study groups for this project include pregnant women who are exposed to cigarette smoke, whether primary or secondary (Exposed cohort/cigarette smoke cohort) at the time of delivery and pregnant women who do not smoke traditional cigarettes and do not have co-morbidities (Unexposed control/healthy control). Patients are recruited at the day of and questionnaire completed about an hour prior to their caesarean procedure. Inclusion and exclusion criteria are outlined in Table 1 and Table 2, additionally, the full data sheet is available in Appendix A, Table A 1.

Table 1: Inclusion criteria for study groups healthy control and cigarette smoke cohort.

Inclusion Criteria	
Unexposed Control Healthy Control	Exposed Cohort Cigarette Smoke Cohort
Healthy singleton pregnant women ≥ 18 years old	Healthy singleton pregnant women ≥ 18 years old
Gestational age > 36 weeks	Gestational age > 36 weeks
Does not smoke	Actively smokes traditional cigarettes
Does not have diabetes	Does not have diabetes
Undergoing caesarean delivery without labour	Undergoing caesarean delivery without labour
Does not have underlying health complications which includes preeclampsia, hypertension, and a diagnosis of asthma	Does not have underlying health complications which includes preeclampsia, hypertension, and a diagnosis of asthma

Table 2: Exclusion criteria for study participants.

Exclusion Criteria	
Age < 18 years old	Diagnosis of chorioamnionitis
Pregnant with multiples	Diagnosis of preeclampsia
Mothers in active labour	Substance use (illicit drug and/or alcohol) during pregnancy
Pre-term, Gestational Age < 36 weeks	Maternal history of asthma

2.1.2 Sample Collection

For this project, the tissue of interest is Amniotic Fluid (AF, n = 32), however, a matched sample of Maternal Serum (MB, n = 33), Umbilical Cord Serum (CB, n = 33), and Placenta (PL, n = 36) are also collected. Notably, for some patients not all complementary tissues were collected, so, there are more for the sera and the placentae. All samples are collected from term and uncomplicated pregnancies during scheduled Caesarean delivery. For the amniotic fluid samples, collected fluid were placed in 50-mL conical base tube and centrifuged at 1000 rpm at 4°C for 5 minutes. Then, the supernatant is placed into a fresh 50-mL conical base tube and centrifuged as above, this process was repeated 3 to 4 times until no visible pellet formed following centrifugation. This was done to remove cell contaminants. The cleared amniotic fluid samples were then transferred into 15-mL conical base tubes or 1.5-mL microcentrifuge tubes. For the sera, blood samples were collected into test tubes and centrifuged at 1000g at 4°C for 10 minutes. The serum supernatant was then collected and placed into 1.5-mL microcentrifuge tubes. For the placenta, tissue samples were collected from different cotyledons using surgical scissors and forceps. Blood contamination was reduced by washing tissue samples with phosphate-buffered saline (PBS). Then,

tissue samples were placed in 2-mL microcentrifuge tubes. Placenta tissue samples were flash frozen in liquid N₂. The collected amniotic fluid, umbilical cord serum, maternal serum, and placenta samples were then shipped to Winnipeg.

2.1.3 Cigarette Smoke Exposure Confirmation

Exposure to cigarette smoke is asked on the patient recruitment questionnaire, however, confirmation of exposure was done by quantifying cotinine abundance using an enzyme-linked immunosorbent assay (ELISA). Cotinine is a longer half-life metabolite of nicotine and is typically used as a biomarker of nicotine exposure (106). The cotinine ELISA kit used is from Calbiotech (El Cajon, CA, USA). This is a competitive ELISA wherein cotinine in the analytes competes with HRP-conjugated cotinine for binding sites on the anti-cotinine antibodies coated onto the wells of the ELISA plate. Therefore, colour development is inversely proportional to cotinine concentration in the analytes. The full protocol is available in the Calbiotech Cotinine ELISA protocol. ELISA was performed according to manufacturer instructions. Samples with detected cotinine are termed cotinine-positive, and those with no detected cotinine are termed cotinine-negative, collectively called cotinine status.

2.1.4 Processing of Placenta Tissue

Protein from each placenta tissue sample was first extracted before sending to Eve Technologies. In brief, placenta tissue was mildly thawed on a glass petri dish surrounded by dry ice. The placenta tissue was divided into 2-3 pieces, and an aliquot (50-150 mg) is placed in a 1.5-mL microcentrifuge tube for protein extraction. Placenta aliquots were washed three times with 1 mL ice-cold PBS. Then, 1 mL of radioimmunoprecipitation assay buffer (RIPA) *without* 1% SDS and with 1X phosphatase inhibitor and 1X protease inhibitor per 70 mg of tissue was placed

inside the microcentrifuge tubes to lyse the placenta aliquots. Each sample was vortexed for about 30 seconds and allowed to rest on ice for 10 minutes. Then, each sample was sonicated 5 times with 10-sec pulses and 10-sec breaks between each pulse. Each sample was centrifuged at 16,000g for 10 mins at 4°C then supernatants were collected into a new 1.5-mL microcentrifuge tube. BCA protein assay was performed for each sample to quantify the amount of extracted protein. Once protein concentrations were assayed, each sample was diluted to 750 µg/mL protein using RIPA *without* 1% SDS and with 1X phosphatase inhibitor and 1X protease as a diluent.

2.2 Cytokine Analysis

Multiplex cytokine assay was performed by Eve Technologies (Calgary, AB) to characterize and quantify the cytokine/chemokine profiles of AF, MB, CB, and PL. The platforms used were the Human Cytokine/Chemokine 96-Plex Discovery Assay® Array (HD96) and the TGFβ 3-Plex Discovery Assay® Multi Species Array (TGFβ1-3) for a total of 99 cytokines/chemokines. The multiplex technology used is polystyrene bead-based that is coated with different fluorophores to identify each analyte (cytokine/chemokine). The Bio-Plex 200 bead analyzer was used to identify the analytes and to measure fluorescence intensity, which is directly proportional to the amounts of each analyte in the sample. Finally, fluorescence intensities are interpolated using a standard curve to quantify each cytokine/chemokine. The list of cytokines quantified in both assays are listed in Appendix B, Table A 2. Diagram of workflow for multiplex cytokine analyses is shown in Figure 1, and individual analyte sensitivities are available in the MilliporeSigma MILLIPLEX® MAP protocol.

2.2.1 Amniotic Fluid and Serum Sample Preparation

Multiplex cytokine assays were performed undiluted for the 96-Plex Discovery Assay. For the 3-Plex Discovery Assay, only the sera were diluted 1:30 which was performed by Eve Technologies. For both assays, AF, MB, and CB samples were thawed once and aliquoted for cytokine assay and for sample storage. 75 µL of each sample were ran for analyses in singlets.

2.2.2 Cytokine Profile Data Pre-Processing

Following multiplex cytokine analyses, Eve Technologies returned data that includes standard fluorescence intensities, standard cytokine/chemokine concentrations, standard curve-interpolated concentrations of the standards, analyte fluorescence intensities, and standard-curve interpolated concentrations of analytes. Total data points collected were 16,038 (99 cytokines/chemokines X 162 samples sent for analysis). Of all data points, 103 were OOR >, 582 were OOR <, and 88 were OOR. These values were defined as “out of range” of the standard curve fit, although fluorescence intensities for those cytokines/chemokines were detected and recorded. OOR >, OOR <, and OOR were assigned values based on a set of rules as follows:

- OOR > is replaced with the highest observed value for that cytokine
- OOR < is replaced with half of the lowest observed value for that cytokine
- OOR is replaced with values based on the two rules discussed prior but manually checking the fluorescence intensity of each to confirm whether it is OOR > or OOR <, then values are replaced accordingly

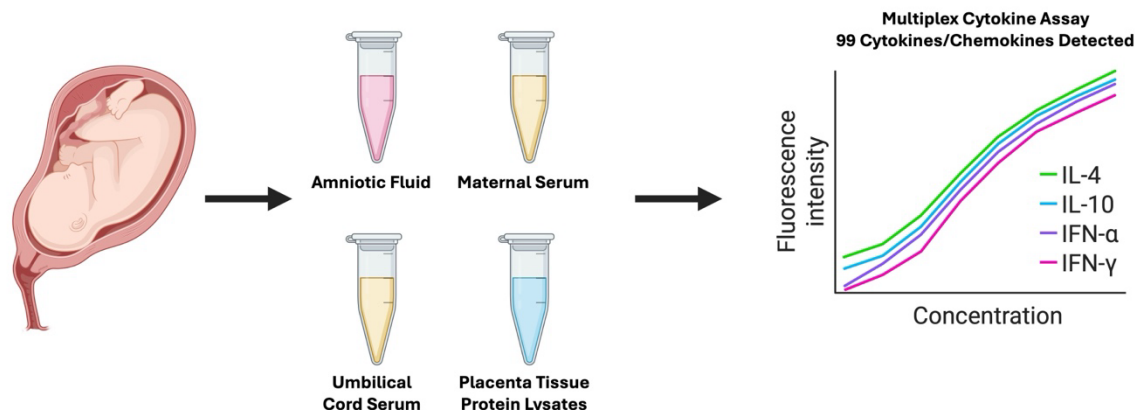


Figure 1. Workflow of cytokine/chemokine profile identification and quantification. Matched samples of amniotic fluid ($n = 32$), maternal serum ($n = 33$), umbilical cord serum ($n = 33$), and placenta tissue ($n = 36$) are collected. Placenta tissue protein was extracted and, together with the amniotic fluid and sera samples, was sent to Eve Technologies (Calgary, AB) for multiplex cytokine assay.

2.3 Cell Experiments

2.3.1 Cell Culture and Barrier Function Measurement

The Calu-3 human airway epithelial cells used for experiments were a gift from Dr. West. Cells were propagated in growth media consisting of DMEM/F-12 media supplemented with 10% v/v fetal bovine serum (FBS) and 1% v/v penicillin/streptomycin (PNS). For all experiments, cells were seeded onto transparent Transwell membranes (13.85 mm diameter, 0.4 μm pore size, 2×10^6 pores/ cm^2 pore density, Greiner Bio-One) at a density of 3×10^5 cells/well and maintained in growth media for 7 days until full confluency and then serum deprived for 24 hours with serum deprivation media consisting of DMEM/F-12 supplemented with 0.5% FBS, 1% v/v PNS, and 1% v/v insulin-transferrin-selenium (ITS). Amniotic fluid samples were diluted to 50% v/v with diluent media consisting of DMEM/F-12 supplemented with 1% v/v PNS. Cells used in experiments are in submerged liquid-liquid interface and are not differentiated into air-liquid interface. The study focuses on *in utero* exposures of the fetal lung which is filled by fluid.

Transepithelial electrical resistance (TEER) was measured as a marker of epithelial barrier function, as previously described (111), using MilliCell ERS-2 Voltohmmeter (Millipore).

2.3.2 Amniotic Fluid Effect on Epithelial Barrier Function

To determine cell tolerance to amniotic fluid, Calu-3 cells were exposed to different concentrations of commercial amniotic fluid: 100%, 50%, 25%, and 12.5%, all v/v (ZenBio Inc). Baseline TEER was measured in serum deprivation media before exposure to amniotic fluid. Then, TEER was measured 24 hours, 48 hours, and 72 hours after exposure. TEER fold change at each time point was calculated by dividing TEER value at each time point per treatment to baseline TEER per treatment. Cells tolerated all dilutions of amniotic fluid; however, the 50% v/v was chosen for experiments since, *in utero*, fetal lung liquid is not made up fully of amniotic fluid (51,52). For all experiments with study participant samples, amniotic fluid was diluted to 50% v/v with diluent media. All TEER measurements were performed as described above. Schematic of experimental design is shown in Figure 2.

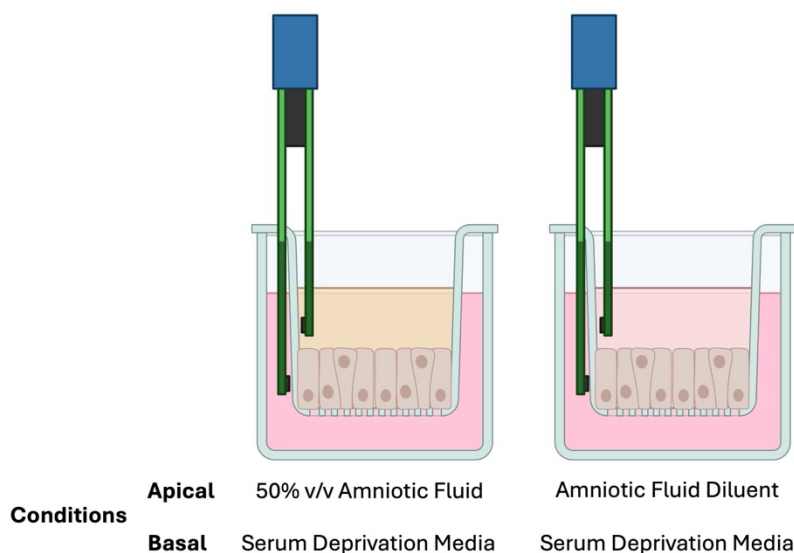


Figure 2. Schematic of experiments measuring Calu-3 epithelial barrier function in response to amniotic fluid.

2.3.3 Amniotic Fluid Effect on Epithelial Wound Healing (Barrier Recovery)

To assess barrier function recovery after wounding, cells were scratched with a 10- μ L pipet tip. Detached cells were washed off 3 times with PBS then cells were treated with 50% v/v amniotic fluid. Baseline TEER and post-wound TEER were measured in serum deprivation media and TEER was measured 24 hours, 48 hours, and 72 hours after wounding. Before exposure to amniotic fluid, cells were washed 3 times with PBS to remove detached cells. TEER fold change was calculated as described above. Wound healing capacity was calculated by determining the slope of fold change increase between 0 (TEER after wounding) and 72 hours. Schematic of the experimental design is shown in Figure 3.

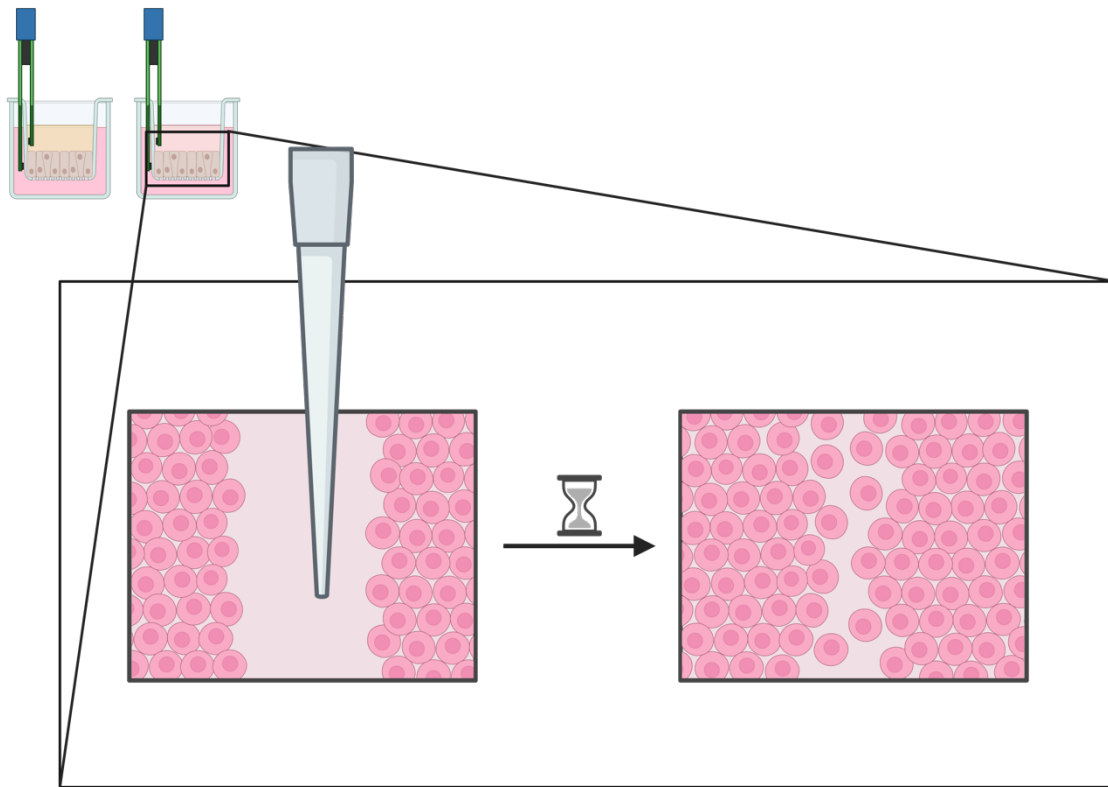


Figure 3. Schematic of wound healing experiments measuring the effect of amniotic fluid on barrier function recovery of Calu-3 cells after wounding.

2.4 Data Analysis

2.4.1 Cytokine Profile Data Analysis

All analyses were performed on R (v 4.4.1). The cytokine/chemokine profiles of each sample type were compared by a principal component analysis (PCA) using the R packages FactoMineR (v 2.11) and factoextra (v 1.0.7). Samples sent for multiplex cytokine analyses were sent in 4 different batches, as participants were recruited over the course of the project. To assess for possible batch effects, a PCA of each sample type were performed to determine whether the cytokine/chemokine profiles would differ based on batch. Out of the 4 sample types, only placenta tissue showed distinct groupings driven by their batches. Batch correction was applied to placenta tissue data using the R package limma (v 3.60.6). Differences in cytokine and chemokine abundances among the 4 sample types were compared by a linear mixed-effects repeated measures model using lmerTest (v 3.1-3). Correlations were calculated by Pearson's correlation method using Hmisc (v 5.2-3). For clustering of cytokine/chemokine profiles of amniotic fluid, Spearman clustering distance, and Complete clustering method using ComplexHeatmap (v 2.20.0) were used. Clusters of cytokines were determined by setting a cut height of $h = 1.2$ on the clustering dendrogram using R (v 4.4.1). To determine protein-protein interactions of the amniotic fluid cytokine/chemokine profiles, functional and pathway enrichment were performed using the STRING database (v 12.0) and EnrichR (112,113). The cytokine/chemokine profiles of cotinine-positive and cotinine-negative amniotic fluid samples were compared by a partial least squares-discriminant analysis (PLS-DA) using mixOmics (v 6.28.0). Differential abundance analyses were done by a least squares linear model using limma (v 3.60.6).

2.4.2 Cotinine ELISA Analysis

Cotinine ELISA standard dose-response curves were estimated using a five-parameter log-logistic function from which cotinine concentrations of samples were interpolated using `drc` (v 3.0-1) on R (v 4.4.1).

2.4.3 Statistical Analysis

All numerical data are presented as means $\pm$ SD, except when otherwise stated. Each “n” represents a study participant. Demographic and clinical measurements were compared by a T-Test except for categorical characteristics, Race and Offspring Sex, which were compared by a χ^2 Test both using `arsenal` (v 3.6.3). Linear mixed-effects repeated measures model using `lmerTest` (v 3.1-3) was used to compare abundances of cotinine between sample types. For barrier function experiments, TEER fold change values over time were compared by a two-way repeated measures ANOVA (factors: cotinine status, time) and TEER fold change at 24 hours were compared by a three-group one-way ANOVA. For barrier recovery after wounding experiments, TEER fold change values over time were compared by a two-way repeated measures ANOVA (factors: cotinine status, time). One-way ANOVA was used to compare wound healing capacity. All ANOVA tests were done using R (v 4.4.1). All post-hoc tests were performed by a pairwise comparison using `emmeans` (v 1.11.0). Individual linear regressions between 24-hour barrier function and cytokine abundances were calculated using R (v 4.4.1). This human discovery research has a small sample size, $n = 37$ total and $n = 7$ for cotinine-positive samples, therefore an unadjusted p value < 0.05 was considered significant.

Chapter 3: Results

3.1 Objective 1 Results

3.1.1 Participant Characteristics

The study participants are of a diverse demographic background. The full demographic and clinical characteristics collected are presented in Table 3. Cotinine-positive and cotinine-negative groups did not differ in any of the demographic and clinical characteristics, although gestational age was on average lower for cotinine-positive participants (ns, $p = 0.117$).

Table 3. Demographic and clinical characteristics of study participants with the comparison of cotinine-negative and cotinine-positive groups.

Characteristic	Unexposed Control Cotinine-Negative (N=30) Median (Q1, Q3)	Exposed Cohort Cotinine-Positive (N=7) Median (Q1, Q3)	<i>p</i> value ^a
Age	34 (29.25, 39)	31 (28.5, 34.5)	0.249
Race			0.763 ^b
BIPOC	10 (40.0%)	2 (33.3%)	
White	15 (60.0%)	4 (66.7%)	
Pregnancy Weight (kg)	69.5 (63.425, 93.05)	92.1 (76.4, 100.3)	0.249
Height (cm)	161.5 (160, 167.45)	167 (163.5, 169.4)	0.26
BMI	28.103 (24.209, 33.528)	35.1 (26.589, 38.501)	0.476
Placental Weight (g)	627.5 (528.737, 750.25)	699.955 (584.977, 778.64)	0.542
Gestational Age (weeks)	38.857 (38.179, 39.143)	38.286 (37.857, 38.5)	0.117
Gravidity	3 (2, 4)	2 (2, 4)	0.825
Term	1 (1, 1)	1 (1, 1)	0.312
Preterm	0 (0, 0)	0 (0, 0)	0.751
Abortion	0 (0, 1.75)	0 (0, 1)	0.645
Living	1 (1, 1)	1 (1, 1.5)	0.181
Offspring Sex			0.238 ^b
F	16 (53.3%)	2 (28.6%)	
M	14 (46.7%)	5 (71.4%)	
Birth Weight (g)	3255 (3102.5, 3787.5)	3440 (3355, 3640)	0.982

^aDifferences were compared by a t-test. ^bDifferences were compared by a χ^2 test. Both tests performed using arsenal (v 3.6.3) on R (v 4.4.1).

3.1.2 The Amniotic Fluid has a Unique Inflammatory Profile

Amniotic fluid was collected from 32 of 37 study participants, many of whom come from a diverse cultural background with a median age of 33 years old (29, 36, IQR). To characterize the amniotic fluid cytokine/chemokine profile and to compare it to other tissues present in pregnancy (umbilical cord serum, maternal serum, and placenta), I analyzed 32 amniotic fluid, 33 umbilical cord serum, 33 maternal serum, and 36 placenta samples for 99 cytokines/chemokines. Analyte concentrations were calculated by Eve Technologies following assay standard curves and established standard operating procedures. All analyzed 99 cytokines/chemokines were detected in all samples, although some values were deemed “out of range” of the assay standard curves. Out of all 16,038 data points collected for the cytokine/chemokine assays, 773 data points were deemed out of range, of which only around 13% were above the dynamic ranges of the standard curves. Additionally, of those that were above the dynamic range, none of the data points were consistent in only one cytokine indicating that none of the samples needed to be diluted and reanalyzed. However, fluorescence intensity was quantified thereby establishing the presence of those cytokines/chemokines within the sample. Cytokine abundances for each sample type collected are presented in Appendix C, Table A 3.

Principal component analysis (PCA) is a powerful technique to determine the overall relationships of multi-dimensional data. It reduces data dimensionality to a set of predictor variables termed principal components (PC) that can be used to predict the characteristics of similar subsets of data within your overall data (114). Therefore, PCA can be used to determine the underlying relationships of the cytokine/chemokine profiles of my samples. PCA of the cytokine/chemokine abundances of all samples revealed the uniqueness of the amniotic fluid

inflammatory profile as compared to the other sample types collected (Figure 4). No overlap of cytokine/chemokine profile was observed for amniotic fluid and placenta with other sample types. Umbilical cord serum and maternal serum inflammatory profiles showed overlap with each other.

For PC1 (Dim1 in Figure 4), the top 10 cytokines that contribute to the variance in PC1 were CTACK, SCF, BAFF, TNF α , TGF- β 1, Perforin, Eotaxin-3, IL-29, sFasL, and TPO. The inflammatory profiles of both umbilical cord serum and maternal serum were characterized by elevated levels of cytokines such as, but not limited to, CTACK, SCF, and BAFF. The abundances of the top 10 cytokines are shown in Figure 5.

For PC2 (Dim2 in Figure 4), the top 10 cytokines that contribute to the variance in PC2 were IFN α 2, IL-28A, MCP-3, G-CSF, MCP-1, sCD137, IL-1 β , IL-9, IL-1RA, and GRO α . The amniotic fluid inflammatory profile was characterized by increased abundances of certain cytokines, G-CSF and MCP-1, for example. The abundances of the top 10 cytokines for each sample type are shown in Figure 6.

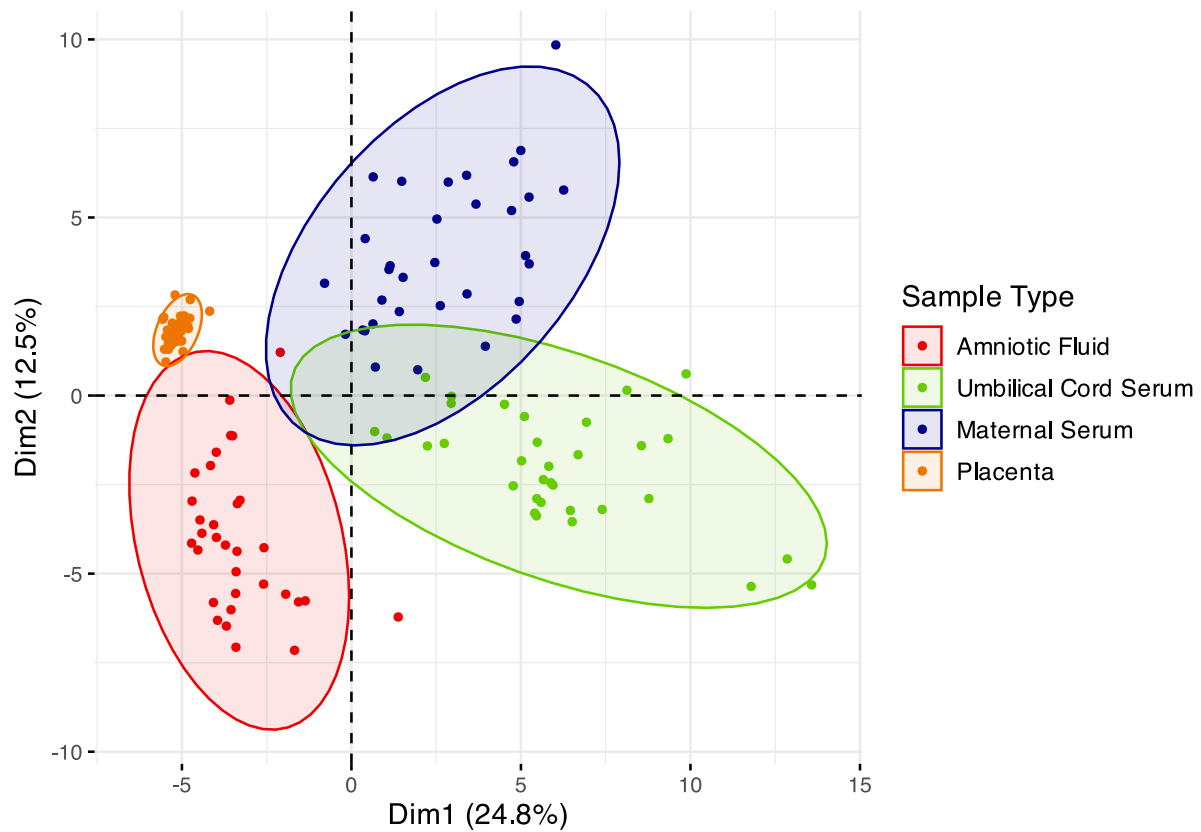


Figure 4. Principal component analysis (PCA) reveals unique cytokine/chemokine profiles for tissues present in pregnancy. Amniotic fluid ($n = 32$), umbilical cord serum ($n = 33$), maternal serum ($n = 33$), and placenta ($n = 36$). Ellipse is drawn over a 95% confidence interval for each group. PCA was performed using FactoMineR (v 2.11) on R (v 4.4.1).

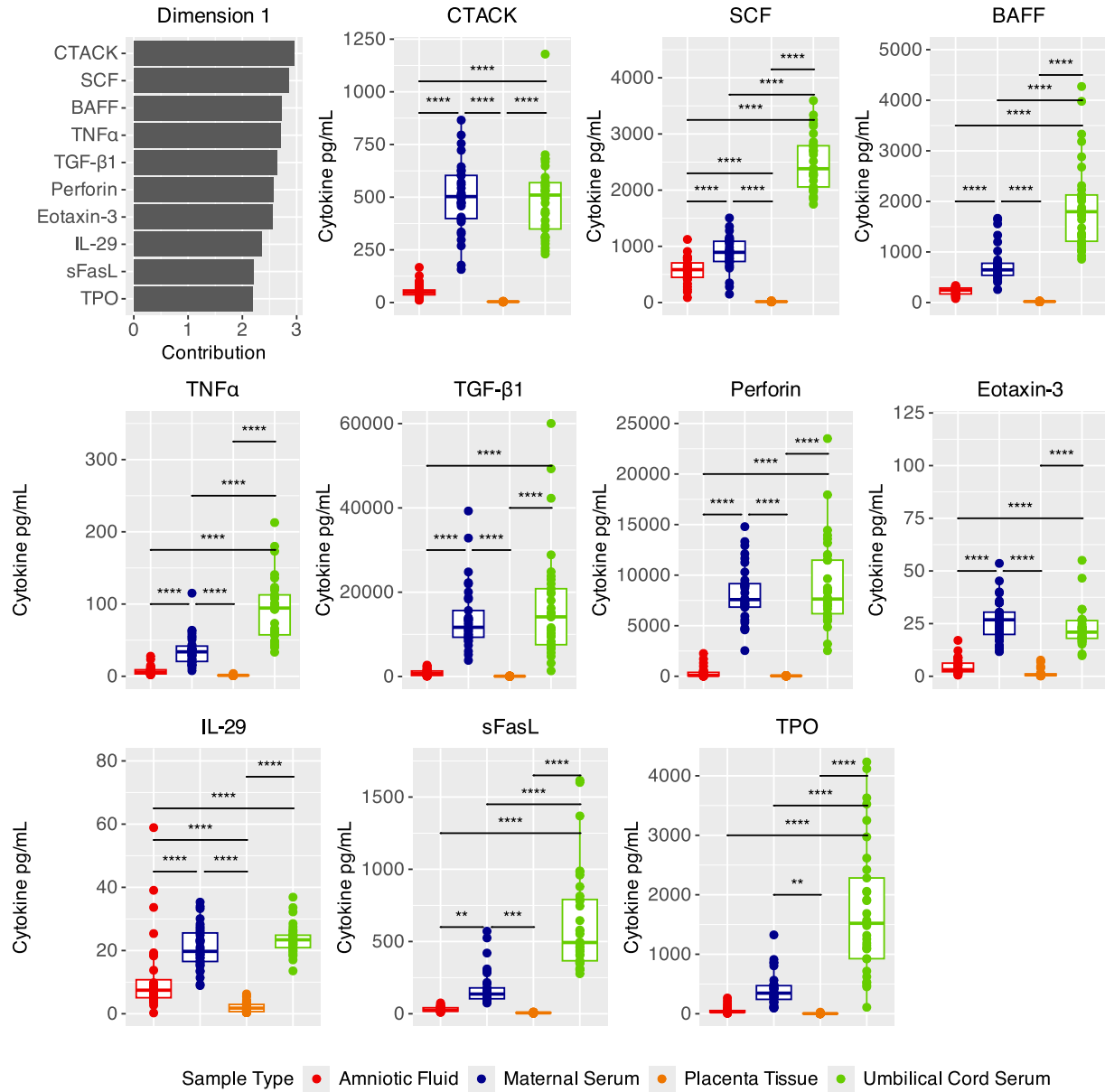


Figure 5. Umbilical cord serum and maternal serum are characterized by abundances of some cytokines. (a) Bar plot showing the level of contribution the top 10 cytokines have in the variation within principal component 1 (Dim1 or x-axis) of the PCA plot shown in Figure 4. (b) – (k) Scatter plot showing the abundances of CTACK, SCF, BAFF, TNF α , TGF- β 1, Perforin, Eotaxin 3, IL-29, sFasL, and TPO for each sample type with boxplots showing medians and interquartile ranges. Amniotic fluid (n = 32), umbilical cord serum (n = 33), maternal serum (n = 33), and placenta (n = 36). Abundance comparisons were performed by a linear mixed-effects repeated measures model using lmerTest (v 3.1-3) followed by pairwise post-hoc testing using emmeans (v 1.11.0) on R (v 4.4.1). **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$

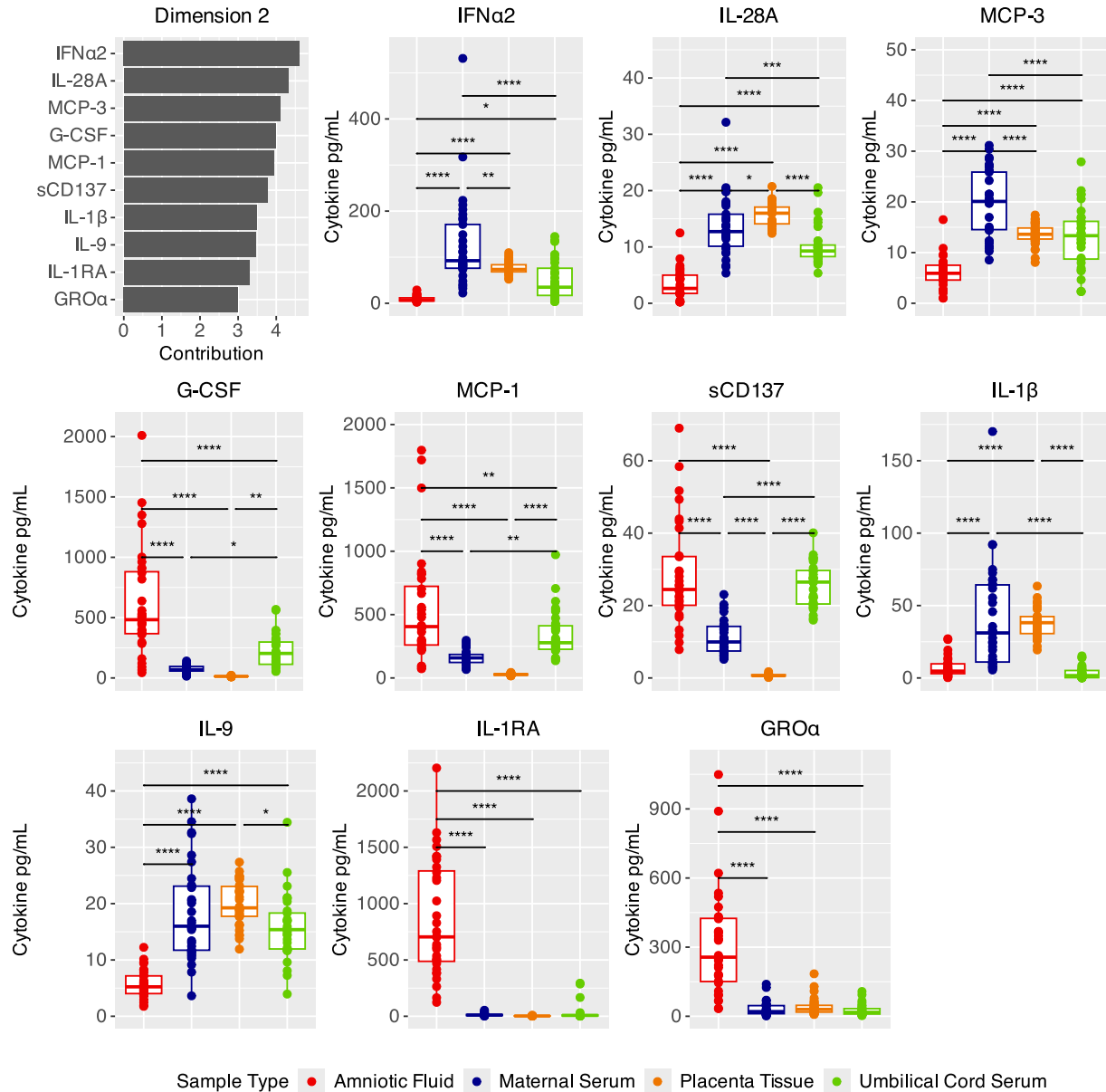


Figure 6. Amniotic fluid and placenta tissue are characterized by abundances of some cytokines. (a) Bar plot showing the level of contribution the top 10 cytokines have in the variation within principal component 2 (Dim2 or y-axis) of the PCA plot shown in Figure 4. (b) – (k) Scatter plot showing the abundances of IFN α 2, IL-28A, MCP-3, G-CSF, MCP-1, sCD137, IL 1 β , IL-9, IL-1RA, and GRO α for each sample type with boxplots showing medians and interquartile ranges. Amniotic fluid (n = 32), umbilical cord serum (n = 33), maternal serum (n = 33), and placenta (n = 36). Abundance comparisons were performed by a linear mixed-effects repeated measures model using lmerTest (v 3.1-3) followed by pairwise post-hoc testing using emmeans (v 1.11.0) on R (v 4.4.1). **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

3.1.3 The Amniotic Fluid Inflammatory Profile is a Network

To characterize the inflammatory profile of the amniotic fluid alone, I correlated the abundances of each cytokine/chemokine among themselves and clustered these abundances using complete clustering method and Spearman clustering distance. A number of pairs of cytokines were highly positively correlated with each other such as LIF and IL-29 ($R^2 = 0.9731$), TGF α and IL-17F ($R^2 = 0.8703$), and IFN ω and LIF ($R^2 = 0.9368$), to name a few (Figure 7). Clustering revealed 5 groups of cytokines whose abundances in amniotic fluid were positively correlated with each other (Figure 7, Figure 8, and Table 4). Functional enrichment analysis of each cluster using the STRING database revealed potential protein-protein interaction networks within each group (Figure 10). As this is a discovery study, clustering and functional enrichment analyses were done on correlation coefficients regardless of correlation significance.

The heatmap (Figure 7) also revealed that clusters 1 and 2 negatively correlate with each other, although this relationship was not captured by the dendrogram. To study this relationship, a correlation plot was generated with only significant ($p < 0.05$) correlations shown (Figure 9). The correlation plot showed groups of cytokines that were negatively correlated with each other. For example, PDGF-AA, PDGF-AB/BB, and IL-27 are all negatively correlated with EGF ($R^2 = 0.2704$, $R^2 = 0.3521$, $R^2 = 0.2485$, respectively) and TGF- β 2 ($R^2 = 0.2803$, $R^2 = 0.3644$, $R^2 = 0.3920$, respectively).

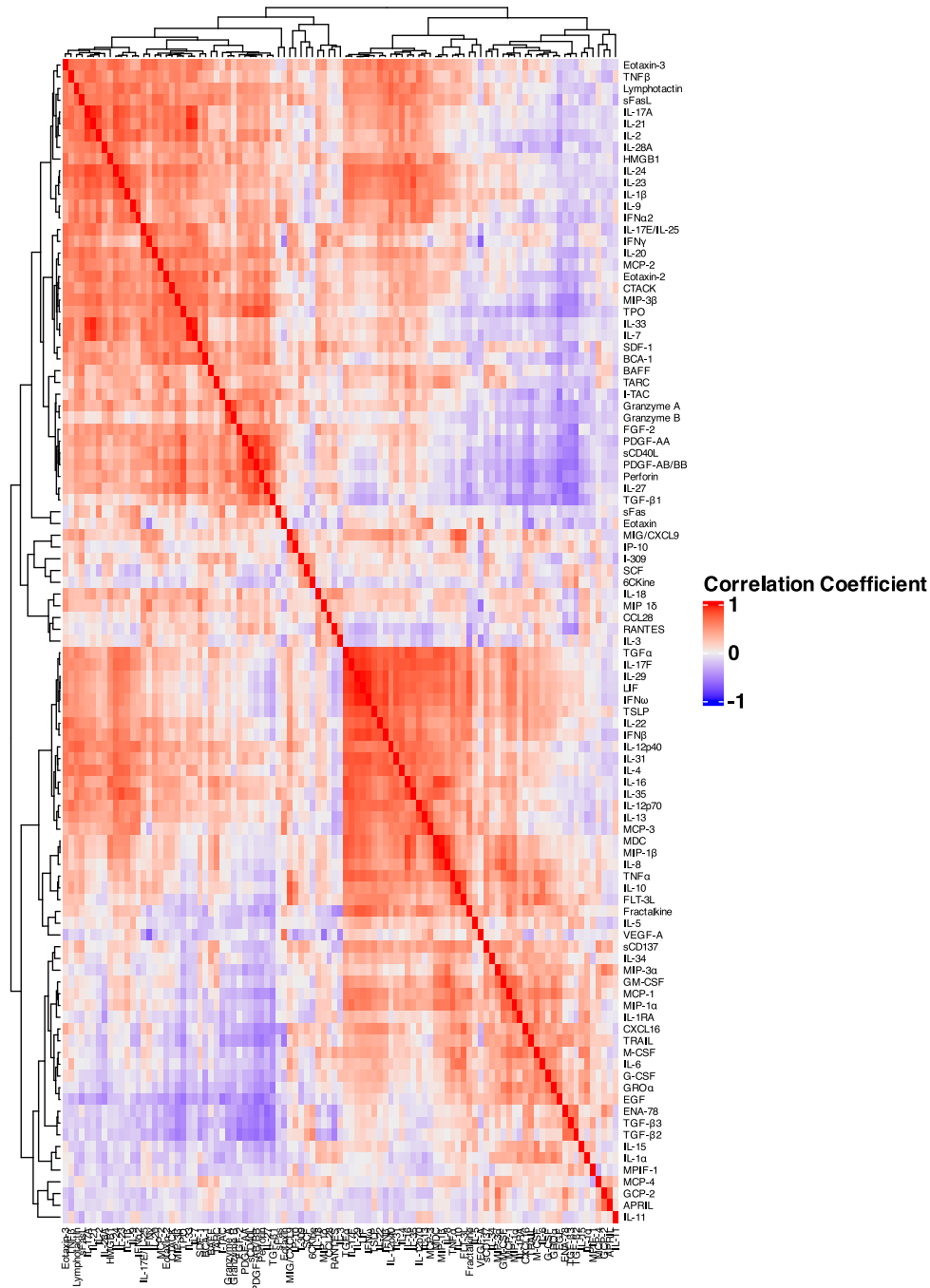


Figure 7. Cytokines and chemokines in the amniotic fluid show positive correlation. Correlation of the cytokine/chemokine profile of amniotic fluid (n = 33) presented as a heatmap showing the correlation coefficient of each cytokine/chemokine among themselves. Profiles were clustered using Spearman clustering distance and complete clustering method. Red and blue represent positive and negative correlation coefficients, respectively. Correlation was calculated using Hmisc (v 5.2-3) and visualized using ComplexHeatmap (v 2.20.0) on R (v 4.4.1).

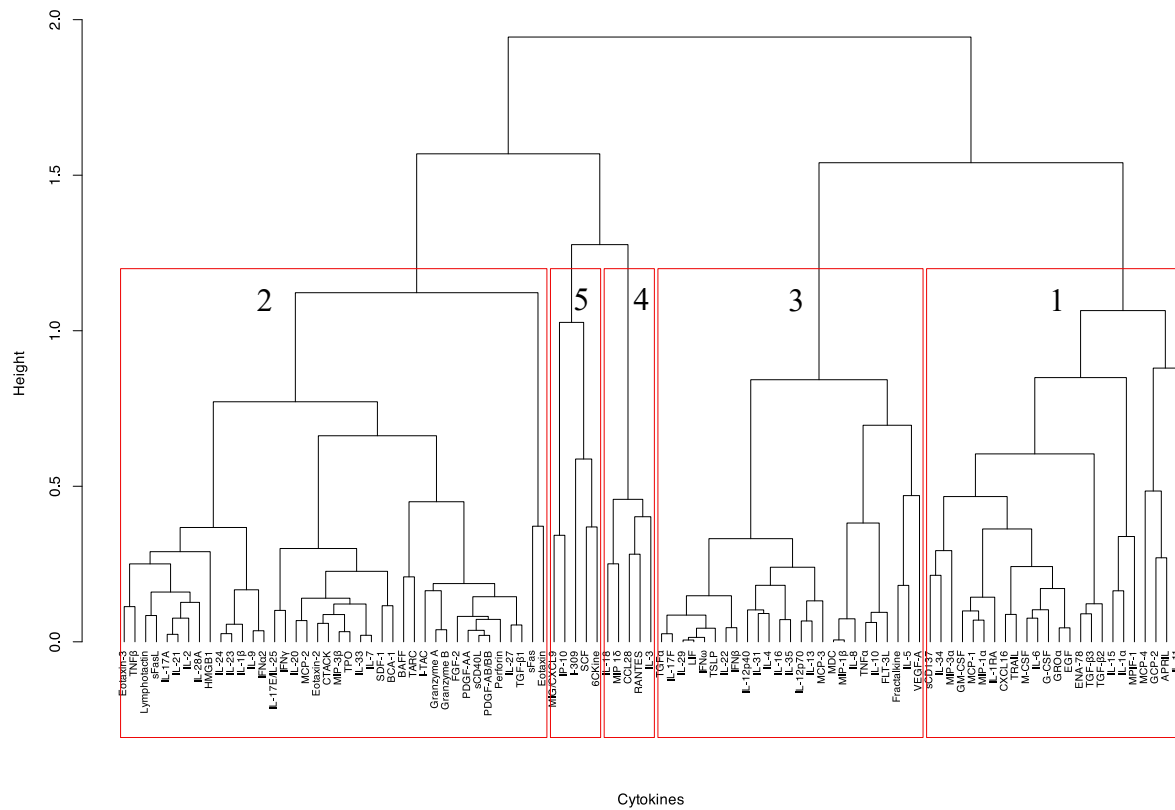


Figure 8. Amniotic fluid cytokine/chemokine profile is characterized by groups with correlated abundances. Correlation clustering dendrogram shows 5 groups of cytokines that cluster closely together. Dendrogram cut height was set at $h = 1.2$ to produce 5 groups (Table 4). Cluster number shown in dendrogram. Clustering was determined using Spearman clustering distance and complete clustering method using ComplexHeatmap (v 2.20.0) on R (v 4.4.1).

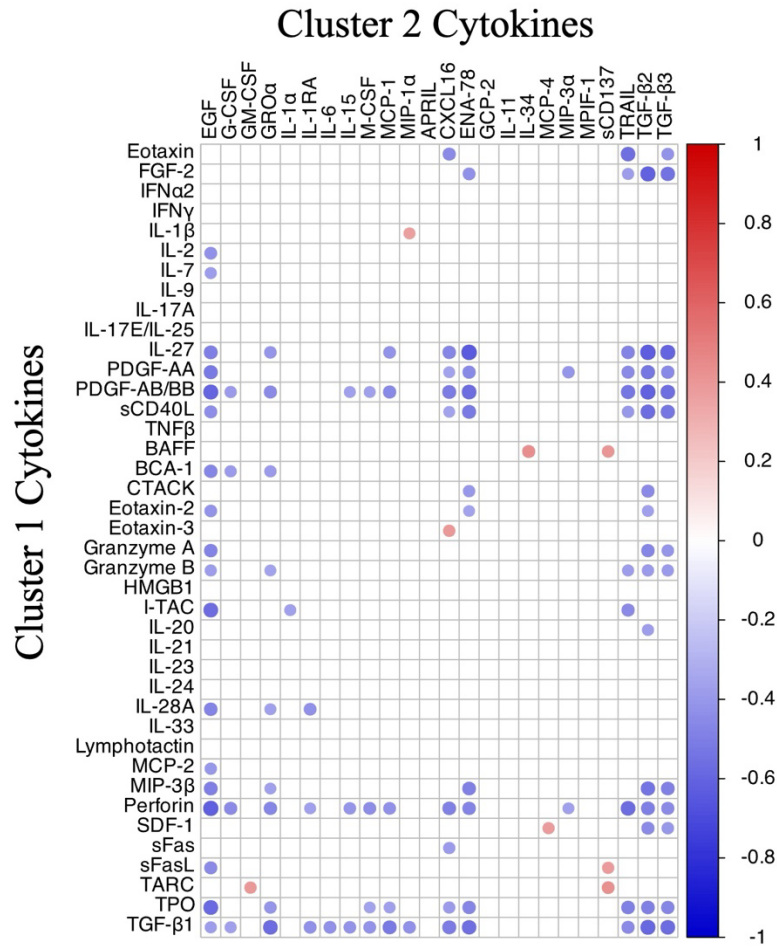


Figure 9. Some amniotic fluid cytokine/chemokine clusters correlate negatively with each other. Correlation plot of cluster 1 and cluster 2 cytokines showing only significant correlations ($p < 0.05$). Red and blue represent negative and positive correlation coefficients, respectively. Correlation was calculated using Spearman correlation using Hmisc (v 5.2-3) on R (v 4.4.1).

Table 4. Cluster groupings of cytokines identified and quantified within the amniotic fluid.

Cluster					
1	2	3	4	5	
EGF	Eotaxin	IL-21	FLT-3L	IL-3	IP-10
G-CSF	FGF-2	IL-23	Fractalkine	IL-18	MIG/CXCL9
GM-CSF	IFN α 2	IL-24	IL-4	RANTES	6CKine
GRO α	IFN γ	IL-28A	IL-5	CCL28	I-309
IL-1 α	IL-1 β	IL-33	IL-8	MIP 1 δ	SCF
IL-1RA	IL-2	Lymphotactin	IL-10		
IL-6	IL-7	MCP-2	IL-12p40		
IL-15	IL-9	MIP-3 β	IL-12p70		
M-CSF	IL-17A	Perforin	IL-13		
MCP-1	IL-17E/IL-25	SDF-1	IL-17F		
MIP-1 α	IL-27	sFas	IL-22		
APRIL	PDGF-AA	sFasL	MCP-3		
CXCL16	PDGF-AB/BB	TARC	MDC		
ENA-78	sCD40L	TPO	MIP-1 β		
GCP-2	TNF β	TGF- β 1	TGF α		
IL-11	BAFF		TNF α		
IL-34	BCA-1		VEGF-A		
MCP-4	CTACK		IFN β		
MIP-3 α	Eotaxin-2		IFN ω		
MPIF-1	Eotaxin-3		IL-16		
sCD137	Granzyme A		IL-29		
TRAIL	Granzyme B		IL-31		
TGF- β 2	HMGB1		IL-35		
TGF- β 3	I-TAC		LIF		
	IL-20		TSLP		

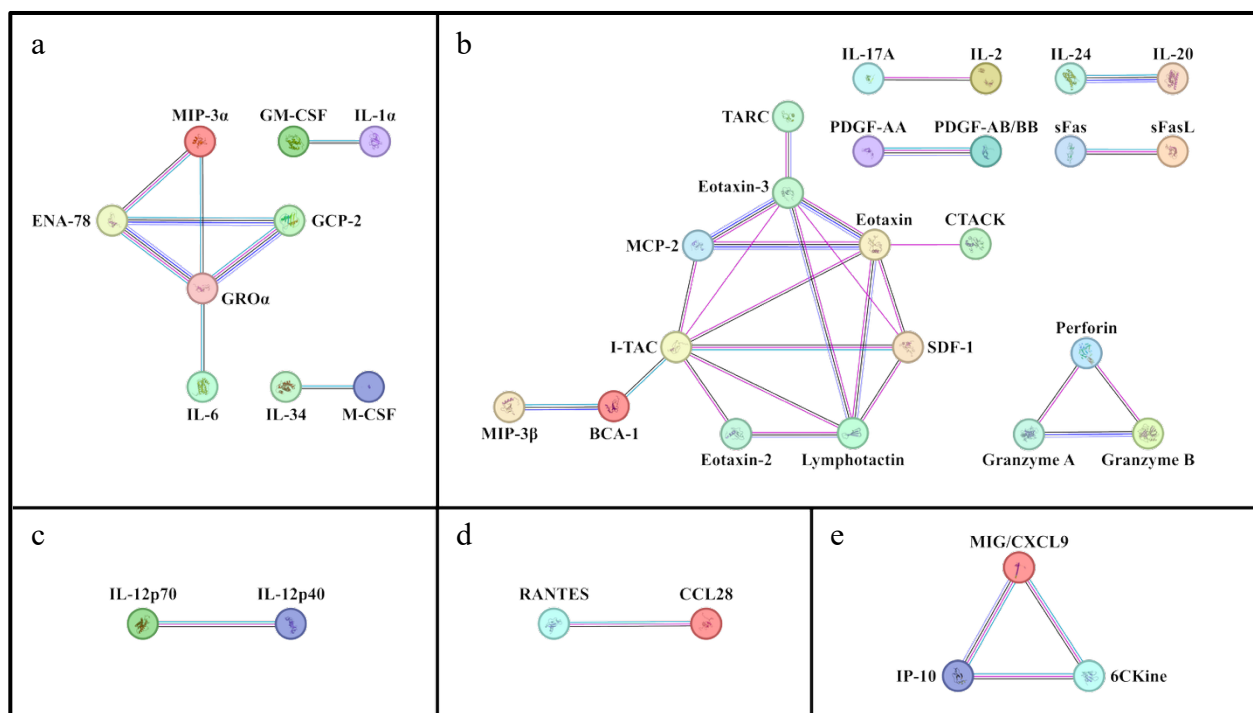


Figure 10. Functional enrichment analyses of cytokine/chemokine clusters predict protein-protein interactions. Interaction network of cytokine clusters was identified using STRING database with Neighbourhood, Experiments, Gene Fusion, Databases, Co-occurrence, and Co-expression settings for active interaction sources. Minimum required interaction score was set at 0.700 (high confidence). (a) Cluster 1, (b) Cluster 2, (c) Cluster 3, (d) Cluster 4, (e) Cluster 5.

To further elucidate the predicted protein-protein interaction networks (Figure 10), the cytokines and chemokines that form a network of at least 3 members in each cluster were analyzed for pathway enrichment using Reactome Pathways built into EnrichR. The group in cluster 1 was involved in chemokine receptor binding chemokines, peptide ligand-binding receptors, and IL-10 signaling. The bigger group in cluster 2 was involved in chemokine receptor binding chemokines, peptide ligand-binding receptors, and Class A 1 (rhodopsin-like receptors), while the smaller group in cluster 2 was involved in ALK signaling in cancer. Lastly, the group in cluster 5 was involved in similar pathways as the core groups in clusters 1 and 2 and in addition it was also involved in G Alpha (I) signaling (Table 5).

Table 5. Predicted protein-protein interactions in cytokine/chemokine clusters are involved in various pathways.

Cluster	Cytokine/Chemokine	Pathway	<i>p</i> value ^a
1	MIP-3 α , ENA-78, GRO α , GCP-2, IL-6	Chemokine Receptors Binding Chemokines	< 0.0001
		Peptide Ligand-Binding Receptors	< 0.0001
		IL-10 Signaling	< 0.0001
2	MIP-3 β , BCA-1, I-TAC, Eotaxin-2, Lymphotactin, SDF-1, Eotaxin, CTACK, Eotaxin-3, MCP-2, TARC	Chemokine Receptors Binding Chemokines	< 0.0001
		Peptide Ligand-Binding Receptors	< 0.0001
		Class A 1 (Rhodopsin-like Receptors)	< 0.0001
2	Perforin, Granzyme A, Granzyme B	Nuclear Events Stimulated by ALK Signaling in Cancer	< 0.0001
		Signaling by ALK Fusions and Activated Point Mutants	< 0.0001
		Signaling by ALK in Cancer	< 0.0001
3	MIG/CXCL9, IP-10, 6CKine	Chemokine Receptors Binding Chemokines	< 0.0001
		Peptide Ligand-Binding Receptors	< 0.0001
		G Alpha (I) Signaling Events	< 0.0001

^a*p* values of association in the identified pathways are taken from Reactome Pathways from EnrichR

3.1.4 The Amniotic Fluid Inflammatory Profile is Altered by Cigarette Smoke Exposure

Our participant recruitment questionnaire asked participants if and how often they smoke traditional cigarettes. However, to confirm cigarette smoke exposure and to quantify the levels thereof, I performed a cotinine ELISA on all samples collected. No cotinine could be detected in any placenta sample. Cotinine was present in amniotic fluid ($n = 7$), umbilical cord serum ($n = 7$), and maternal serum ($n = 7$) as shown in Figure 11. Not all amniotic fluid collected had matched sera samples, only 6 samples had complementary sera. Nevertheless, the levels of cotinine detected in amniotic fluid is significantly different from that of umbilical cord serum ($p < 0.05$) but not maternal serum (ns, $p = 0.0826$). On average, amniotic fluid had higher concentrations of cotinine (49.00 ± 10.95 ng/mL) than umbilical cord serum (33.85 ± 22.23 ng/mL) and maternal serum (38.45 ± 23.69 ng/mL).

Cotinine-positive amniotic fluids ($n = 7$) were then considered to be exposed to cigarette smoke while samples that were cotinine-negative ($n = 25$) were considered to not be exposed to cigarette smoke. PLS-DA comparison of the inflammatory profiles between cotinine-positive and cotinine-negative samples identified cytokines that were of high importance in discriminating between the two groups based on VIP scores (Figure 13). Cotinine-positive amniotic fluid had an altered cytokine/chemokine profile characterized by increased abundances of cytokines such as MIG/CXCL9, IP-10, IFN γ , IL-10, and IL-6 as shown in Figure 12 and Figure 13.

Differential abundance analysis of the cytokine/chemokine profiles of cotinine-positive and cotinine-negative amniotic fluid samples revealed cytokines that were significantly abundant in cotinine-positive samples: MIG/CXCL9 (4-fold), IP-10 (12-fold), IFN γ (2-fold), IL-10 (2-fold), and IL-6 (2-fold) compared to cotinine-negative. As well, some cytokines were significantly abundant in cotinine-negative samples: CTACK (2-fold), CCL28 (1.2-fold), and Eotaxin-2 (2-fold)

compared to cotinine-positive as shown in Figure 14 and Table 6. Additionally, the full comparison of abundances between the two groups are presented in Appendix D, Table A 4.

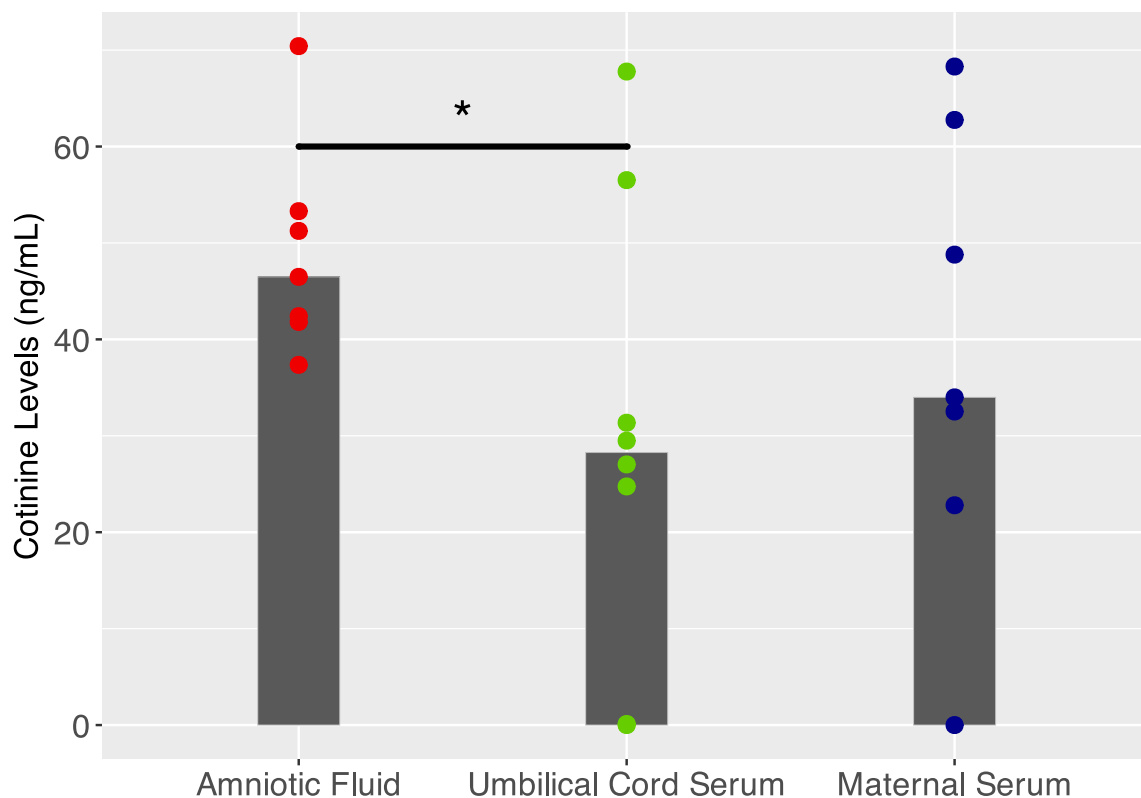


Figure 11. Cotinine bioaccumulates in amniotic fluid ($n = 7$). Cotinine is also detected in umbilical cord serum ($n = 7$), and maternal serum ($n = 7$). Bar plots show medians and individual data points. Abundance comparisons were performed by a linear-mixed effects repeated measures model using lmerTest (v 3.1-3) followed by pairwise post-hoc testing using emmeans (v 1.11.0) on R (v 4.4.1). * $p < 0.05$.

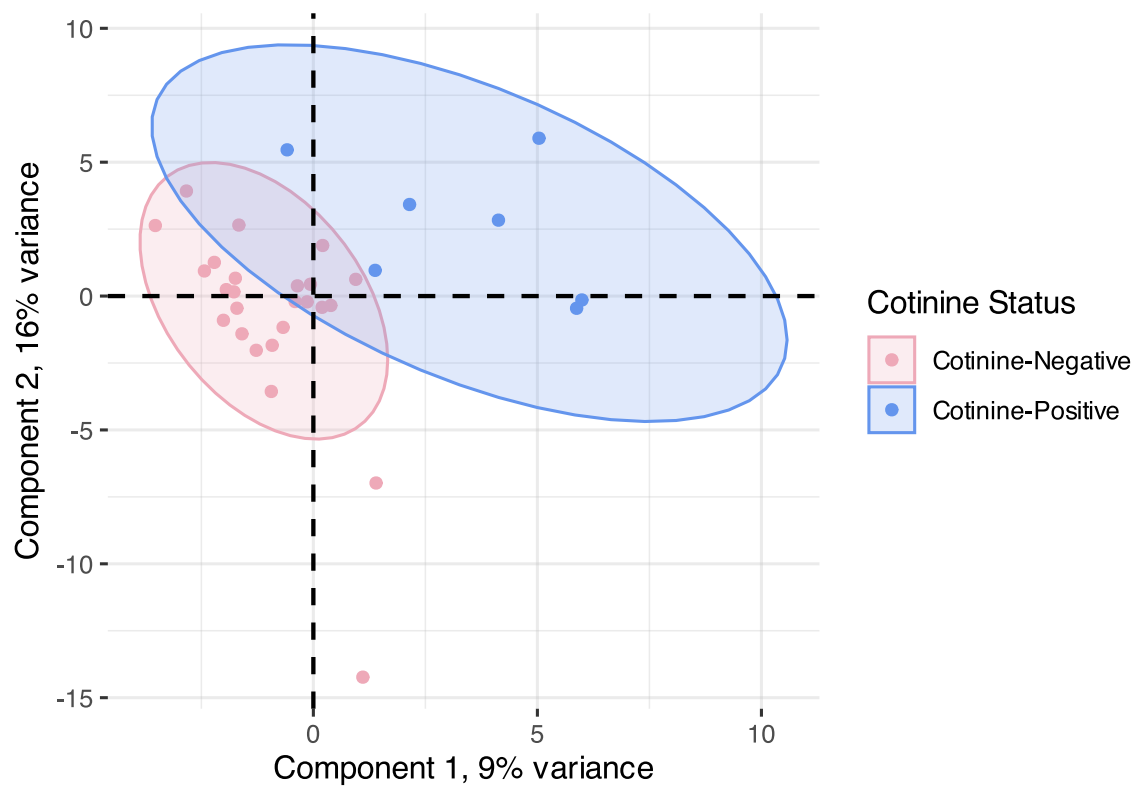


Figure 12. The cytokine/chemokine profile of amniotic fluid is altered by cigarette smoke exposure. Partial least-squares discriminant analysis (PLS-DA) showing the different cytokine/chemokine profiles of amniotic fluid of cotinine-positive ($n = 7$, blue) and of cotinine-negative ($n = 25$, pink). Ellipse drawn at a 95% confidence interval for each group. PLS-DA was performed using mixOmics (v 6.28.0) on R (v 4.4.1).

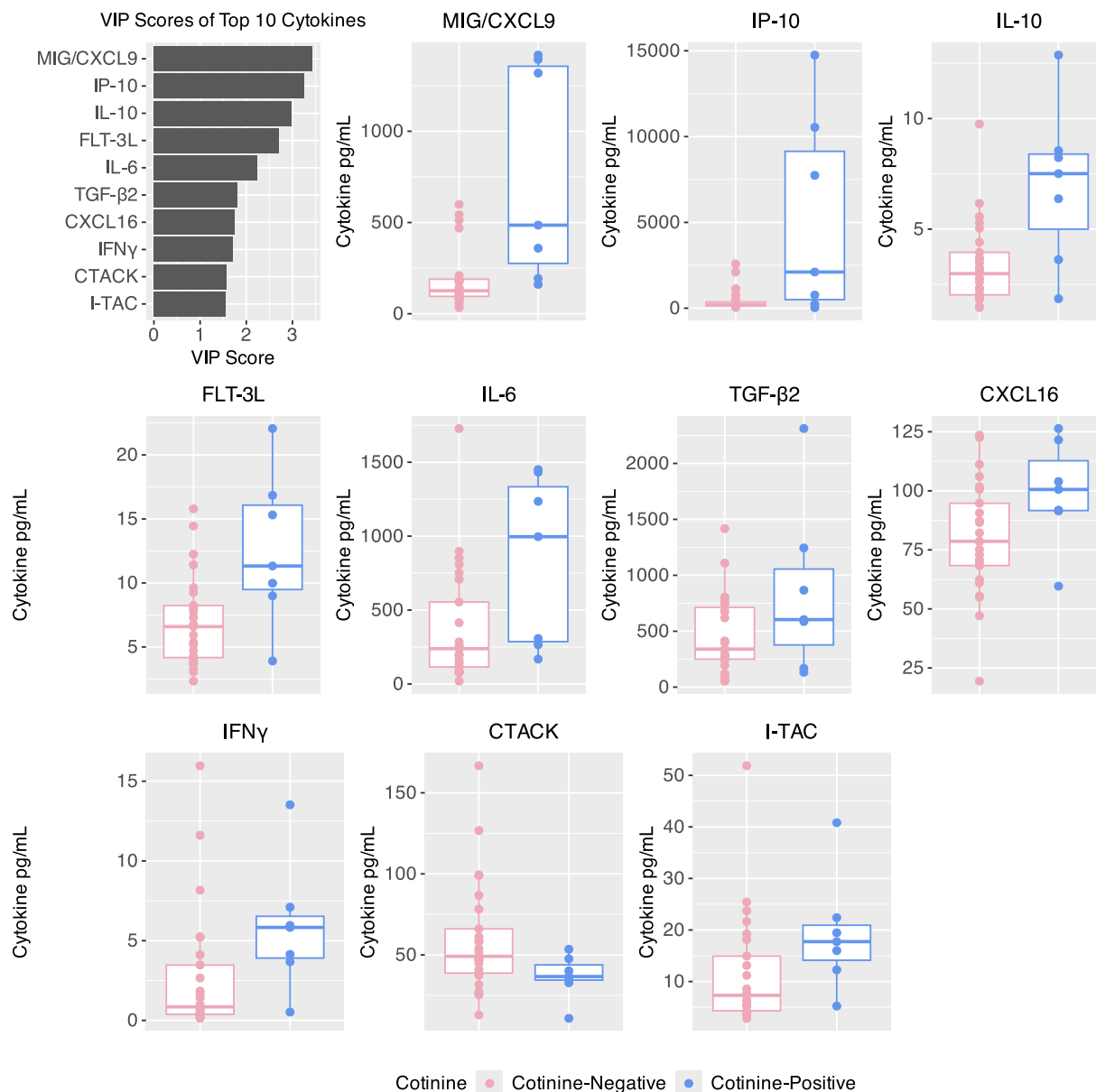


Figure 13. Amniotic fluid exposed to cigarette smoke is characterized by higher abundances of some cytokines. (a) Top 10 cytokines with the highest VIP scores based on the PLS-DA. (b) – (k) Scatter plots of the abundances of MIG/CXCL9, IP-10, IL-10, FLT-3L, IL-6, TGF- β 2, CXCL16, IFN γ , CTACK, and I-TAC for both cotinine-positive ($n = 7$, blue) and cotinine-negative ($n = 25$, pink) samples. PLS-DA was performed using mixOmics (v 6.28.0) on R (v 4.4.1).

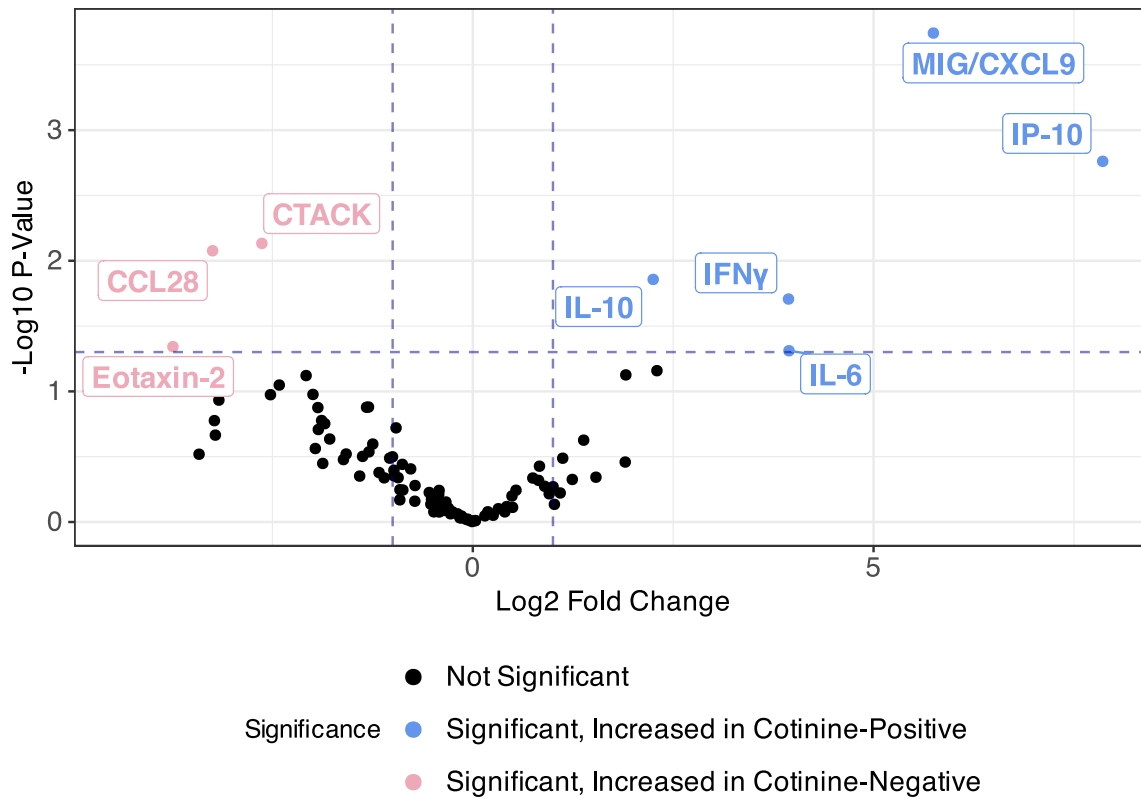


Figure 14. Differential abundance analysis shows some cytokines are significantly elevated in cotinine-positive amniotic fluid. Volcano plot showing the cytokines that are significantly more abundant in either the cotinine-positive (blue) or cotinine-negative (pink) samples. P values shown in plot are unadjusted and a $p < 0.05$ are significant. Differential abundance analysis performed by a linear model using limma (v 3.60.6) on R (v 4.4.1).

Table 6. Abundances and unadjusted *p* values of significantly different cytokines between cotinine-positive and cotinine-negative amniotic fluid samples.

Cytokine	Cotinine-Positive (Mean $\pm$ SD pg/mL)	Cotinine-Negative (Mean $\pm$ SD pg/mL)	Unadjusted <i>p</i> value
MIG/CXCL9	761.11 $\pm$ 586.28	187.65 $\pm$ 160.86	0.0002
IP-10	5167.28 $\pm$ 5871.21	440.94 $\pm$ 623.31	0.002
CCL28	256.52 $\pm$ 217.11	317.44 $\pm$ 151.56	0.008
CTACK	36.81 $\pm$ 13.50	59.27 $\pm$ 34.61	0.007
IL-10	7.00 $\pm$ 3.58	3.4 $\pm$ 1.87	0.01
IFN γ	5.82 $\pm$ 4.01	2.72 $\pm$ 3.95	0.02
IL-6	836.66 $\pm$ 572.74	374.52 $\pm$ 394.07	0.05
Eotaxin-2	24.72 $\pm$ 26.86	48.43 $\pm$ 65.12	0.05

3.2 Objective 2 Results

3.2.1 Amniotic Fluid Alters Calu-3 Barrier Function at 24 Hours of Exposure

Exposure of Calu-3 cells to 50% v/v amniotic fluid, regardless of cotinine status, resulted in an initial increase in barrier function (1.16-fold) after 24 hours compared to pre-exposure levels. However, modulation of barrier function gradually decreased characterized by similar barrier function to control at the 72-hour time point relative to pre-exposure levels (amniotic fluid exposure = 1.30-fold, control = 1.31-fold, Figure 15).

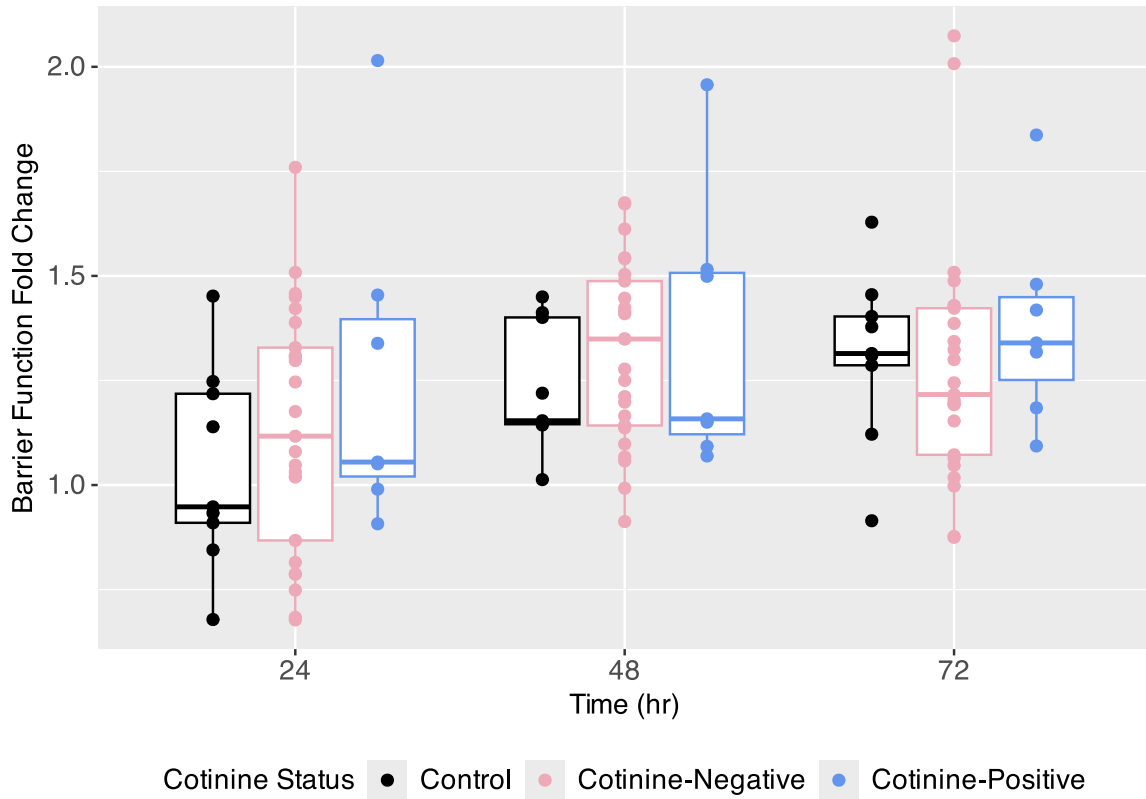


Figure 15. Calu-3 barrier function increases at 24 hours after exposure to 50% v/v amniotic fluid then attenuates back to control levels over 72 hours. Data points are shown for each sample, cotinine-positive (n = 7, blue), cotinine-negative (n = 25, pink), and control (n = 9, black). Boxplots show median and interquartile range.

Since barrier function weaned to control levels over 72 hours and barrier function could be affected by cell paracrine and autocrine signalling, I focused on the 24-hour time point where there is most variability in response to amniotic fluid exposure. Responses ranged from 0.68- to 1.76-fold and 0.91- to 2.02-fold for cotinine-negative and cotinine-positive samples respectively. On average, after 24 hours of exposure to 50% v/v amniotic fluid, Calu-3 cells exposed to cotinine-positive samples had a higher increase in barrier function (1.26-fold) than those exposed to cotinine-negative (1.13-fold) and control (1.04-fold) conditions. These differences in response were not significant (ns, $p = 0.363$) as shown in Figure 16.

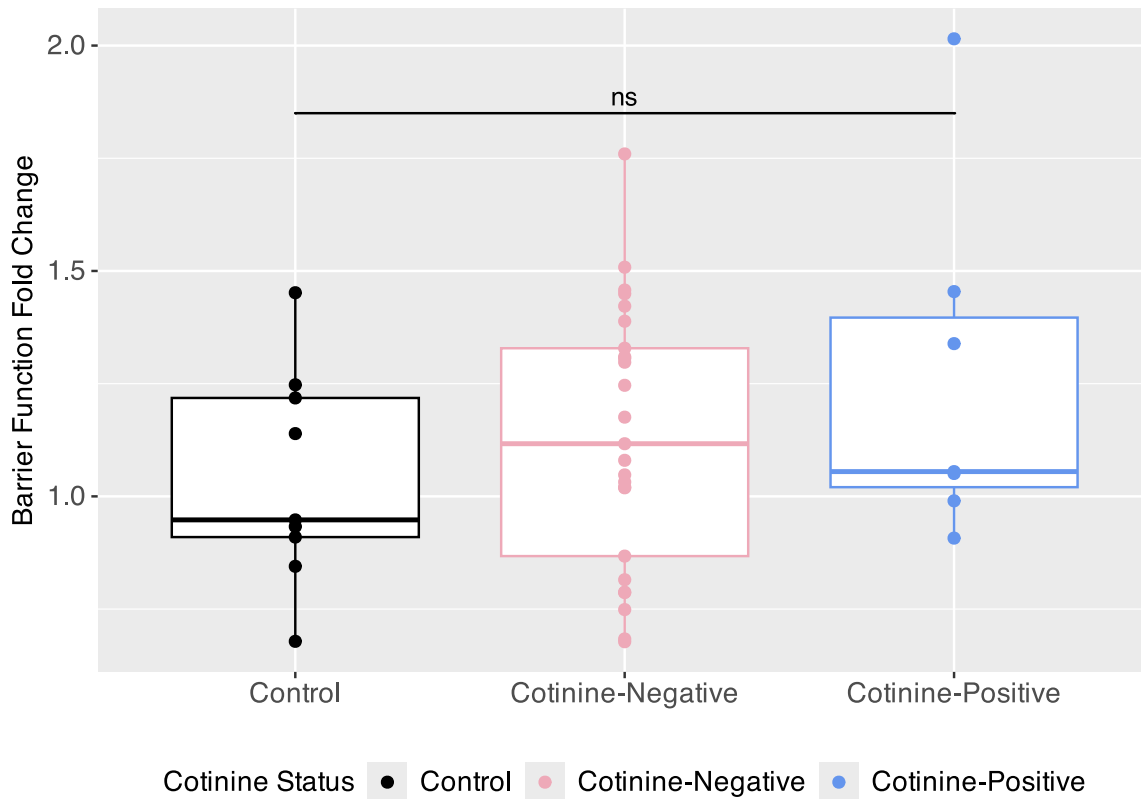


Figure 16. Calu-3 barrier function is altered by exposure to 50% v/v amniotic fluid after 24 hours. Cells exposed to cotinine-positive amniotic fluid ($n = 7$, blue) on average achieved a higher barrier function than those exposed to cotinine-negative ($n = 25$, blue) and control ($n = 9$, black). Scatter plot shows individual data points and boxplots show medians and interquartile ranges. Differences were compared by a one-way ANOVA using rstatix (v 0.7.2) on R (v 4.4.1). ns, $p = 0.363$

Correlation of the barrier function of cells after 24 hours of amniotic fluid exposure to their cytokine/chemokine abundances yielded four cytokines that were significantly negatively correlated to barrier function. These cytokines were Fractalkine, IFN α 2, IL-9, and IL-13 as shown in Figure 17. No cytokine was significantly positively correlated to barrier function.

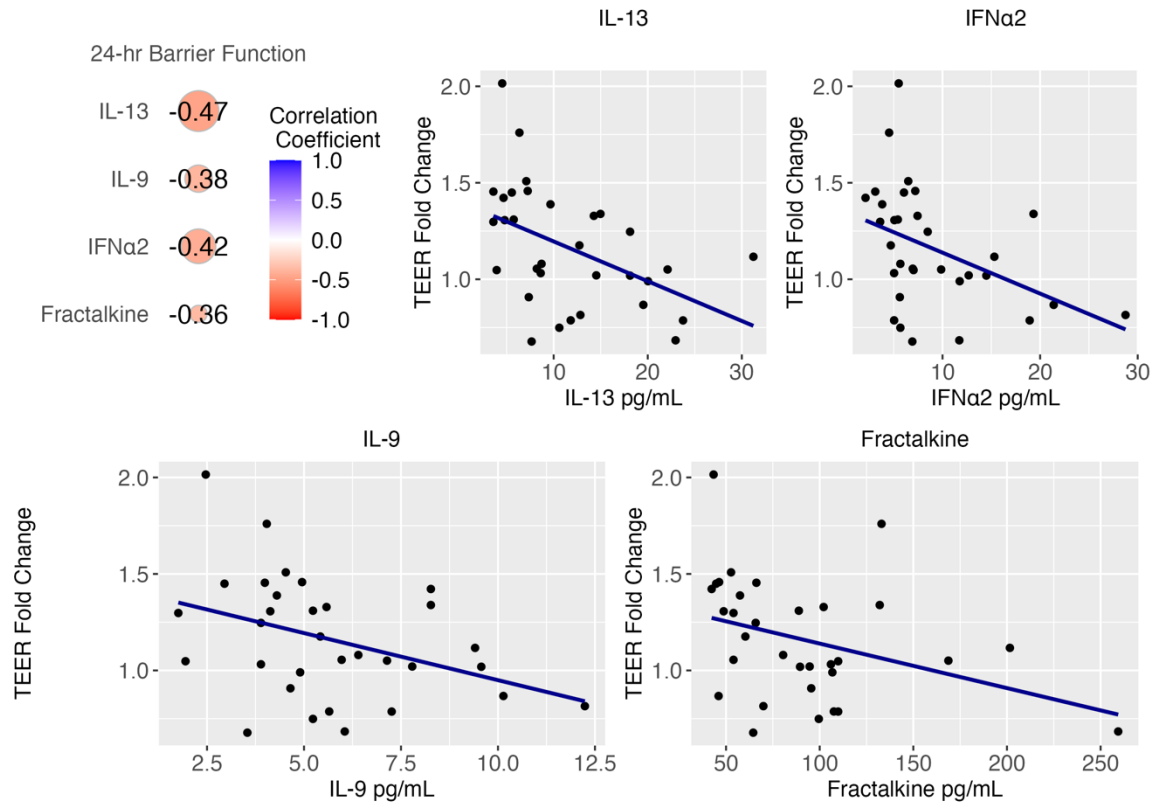


Figure 17. Fractalkine, IFN α 2, IL-9, and IL-13 abundances in amniotic fluid are significantly negatively correlated to Calu-3 barrier function following 24 hours of exposure to 50% v/v amniotic fluid. (a) Correlation coefficients of IL-13, IL-9, IFN α 2, and Fractalkine with barrier function after 24 hours of exposure, negative correlation is red, positive correlation is blue. (b) IL-13 abundance is negatively correlated with barrier function ($R^2 = 0.218$, $p = 0.0070$). (c) IFN α 2 abundance is negatively correlated with barrier function ($R^2 = 0.173$, $p = 0.0179$). (d) IL-9 abundance is negatively correlated with barrier function ($R^2 = 0.147$, $p = 0.0301$). (e) Fractalkine abundance is negatively correlated with barrier function ($R^2 = 0.129$, $p = 0.0438$). p values are unadjusted, $n = 32$ for all. Correlations were performed by Pearson correlation method using Hmisc (v 5.2-3) and individual linear regressions were performed, both on R (v 4.4.1).

3.2.2 Amniotic Fluid Augments Calu-3 Wound Healing

Measuring TEER over 72 hours from physical wounding of the Calu-3 cell monolayer was used to assess recovery of barrier function over said time. On average, 72 hours after wounding the cell layer via a scratch, cells exposed to cotinine-positive amniotic fluid recovered better (1.42-fold) than those exposed to cotinine-negative (1.05-fold) and control (1.12-fold) conditions. Regardless of cotinine status, amniotic fluid seemed to improve TEER recovery after 72 hours (1.16-fold) compared to control (1.12-fold). These differences in wound healing over 72 hours were not significant (Figure 18, $p = 0.193$, two-way ANOVA interaction p value).

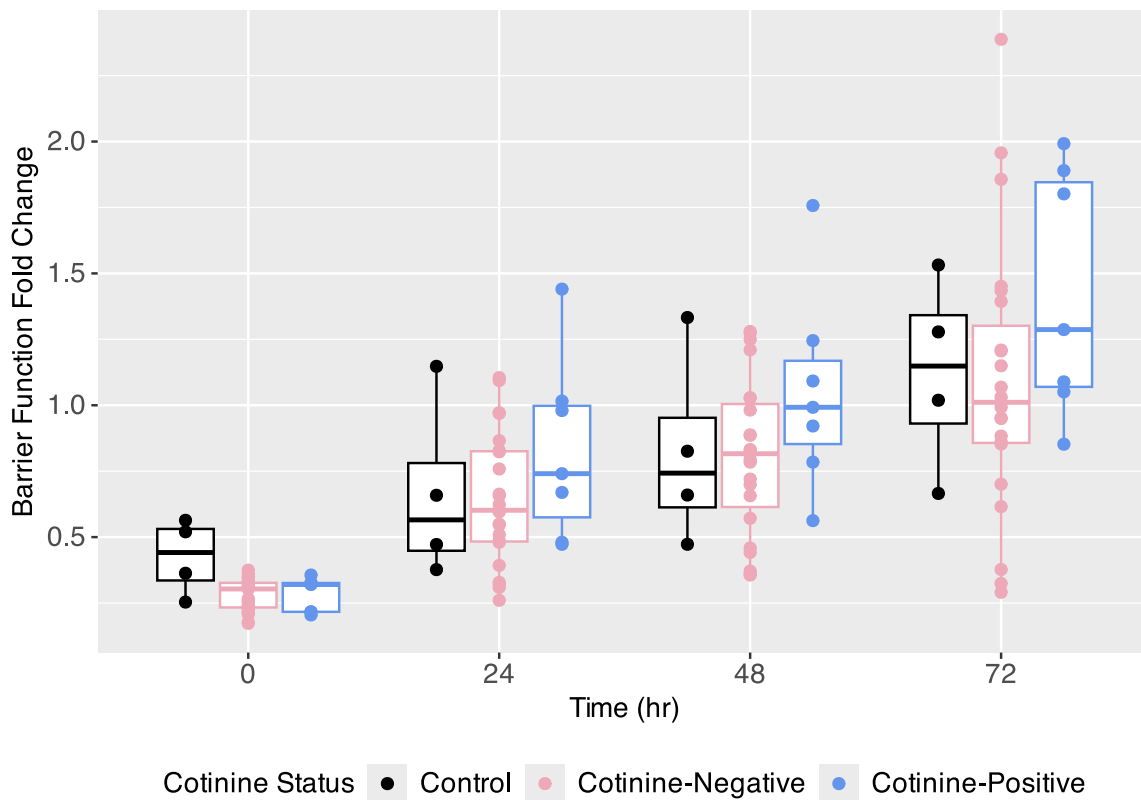


Figure 18. Amniotic fluid augments the recovery of barrier function of Calu-3 cells after wounding. Regardless of cotinine status, cells exposed to amniotic fluid ($n = 32$) recovered better than control conditions. Time = 0 represents barrier function after wounding. Differences in recovery of barrier function were compared by a two-way repeated measures ANOVA (factors: cotinine status, time) using R (v 4.4.1, $p = 0.193$).

To assess whether the amniotic fluid cytokines/chemokines were associated with initial response to wounding, the correlation of the TEER fold change at 24 hours to cytokine/chemokine abundances were calculated. This showed a number of cytokines that were both positively correlated (TNF α , MIP-1 α , and MCP-1) and negatively correlated (IL-28A, and Perforin) to barrier function ($P < 0.05$) as shown in Figure 19.

Since barrier function was not fully recovered by 24 hours (TEER fold-change < 1), I calculated wound healing capacity to correlate with cytokine abundances. This was assessed by the slope of TEER recovery as a function of time from 0 to 72 hours after wounding. On average, cells exposed to cotinine-positive amniotic fluid had a higher wound healing capacity ($0.0159 \pm 0.0058 \text{ } \Omega \cdot \text{hr}^{-1}$) than those exposed to cotinine-negative ($0.0112 \pm 0.0067 \text{ } \Omega \cdot \text{hr}^{-1}$) and control ($0.0097 \pm 0.0053 \text{ } \Omega \cdot \text{hr}^{-1}$) conditions. However, these differences were not significant (Figure 20, $p = 0.196$, one-way ANOVA).

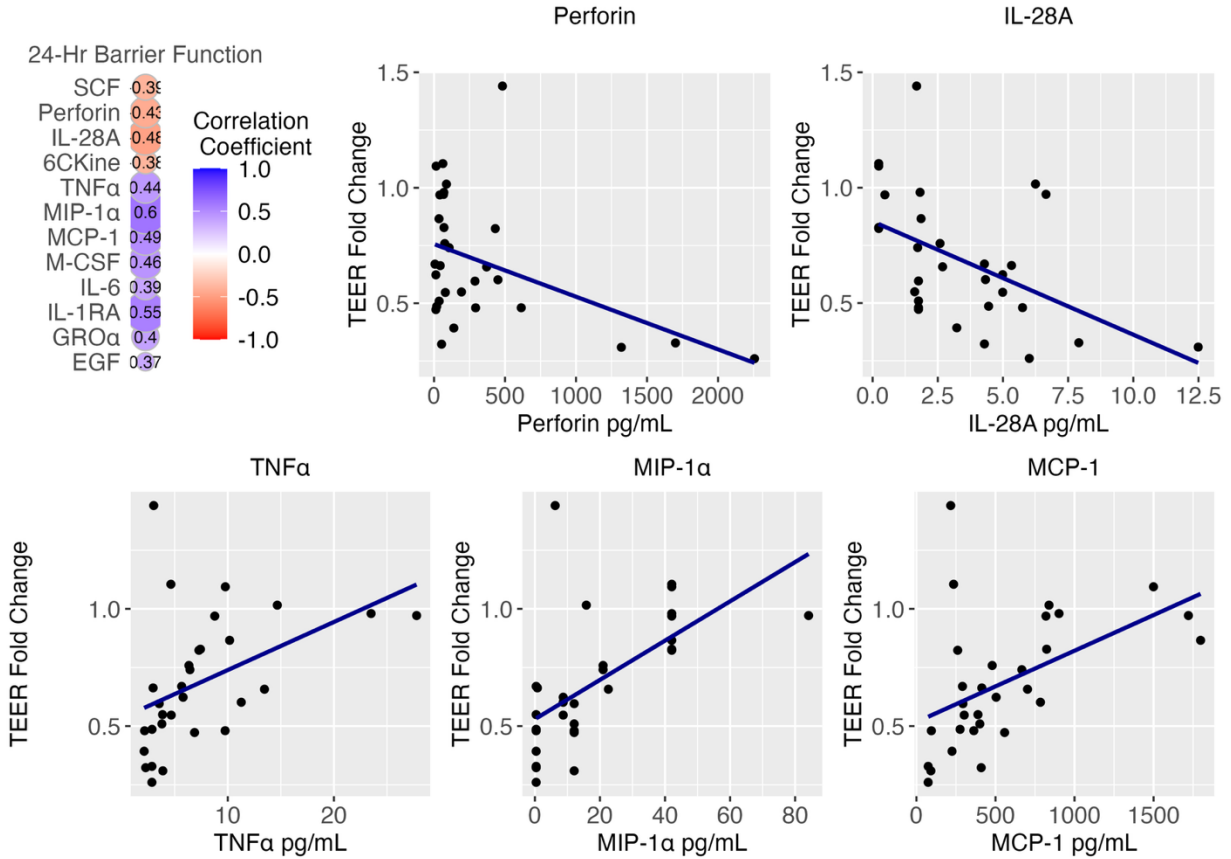


Figure 19. Abundances of certain cytokines and chemokines in amniotic fluid are significantly correlated to Calu-3 barrier function 24 hours after wounding and exposure to 50% v/v amniotic fluid. (a) Correlation coefficients of cytokines and barrier function 24 hours after wounding, negative correlation is red, positive correlation is blue. (b) Perforin is negatively correlated with barrier function ($R^2 = 0.1867$, $p = 0.0171$). (c) IL-28A is negatively correlated with barrier function ($R^2 = 0.2335$, $p = 0.0068$). (d) TNFα is positively correlated with barrier function ($R^2 = 0.1953$, $p = 0.0449$). (e) MIP-1α is positively correlated with barrier function ($R^2 = 0.3639$, $p = 0.0004$). (f) MCP-1 is positively correlated with barrier function ($R^2 = 0.2413$, $p = 0.0058$). p values are unadjusted, $n = 30$ for all. Correlations were performed by Pearson correlation method using Hmisc (v 5.2-3) and individual linear regressions were performed, both on R (v 4.4.1).

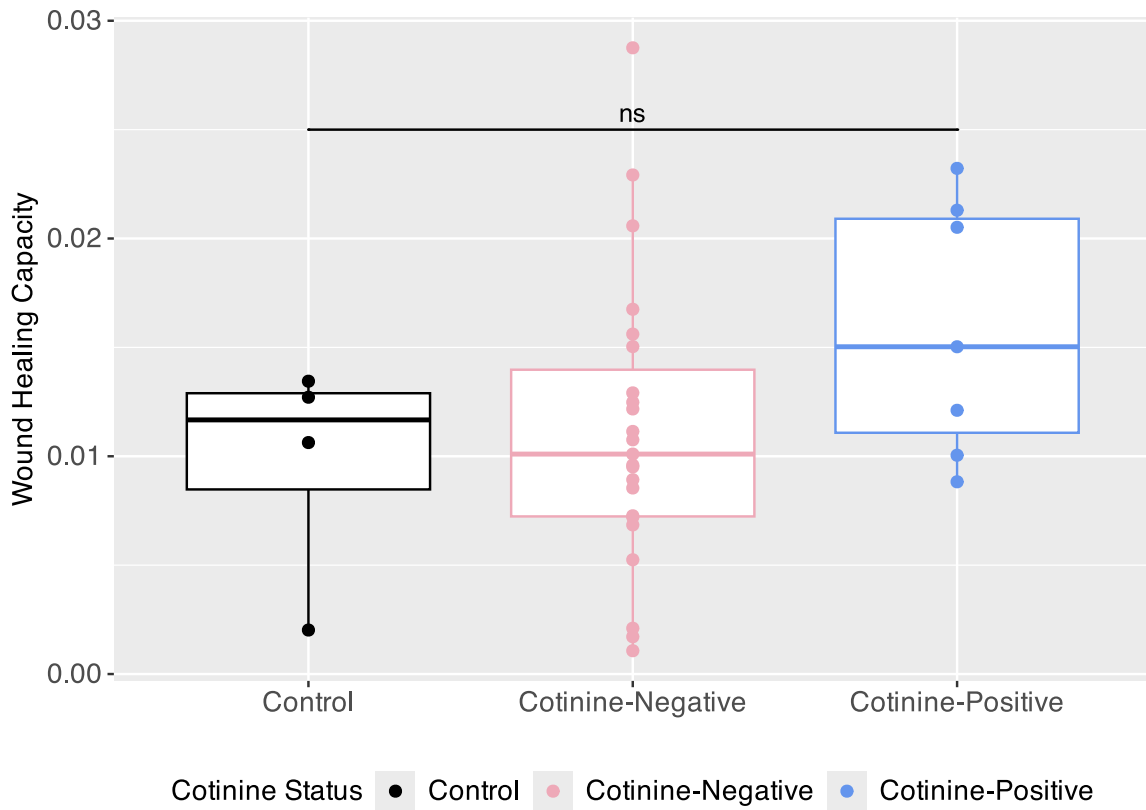


Figure 20. Amniotic fluid augments the wound healing capacity of Calu-3 cells. Cells exposed to cotinine-positive samples ($n = 7$) achieved a higher wound healing capacity than those exposed to cotinine-negative ($n = 25$) and control ($n = 4$) conditions. Differences were compared by a one-way ANOVA using R (v 4.4.1). $p = 0.196$

Finally, to assess whether wound healing capacity was associated with the abundances of cytokines and chemokines, I calculated the correlation between the two. This revealed many cytokines both positively and negatively correlated to wound healing capacity ($p < 0.05$). For example, EGF and CXCL16 were both positively correlated to wound healing capacity while Granzyme A and Eotaxin-2 were both negatively correlated (Figure 21). It is important to emphasize that some negative correlations were driven by a couple data points such as in Granzyme A and Eotaxin-2.

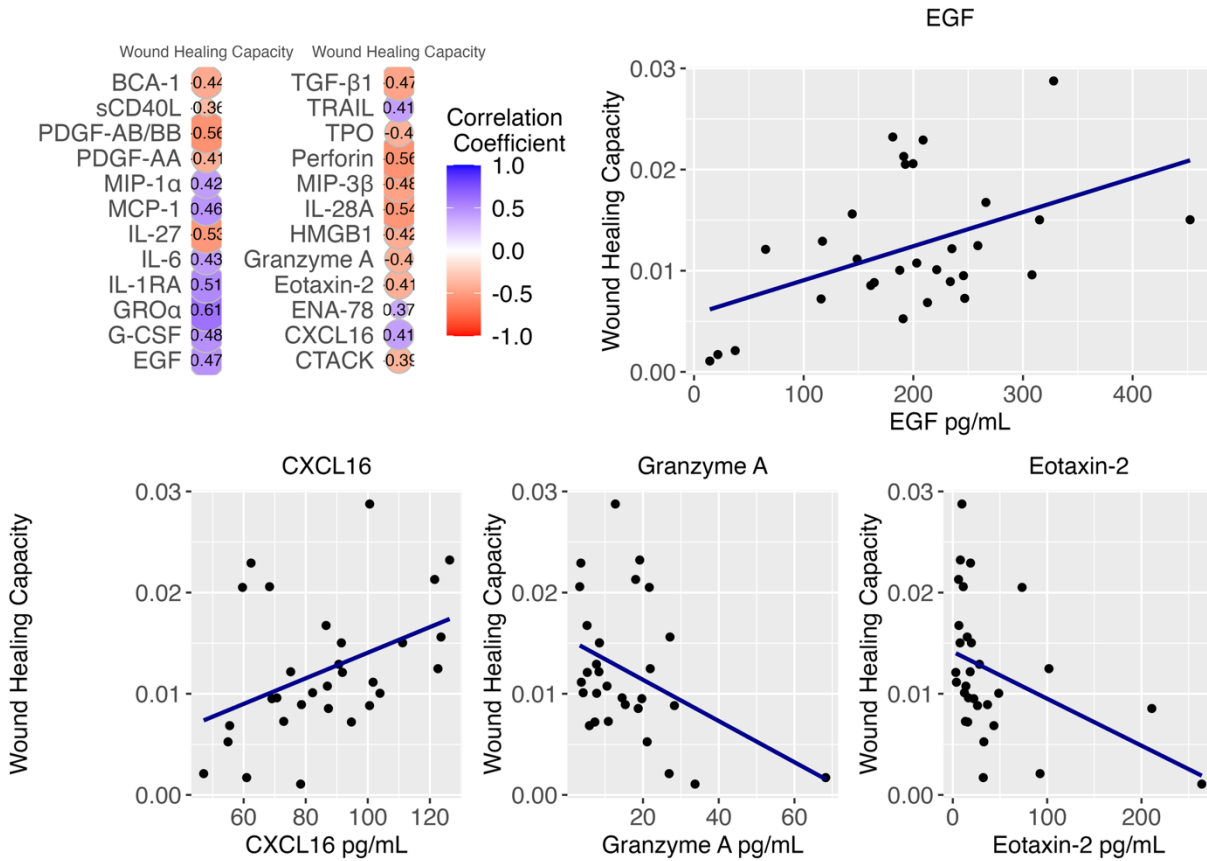


Figure 21. Abundances of cytokines and chemokines are positively and negatively correlated with wound healing capacity ($p < 0.05$). (a) Correlation plot showing cytokines and their respective correlation coefficients to wound healing capacity, red and blue colour represent negative and positive correlation, respectively. (b) EGF is positively correlated with wound healing capacity ($R^2 = 0.22$, $p = 0.0089$). (c) CXCL16 is positively correlated with wound healing capacity ($R^2 = 0.1714$, $p = 0.0229$). (d) Granzyme A is negatively correlated with wound healing capacity ($R^2 = 0.1574$, $p = 0.0300$). (e) Eotaxin-2 is negatively correlated with wound healing capacity ($R^2 = 0.1683$, $p = 0.02433$). p values are unadjusted, $n = 30$ for all. Correlations were performed by Pearson correlation method using Hmisc (v 5.2-3) and individual linear regressions were performed, both on R (v 4.4.1).

Chapter 4: Discussion

4.1 Summary of Study Findings

I have determined that amniotic fluid, umbilical cord serum, maternal serum, and placenta are abundant in cytokines and chemokines. Furthermore, the cytokine/chemokine profile of amniotic fluid is unique compared to the two sera and placenta. To my knowledge, this is the first study to identify and quantify the amniotic fluid cytokine/chemokine profile and to characterize the profile in comparison to other tissues present in pregnancy. I have observed that *in utero* cigarette smoke exposure leads to bioaccumulation of cotinine in the amniotic fluid and is associated with an altered amniotic fluid cytokine/chemokine profile. Previous research has linked changes to abundances of specific cytokines in the amniotic fluid with detrimental effects to offspring lungs, but whether those changes can come from the environment remained unexplored. My study is the first to link environmental factors, specifically cigarette smoke exposure, to changes in the amniotic fluid cytokine/chemokine profile. Lastly, I have observed that airway epithelial barrier function is negatively correlated with abundances of a number of cytokines/chemokines after 24 hours of exposure. Additionally, amniotic fluid augments the wound healing capacity of airway epithelial cells and wound healing capacity is both positively and negatively correlated with abundances of specific amniotic fluid cytokines/chemokines. My study is the first to elucidate associations between specific amniotic fluid cytokines/chemokines and airway epithelial cell function. My data provide evidence of a new paradigm linking *in utero* exposures with the developing lung through changes in amniotic fluid cytokine and chemokine composition.

4.2 Relevance to Developmental Origins of Health and Disease

In utero exposures to environmental insults, specifically cigarette smoke, is associated with an increased risk for chronic lung disease such as asthma and with a reduction in lung function both in early life and adulthood (16,19–22,27,115). At birth, the branching and formation of airways is complete and although there are functional alveoli, most of the alveoli present in adulthood are formed postnatally (116,117). In the absence of lung pathologies, a person's theoretical maximal lung function, as measured by FEV₁, is achieved in early adulthood followed by a gradual decline with age. The development of and rate of decline of lung function is determined by multiple factors including both prenatal and postnatal exposures, such as cigarette smoke exposure (both *in utero* and in early/adult life), maternal asthma, respiratory infection, and various social determinants of health (7,9,19,21,118,119). Therefore, adverse events in both the prenatal and early life stages can have lifelong detrimental structural and functional effects on the lungs that can lead to chronic lung disease in later life.

Fetal-maternal interactions via cytokines have been shown to alter lung development, lung allergic response, and may be associated with later development of asthma (74,120–122). In the context of *in utero* cigarette smoke exposure, the offspring immune response is altered, characterized by an attenuated toll-like-receptor pathway immune activation (reduced production of IL-6 and TNF α) and increased production of Th2 cytokines such as IL-13 and IL5. These studies were done using immune cells isolated from umbilical cord blood (78,79). *In utero* exposure to amniotic fluid IL-5 leads to airway hyperresponsiveness by augmenting the bronchoconstriction caused by the response of the vagus nerve to allergen challenge. Furthermore, *in utero* inflammatory exposures such as chorioamnionitis are associated with fetal lung inflammation and disruption of alveolar development. This effect is driven by both IL-1 and TNF α signaling in fetal

lungs. Both airway hyperresponsiveness and lung inflammation are hallmarks of asthma (31,74). Interestingly, these findings implicate that intra-amniotic exposure of fetal lungs to increased levels of those cytokines are the drivers of the observed lung inflammation and airway hyperresponsiveness. Therefore, the amniotic fluid may be a novel pathway of communication between fetal lungs and the maternal environment. This is supported by the fact that only about 11% of fetal circulation flows to the fetal lungs (93). Although *in utero* cigarette smoke exposure has been associated with alterations to the abundances of certain cytokines in the umbilical cord blood, namely IL-4 and IFN γ , the cytokine/chemokine profile of the amniotic fluid and whether it is altered by cigarette smoke exposure remains unknown (121). Therefore, I sought to characterize the amniotic fluid cytokine/chemokine profile, to compare it to other tissues present in pregnancy (umbilical cord serum, maternal serum, and placenta tissue), and to determine whether cigarette smoke exposure alters this cytokine/chemokine profile.

Multiplex cytokine/chemokine analysis revealed that cytokines and chemokines are highly abundant in amniotic fluid, and its composition is unique compared to the two sera and placenta profiles. Furthermore, the cytokine/chemokine profile of amniotic fluid is not similar to the two sera and placenta. This suggests that the source of amniotic fluid cytokines could be either a blend of multiple sources, or it belongs in a class of its own. The source of the cytokines and chemokines in the amniotic fluid can come from both the fetal and maternal tissues, although this cannot be fully determined within the context of my project. Nevertheless, if the cytokines are mainly from maternal tissues, then we expect the profiles of amniotic fluid and maternal serum to show similarities. Likewise, if the cytokines are mainly from fetal tissues, then we expect the profiles of amniotic fluid and umbilical cord serum to show similarities. Some cytokines are very highly

abundant in amniotic fluid such as G-CSF, MCP-1, IL-1RA, and GRO α . My results show the abundance of EGF in amniotic fluid which could be contributing to the EGF fetal lungs require for the proliferation of epithelial cells (123). Interestingly, I detected cytokines that have been explored as biomarkers of adverse neonatal outcomes, such as IL-6 and IL-8, albeit not at the levels associated with bronchopulmonary dysplasia and fetal lung injury (48,72). In the amniotic fluid, I was able to detect IL-1 β , IL-16, TNF α , IFN γ , and EGF, all of which are present at term. The levels of IL-1 β , IL-16, and TNF α that I detected were lower than what was found in a different study from likewise uncomplicated pregnancies. Elevations in the abundances of those three cytokines in amniotic fluid is indicative of infection in patients with preterm premature rupture of the membranes and none of my study participants have received that diagnosis (47).

I have detected IL-1 β , IL-5, SDF-1, and TGF- β (1,2,3) in my amniotic fluid samples. Intra-amniotic delivery of IL-1 β is causative of fetal lung inflammation (69). Although I have detected IL-1 β in my amniotic fluid samples, it is unclear whether the abundance I have observed is at an inflammatory dose. Furthermore, the levels IL-5 I have observed in the amniotic fluid are at several orders of magnitude lower than what was found to be associated with airway hyperresponsiveness (74). SDF-1 expression in fetal lungs is important in pulmonary vasculature development and its deficiency leads to defects in vasculature diameter and complexity (124). My results show the presence of SDF-1 in amniotic fluid, although whether its abundance is similar to SDF-1 in lung development remains unstudied. Lastly, TGF- β (1,2,3) have been shown to play a big role in normal lung development. It is involved in epithelial cell differentiation during lung organogenesis. However, overexpression of TGF- β has been associated with inhibiting airway branching morphogenesis, which is detrimental to lung development (125). I detected all three isoforms of

TGF- β , albeit at much lower concentration than what has been found to inhibit branching morphogenesis (126). My findings suggest that sub-pathological expression of these cytokines in the amniotic fluid occurs in uncomplicated pregnancies. In total, 99 cytokines were detected at various levels within the amniotic fluid, emphasizing the role amniotic fluid may play in signaling pathway transduction for fetal lung development.

The profiling of umbilical cord serum, maternal serum, and placenta tissue also revealed the rich abundance of cytokines and chemokines within them. Comparing the profiles of amniotic fluid, the two sera, and the placenta via a PCA revealed the uniqueness of their cytokine/chemokine profiles, especially the amniotic fluid and placenta which are both characterized by low abundances of some cytokines such as CTACK, BAFF, TNF α , and TGF- β 1. Furthermore, the cytokine/chemokine profiles of umbilical cord serum and maternal serum showed similarities, characterized by similar abundances of cytokines such as CTACK, Perforin, and IL-29. The cytokine profiles of amniotic fluid and maternal serum are distinct. In the amniotic fluid, the abundances of IL-6, IL-8, and IL-1RA are all higher than maternal serum. This finding agrees with previous work that has observed the same phenomenon (127).

Characterization and quantification of the cytokine/chemokine profiles of amniotic fluid at term for uncomplicated pregnancies revealed the rich abundance of cytokines and chemokines that may influence the fetal lung epithelia. The uniqueness of the cytokine/chemokine profile of amniotic fluid as shown by the PCA, suggests multiple sources of cytokines both from the fetal and maternal tissues, or it is its own unique composition. Furthermore, the similarities in abundances of some cytokines in the amniotic fluid and placenta and the similarities in profiles

between the two sera suggest a degree of communication between these tissues. Since previous evidence have linked increased levels of certain cytokines in the amniotic fluid to detrimental effects to fetal lungs such as lung inflammation and alveolar injury, changes in the cytokine/chemokine profile of the amniotic fluid may influence lung development or pulmonary cellular function consistent with future lung disease (31,69,74).

4.3 The Amniotic Fluid as a Medium of Communication

Correlation of the amniotic fluid cytokine/chemokine profiles revealed clusters of cytokines that are positively correlated with each other. Functional and pathway enrichment of these clusters suggest that these cytokines not only interact with each other but are also involved in multiple signaling pathways such as ligands binding to chemokine receptors, IL-10 signaling, and G-protein coupled receptor signaling (113,128). Cytokines and chemokines are the messengers of the immune system and are involved in both the induction and reduction of an inflammatory response. Some cytokines are classified as pro-inflammatory, anti-inflammatory, or both depending on their function and the context in which they are expressed. Most importantly, cytokines are often released in tandem whether for a pro-inflammatory or anti-inflammatory response (129). This provides evidence that the amniotic fluid can act as a medium of communication by transporting signaling molecules such as cytokines and chemokines from their source to their target.

My results show that IL-4, IL-5, and IL-13 are positively correlated with each other and are also grouped together in one cluster. In the context of chronic lung disease such as asthma, cytokines and chemokines are involved in the induction of airway inflammation which is a central feature of asthma. Asthma is a heterogeneous disease and as such can be classified into multiple

phenotypes like type 2-high and type 2-low asthma. Type 2-high asthma is mainly driven by the signaling pathways of IL-4, IL-5, and IL-13 released by T helper type 2 cells (130). Interestingly, the abundances of these three cytokines are lower than their abundances in the two sera which could indicate the site of production of these cytokines which is likely in circulation and can then be secreted into the lumen of the fetal lung and into the amniotic fluid.

My results show the clustering and positive correlation of IL-25 and IL-33, but not TSLP. As well, GM-CSF, M-CSF, TGF- β 2 and TGF- β 3 show clustering and positive correlation with each other, although TGF- β 1 was clustered with a different group of cytokines. Airway epithelial cells can release multiple cytokines (TSLP, IL-25, IL-33, GM-CSF, M-CSF, and TGF- β) in response to allergens. The release of these cytokines ultimately results in hypersecretion of mucus and airway inflammation characterized by high levels of eosinophils (108,130). Overall, my findings suggest that amniotic fluid cytokines and chemokines interact with each other, are co-expressed in the amniotic fluid, and may induce fetal lung inflammation.

My results show the abundance of TGF- β 1, EGF, GRO α , IL-6, and IL-8 in the amniotic fluid. Chronic airway inflammation coupled with mechanical stress such as bronchoconstriction and bronchospasm result in the release of TGF- β 1, EGF, and GM-CSF. These cytokines are associated with recruitment of immune cells to the airways and in the thickening of the airway wall (100). In fetal lungs, mechanical stress can come from fetal breathing movements. The downstream effects of these cytokines are the aberrant proliferation of airway fibroblasts and smooth muscles. The persistent recruitment of immune cells as well as their release of IL-13 into the airways stimulate airway smooth muscles to proliferate. The interaction of smooth muscles

with epithelial cells contributes to airway inflammation characterized by increased expression of CXCL1 (GRO α), IL-6, and IL-8 (100,131). Epithelial release of IL-6 can further activate and recruit immune cells, thereby contributing to a positive feedback loop of chronic airway inflammation (39). However, it is unclear whether the abundances of these cytokines that I have observed in the amniotic fluid are at a pathological concentration or at levels that can lead to permanent alterations in lung development. The presence of such cytokines does not automatically equate to changes in lung development consistent with future disease.

4.4 Amniotic Fluid is Influenced by the Maternal Environment

I focused on *in utero* cigarette smoke exposure as a maternal environmental factor that could alter the cytokine/chemokine profile of amniotic fluid. Therefore, I first confirmed whether my study participants are exposed to cigarette smoke, and if so, to what degree. My results show that 7 out of 37 participants are exposed to cigarette smoke as determined by a cotinine ELISA. In those exposed to cigarette smoke, I detected cotinine in the amniotic fluid, umbilical cord serum, and maternal serum, but not in placenta tissue. While other studies have quantified cotinine in these tissues before, to my knowledge, this is the first study to compare cotinine concentrations in matched samples taken at birth (132–134). Furthermore, I observed the bioaccumulation of cotinine in amniotic fluid, with average levels higher than the two sera. This could be because cotinine can be transported to the amniotic fluid through the placenta thereby entering fetal circulation and eventually reaching the amniotic fluid via fetal urine production. Furthermore, fetal swallowing of and subsequent urination into amniotic fluid could contribute to the bioaccumulation of cotinine (40,41).

My clinical measurements of participant characteristics did not show significant differences between cotinine-positive and cotinine-negative participants. However, on average, gestational age of cotinine-positive participants trended lower than cotinine-negative and I observed the opposite trend for birthweight. *In utero* cigarette smoke exposure is associated with a number of adverse outcomes for the offspring such as preterm birth and low birthweight (4). This might be partly because I am underpowered to detect statistical differences with a small sample size.

Comparison of the cytokine/chemokine profiles of amniotic fluid between cotinine-positive and cotinine-negative participants showed altered profiles associated with exposure to cigarette smoke. Cotinine-positive amniotic fluid is characterized by higher abundances of some cytokines such as MIG/CXCL9, IP-10, IL-6, IL-10, and IFN γ . While some studies have linked the environmental influence on amniotic fluid composition such as maternal diet, to my knowledge, this is the first study to associate cigarette smoke exposure to alterations in the cytokine/chemokine profile of amniotic fluid (49,50).

Studies on cigarette smoke exposure during pregnancy has linked nicotine with altered fetal lung development through interactions with $\alpha 7$ nAChR expressed by airway epithelial cells (24,25,135). In humans, cotinine is the major, longer half-life, metabolite of nicotine and is typically used as a biomarker of nicotine, the most bioactive compound in cigarettes. Similar to nicotine, cotinine binds to $\alpha 7$ nAChR and can act as an agonist, albeit at a much lower potency (106). Nevertheless, since cotinine bioaccumulates in the amniotic fluid and by inference, nicotine is also present in the amniotic fluid, both could potentially directly influence fetal lung

development by activating $\alpha 7nAChr$ in the airway epithelium. Therefore, there is a potential that cigarette smoke exposure can alter the cytokine/chemokine profile of amniotic fluid and can affect lung development, and that the bioaccumulation of cigarette smoke metabolites in the amniotic fluid may influence lung development as well.

4.5 Amniotic Fluid Affects Airway Epithelial Barrier Function

The airway epithelial barrier provides protection to the rest of the lung tissue from environmental insults, and dysfunction of this barrier, characterized by the loss of tight junctions and increased permeability, is associated with asthma pathogenesis and severity (108,130,136). Therefore, I sought to elucidate the effect of the amniotic fluid cytokine/chemokine profile on the barrier function of airway epithelial cells. In Calu-3 cells, I measured TEER as a surrogate for barrier function over 72 hours. However, I chose to focus my analyses on the 24-hour barrier function. It is highly likely that the barrier function response to the cytokines and chemokines in the amniotic fluid is most apparent 24 hours after exposure since cytokines/chemokines have a very short half-life, no longer than a day. In addition, epithelial cells release their own cytokines that can also affect barrier function through paracrine signaling (39,129,130).

My results suggest that amniotic fluid, as a whole, can increase barrier function but the level of increase achieved is significantly negatively correlated with the abundances of IL-13, IFN α 2, IL-9, and Fractalkine. This observation agrees with previous research associating increased IL-9 and IL-13 with the reduced production of tight junction proteins and a subsequent failure to form a functional barrier, an increase in epithelial permeability, and a disbalance of the epithelial cell types (39,137,138). Furthermore, IFN α has been associated with reducing airway epithelial proliferation and differentiation, and Fractalkine has been implicated as playing a major role in

inducing airway inflammation in response to allergens (139–141). My results suggest that these two cytokines affect the barrier function negatively when airway epithelial cells are exposed to them, thereby contributing to barrier dysfunction associated with chronic lung disease (39).

4.6 Amniotic Fluid Augments Airway Epithelial Barrier Recovery After Wounding

After wounding the Calu-3 cell layer, recovery of the barrier was better when exposed to amniotic fluid compared to control over 72 hours. Likewise, the initial barrier response to the amniotic fluid cytokines and chemokines was measured as TEER fold change at 24-hours, as above. TEER at 24 hours is both positively and negatively associated to many cytokines. For example, Perforin and IL-28A are both negatively correlated with TEER, however, TNF α , MIP-1 α , and MCP-1 are positively correlated with TEER. In the context of disease, Perforin is involved in apoptosis and is increased in the airways with chronic lung disease. IL-28A is linked to epithelial barrier dysfunction by reducing the expression of tight junction proteins (142,143). So, the negative correlation of Perforin and IL-28A to TEER is unsurprising.

Interestingly, TNF α , MIP-1 α , and MCP-1 are significantly positively correlated with barrier function 24 hours following wounding. Although TNF α , MIP-1 α , and MCP-1 have been associated with epithelial barrier dysfunction characterized by reduced production of tight junction proteins and by inducing airway inflammation, my findings suggest that they are positively correlated with wound repair. It is important to note that wound repair involves both cell migration and cell proliferation and to determine whether these cytokines are correlated with either process requires a different experiment that is outside the scope of the experiments presented here. More importantly, examining wound healing over the course of 72 hours would better capture its relationship with amniotic fluid cytokines and chemokines. Nevertheless, since my cell model

utilizes a liquid-liquid interface with undifferentiated cells in air-liquid interface, this is a counterintuitive response which may be due to other components in the amniotic fluid such as other proteins and lipids or other confounding variables that I have not yet studied (39,42,45,47,144).

However, since TEER was not recovered by 24 hours, I sought to examine whether amniotic fluid exposure is associated with wound healing capacity. Wound healing capacity is determined as the slope of TEER recovery from wounding (0 hours) to 72 hours. In Calu-3 cells, my results suggests that amniotic fluid augments wound healing capacity. This may be due, in part, by the growth factors in amniotic fluid. My results show that EGF is abundant in amniotic fluid and is positively correlated with wound healing. EGF has been shown to be vital for epithelial repair (136). Consequently, TGF- β 1 is negatively correlated with wound healing capacity, and it has been identified as having an antagonistic effect on EGF signaling (136). Interestingly, MIP-1 α and MCP-1 are both positively correlated to wound healing capacity. Again, this is a contradictory outcome than previous studies that have associated both to a pro-inflammatory response. Specifically, MIP-1 α is expressed at higher levels in people with asthma. Taken together, these two cytokines could have a detrimental effect to the overall epithelial barrier integrity (39,136,144).

Indeed, the complex cytokine and chemokine profile of amniotic fluid can influence epithelial wound healing. However, in all of my cell experiments, it is difficult to associate epithelial barrier function and wound healing to the effect of a single cytokine. The production of cytokines and chemokines is a tightly regulated process and the sources of these signaling molecules can come from many different cell types (145). These cytokines have many functions

and can sometimes be synergistic or antagonistic. For example, production of TNF α can activate other immune cells that can then further release other pro-inflammatory cytokines that can result in further activation of immune cells (129,145). Although not significant, my results show that TNF α is positively correlated with wound healing. Within the context of my cell experiments, the measured barrier function and wound healing could be a result of the collective effect of all cytokines and chemokines present. Therefore, examining wound healing capacity over 72 hours could capture the overall compounded effect of all cytokines and chemokines in the amniotic fluid. However, it is important to remember that the composition of amniotic fluid is not only cytokines and chemokines. Rather, it is a complex mixture of other proteins, lipids, solutes, and other biomolecules including nicotine and cotinine (42,45,107). Hyaluronic acid is abundant in amniotic fluid and has been shown to improve re-epithelialization following injury (146). However, studies have shown that both LPS-induced and spontaneous TNF α production in the amniotic fluid are negatively proportional to levels of hyaluronic acid (147). Therefore, there is a potential that other amniotic fluid components, including cotinine itself, are contributing to my observed epithelial barrier function and wound healing capacity either in addition to or independent of amniotic fluid cytokines and chemokines.

4.7 Significance

I have quantified and characterized the amniotic fluid cytokine/chemokine profile at term for uncomplicated pregnancies and have compared the profiles with those from other tissues present in pregnancy: umbilical cord serum, maternal serum, and placenta. Furthermore, I have shown that the cytokines and chemokines present within the amniotic fluid can interact with each other and form signaling networks. Many of the amniotic fluid cytokines are related to chronic lung disease pathology and severity. Furthermore, I have shown that the amniotic fluid

cytokine/chemokine profile can be altered by the maternal environment, specifically cigarette smoke exposure. Lastly, I have shown that airway epithelial barrier function and wound recovery are altered by amniotic fluid and are associated with the abundances of a number of cytokines and chemokines. My findings suggest that the amniotic fluid cytokine/chemokine profiles can affect the fetal airway epithelial barrier function which may lead to permanent alterations in fetal lung development and can contribute to the development of chronic lung disease.

4.8 Limitations

My study involved 37 participants of which I was able to collect 32 amniotic fluid samples. I confirmed 7 that were exposed to cigarette smoke and the rest were non-exposed. Given the small sample size of the cigarette smoke-exposed group, I am underpowered to accurately detect statistical difference in both the clinical measures collected and the experiments performed. Furthermore, though the recruitment questionnaire asked about cigarette smoke exposure, I am unable to specifically evaluate the dose of long-term cigarette smoke exposure. To circumvent this limitation, I performed a cotinine ELISA to quantify the amount of cigarette smoke exposure in the previous 19-20 hours (106). Nevertheless, analyses of the cytokine profiles between the two groups revealed cytokines that are significantly expressed differentially, albeit with unadjusted *p* values. Furthermore, my functional and pathway enrichment analyses are biased since I only studied cytokines and chemokines. The amniotic fluid is rich in biomolecules such as other proteins, enzymes, ions, oxidized lipids, and phospholipids to name a few (42,45,47). Regardless, it is interesting that my data show clusters of cytokines/chemokines that are correlated within a pathway suggesting interactive communication networks. Since this is a discovery study with a small sample size, I opted to not adjust *p* values for statistical analyses which may affect generalizability of the results. All study participants live in Edmonton, Alberta and their

environmental exposures leading up to sample collection could be confounding variables in my research. Seasonality of sample collection, environmental pollution, living conditions, wildfire smoke exposure, and other social determinants of health could all potentially impact the results of my study. Since I am studying the relationship between *in utero* cigarette smoke exposure (an environmental exposure) and the cytokine/chemokine profile of amniotic fluid, other environmental exposures such as the ones listed above could influence outcomes of the study.

All tissues collected for this sample were taken following scheduled caesarean delivery to avoid changes in cytokine/chemokine profiles related to active labour. However, I am unable to determine whether the cytokines and chemokines detected in the amniotic fluid are released by maternal cells or fetal cells. These cytokines and chemokines are nevertheless exposed to the fetal lung via fetal breathing movements. Multiple amniocenteses over pregnancy can potentially mitigate this limitation. However, amniocentesis carries risk and complications to the pregnant individual and the fetus. Furthermore, it is typically only done for genetic testing should congenital anomalies be detected by ultrasound imaging of the fetus (148). Therefore, to completely mitigate this limitation, a non-human model would be needed.

Lastly, the study focuses on possible effects of the amniotic fluid cytokine/chemokine profile on fetal airway epithelial barrier function, however, my cell model uses Calu-3 epithelial cells which are of adult origin. The cells were chosen for ease of transwell culture propagation and were used to measure a physiological response, barrier function, to amniotic fluid. In the future, the transwell culture model can be done using fetal airway epithelial cells to confirm the barrier function effects of amniotic fluid.

4.8 Future Directions

A non-human primate animal model of *in utero* cigarette exposure can potentially circumvent the limitations of this study as discussed above. A full schematic of this proposed experiment is shown in Figure 22. The rhesus macaque (*Macaca mulatta*) is a non-human primate commonly used in reproductive and maternal-fetal interaction research for their genetic and physiological similarity with humans (149). The use of a rhesus macaque model presents a unique opportunity to perform controlled experiments wherein environmental influence on the results can be minimized. To understand the impact of *in utero* cigarette smoke exposure on the amniotic fluid cytokine/chemokine profile, pregnant rhesus macaques can be exposed to cigarette smoke at a specific, clinically relevant dose, over gestation. Furthermore, multiple amniocentesis can be performed to characterize the amniotic fluid cytokine/chemokine profiles over pregnancy to study the role of amniotic fluid in signal transduction. At term, amniotic fluid, umbilical cord serum, maternal serum, and placenta tissue can be collected and cytokine/chemokine profiles analyzed as in this project. Since potential environmental confounding variables are eliminated in this model, this would provide stronger evidence on the changes in cytokine/chemokine profiles driven by cigarette smoke exposure.

This controlled exposure experiment can also help us understand amniotic fluid flow within the fetal compartment. Doppler ultrasound, including colour ultrasound imaging, has been successfully used to measure fetal rhesus renal artery diameter, length, vascular branching, and hemodynamic parameters (150). This same technique can be adapted to assess fetal lung development as measured by airway diameters and branching patterns and can be used to quantify amniotic fluid influx and fetal lung liquid efflux during fetal breathing movements. By measuring

the fluid flow into and out of the fetal lungs, the volumetric contribution of amniotic fluid to fetal lung liquid can be elucidated. In addition, this model presents an opportunity to characterize other biomolecules in amniotic fluid, through lipidomic or proteomic profiling, that could influence fetal lung development such as other proteins, amino acids, and lipids (42,44,45). Interestingly, the amniotic membrane that houses the amniotic fluid has been found to be a secondary source of pulmonary surfactant which is vital for lung function (151). The proposed rhesus macaque model will add immense knowledge on the role amniotic fluid plays in fetal lung development.

To mechanistically associate amniotic fluid components to lung development, cytokines, chemokines, other proteins, and lipids that are identified as having been modified by environmental (*in utero*) exposures can be studied individually. For example, a specific cytokine can be transgenically upregulated, deleted, and exogenously administered in the pregnant parent generation and followed by examining lung development over gestation and postnatally as well.

As for the effects of amniotic fluid on airway epithelial barrier function, precision cut lung slices (PCLS) of the rhesus fetuses can be harvested for an *in vitro* model. PCLS provides a unique opportunity to model the lung environment capturing the interactions of the airway epithelium and other cell types present in the airways (152). PCLS can be exposed to amniotic fluid, as in this project, and immunofluorescence staining for tight junction markers and multiplex cytokine analysis after exposure can elucidate the impact of amniotic fluid on the epithelial barrier function. In addition, PCLS can be wounded and then exposed to amniotic fluid. Time-lapse and video imaging can then be utilized to study the wound healing behaviour of airway epithelial cells and the possible impact of amniotic fluid on its capacity to heal. This would also help us understand

the influence of amniotic fluid on the airway architecture and can be useful in studying the pathogenesis of chronic lung diseases (152,153). Although these experiments cannot link airway epithelial barrier function to single cytokines, the controlled exposure nature of the rhesus macaque model can show the compounded effect of all amniotic fluid components. Furthermore, the PCLS experiments can be adapted to study the influence of the amniotic fluid milieu individually (i.e. PCLS exposure to only one analyte) or in groups (i.e. PCLS exposure to proteins identified as having interactions or characterized as part of a signal transduction pathway) on airway physiology.

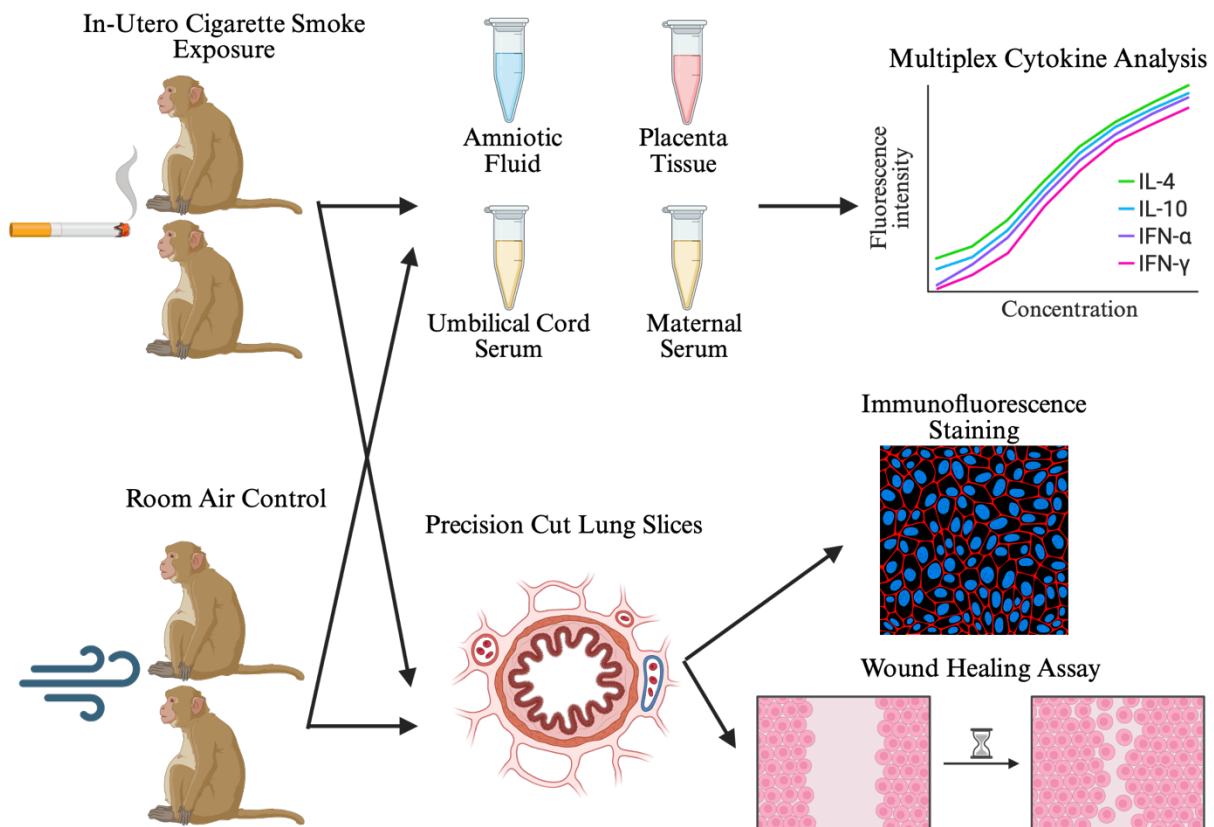


Figure 22. Experimental design for the *in utero* cigarette smoke exposure in a rhesus macaque model to assess the influence of the amniotic fluid milieu on offspring airway epithelial barrier function.

Appendices

Appendix A

Table A 1. Patient demographic and clinical measures data sheet.

DATA SHEET

Study Log number:	Collection Date:
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Maternal History	Age:	Race:
Pre-Pregnancy weight:	Height:	BMI:
Smoker: Yes or No Number of years: Packs per day:	Smoking Hx notes:	
Asthma: Yes or No	Asthma Hx notes:	
Diabetes: Yes or No Type 2 Or Gestational	Diabetes Hx notes:	

Obstetrical History		G ____ T ____ P ____ A ____ L ____	
Gestational Age:	BP (early gest.):	BP (Term):	
Placental weight:	Pathology: Yes or No	BP (last 24hrs):	
Proteinuria:	Hematocrit:	Anesthesia:	
Urate:	Hemoglobin:	Spinal or General or Epidural	
Pertinent Obstetrical Hx:			
Medication Hx during pregnancy:			

Fetal History		Male or Female		Birth weight:	
NICU Admission:	Apgars:	Cord gases:		Temp:	
Yes or No	1min ____ 5min ____	A ____ V ____			

Appendix B

Table A 2. List of cytokines and chemokines analyzed in multiplex cytokine analyses.

Cytokine Symbol	Name ^a	Gene Symbol ^a	HGNC ID ^a
EGF	Epidermal Growth Factor	EGF	3229
Eotaxin	C-C Motif Chemokine Ligand 11	CCL11	10610
FGF-2	Fibroblast Growth Factor 2	FGF2	3676
FLT-3L	Fms Related Receptor Tyrosine Kinase 3 Ligand	FLT3LG	3766
Fractalkine	C-X3-C Motif Chemokine Ligand 1	CX3CL1	10647
G-CSF	Colony Stimulating Factor 3	CSF3	2438
GM-CSF	Colony Stimulating Factor 2	CSF2	2434
GRO α	C-X-C Motif Chemokine Ligand 1	CXCL1	4602
IFN α 2	Interferon Alpha 2	IFNA2	5423
IFN γ	Interferon Gamma	IFNG	5438
IL-1 α	Interleukin 1 Alpha	IL1A	5991
IL-1 β	Interleukin 1 Beta	IL1B	5992
IL-1RA	Interleukin 1 Receptor Antagonist	IL1RN	6000
IL-2	Interleukin 2	IL2	6001
IL-3	Interleukin 3	IL3	6011
IL-4	Interleukin 4	IL4	6014
IL-5	Interleukin 5	IL5	6016
IL-6	Interleukin 6	IL6	6018
IL-7	Interleukin 7	IL7	6023
IL-8	C-X-C Motif Chemokine Ligand 8	CXCL8	6025
IL-9	Interleukin 9	IL9	6029
IL-10	Interleukin 10	IL10	5962
IL-12p40	Interleukin 12B	IL12B	5970
IL-12p70	Interleukin 12A	IL12A	5969
IL-13	Interleukin 13	IL13	5973
IL-15	Interleukin 15	IL15	5977
IL-17A	Interleukin 17A	IL17A	5981
IL-17E/IL-25	Interleukin 25	IL25	13765
IL-17F	Interleukin 17F	IL17F	16404
IL-18	Interleukin 18	IL18	5986
IL-22	Interleukin 22	IL22	14900
IL-27	Interleukin 27	IL27	19157
IP-10	C-X-C Motif Chemokine Ligand 10	CXCL10	10637
M-CSF	Colony Stimulating Factor 1	CSF1	2432
MCP-1	C-C Motif Chemokine Ligand 2	CCL2	10618

MCP-3	C-C Motif Chemokine Ligand 7	CCL7	10634
MDC	C-C Motif Chemokine Ligand 22	CCL22	10621
MIG/CXCL9	C-X-C Motif Chemokine Ligand 9	CXCL9	7098
MIP-1 α	C-C Motif Chemokine Ligand 3	CCL3	10627
MIP-1 β	C-C Motif Chemokine Ligand 4	CCL4	10630
PDGF-AA	Platelet Derived Growth Factor Subunit A	PDGFA	8799
PDGF-AB/BB	Platelet Derived Growth Factor Subunit B	PDGFB	8800
RANTES	C-C Motif Chemokine Ligand 5	CCL5	10632
sCD40L	Cd40 Ligand	CD40LG	11935
TGF α	Transforming Growth Factor Alpha	TGFA	11765
TNF α	Tumor Necrosis Factor	TNF	11892
TNF β	Lymphotoxin Alpha	LTA	6709
VEGF-A	Vascular Endothelial Growth Factor A	VEGFA	12680
6CKine	C-C Motif Chemokine Ligand 21	CCL21	10620
APRIL	TND Superfamily Member 13	TNFSF13	11928
BAFF	TNF Superfamily Member 13B	TNFSF13B	11929
BCA-1	C-X-C Motif Chemokine Ligand 13	CXCL13	10639
CCL28	C-C Motif Chemokine Ligand 28	CCL28	17700
CTACK	C-C Motif Chemokine Ligand 27	CCL27	10626
CXCL16	C-X-C Motif Chemokine Ligand 16	CXCL16	16642
ENA-78	C-X-C Motif Chemokine Ligand 5	CXCL5	10642
Eotaxin-2	C-C Motif Chemokine Ligand 24	CCL24	10623
Eotaxin-3	C-C Motif Chemokine Ligand 26	CCL26	10625
GCP-2	C-X-C Motif Chemokine Ligand 6	CXCL6	10643
Granzyme A	Granzyme A	GZMA	4708
Granzyme B	Granzyme B	GZMB	4709
HMGB1	High Mobility Group Box 1	HMGB1	4983
I-309	C-C Motif Chemokine Ligand 1	CCL1	10609
I-TAC	C-X-C Motif Chemokine Ligand 11	CXCL11	10638
IFN β	Interferon Beta 1	IFNB1	5434
IFN ω	Interferon Omega 1	IFNW1	5448
IL-11	Interleukin 11	IL11	5966
IL-16	Interleukin 16	IL16	5980
IL-20	Interleukin 20	IL20	6002
IL-21	Interleukin 21	IL21	6005
IL-23	Interleukin 23 Subunit Alpha	IL23A	15488
IL-24	Interleukin 24	IL24	11346
IL-28A	Interferon Lambda 2	IFNL2	18364
IL-29	Interferon Lambda 1	IFNL1	18363
IL-31	Interleukin 31	IL31	19372

IL-33	Interleukin 33	IL33	16028
IL-34	Interleukin 34	IL34	28529
IL-35	Interleukin 12, Subunit P35	IL12A.35	5969
LIF	Lif Interleukin 6 Family Cytokine	LIF	6596
Lymphotactin	X-C Motif Chemokine Ligand 1	XCL1	10645
MCP-2	C-C Motif Chemokine Ligand 8	CCL8	10635
MCP-4	C-C Motif Chemokine Ligand 13	CCL13	10611
MIP 1 δ	C-C Motif Chemokine Ligand 15	CCL15	10613
MIP-3 α	C-C Motif Chemokine Ligand 20	CCL20	10619
MIP-3 β	C-C Motif Chemokine Ligand 19	CCL19	10617
MPIF-1	C-C Motif Chemokine Ligand 23	CCL23	10622
Perforin	Perforin 1	PRF1	9360
sCD137	Tnf Receptor Superfamily Member 9	TNFRSF9	11924
SCF	Kit Ligand	KITLG	6343
SDF-1	C-X-C Motif Chemokine Ligand 12	CXCL12	10672
sFas	Fas Cell Surface Death Receptor	FAS	11920
sFasL	Fas Ligand	FASLG	11936
TARC	C-C Motif Chemokine Ligand 17	CCL17	10615
TPO	Thyroid Peroxidase	TPO	12015
TRAIL	TNF Superfamily Member 10	TNFSF10	11925
TSLP	Thymic Stromal Lymphopoietin	TSLP	30743
TGF- β 1	Transforming Growth Factor Beta 1	TGFB1	11766
TGF- β 2	Transforming Growth Factor Beta 2	TGFB2	11768
TGF- β 3	Transforming Growth Factor Beta 3	TGFB3	11769

^aCytokine names, gene symbols, and HGNC ID were taken from the HUGO Gene Nomenclature Committee database (154).

Appendix C

Table A 3. Average abundances of cytokines and chemokines for all samples collected. Data is presented as mean $\pm$ SD

Cytokine	Amniotic Fluid (N = 32) pg/mL	Umbilical Cord Serum (N = 33) pg/mL	Maternal Serum (N = 33) pg/mL	Placenta Tissue (N = 36) pg/mL
EGF	190.58 $\pm$ 93.46	144.47 $\pm$ 148.61	70.13 $\pm$ 56.68	1.84 $\pm$ 0.8
Eotaxin	10.32 $\pm$ 2.74	28.89 $\pm$ 18.41	23.62 $\pm$ 13.69	17.9 $\pm$ 2.18
FGF-2	154.85 $\pm$ 105.7	211.58 $\pm$ 145.18	216.45 $\pm$ 128.25	2612.23 $\pm$ 554.25
FLT-3L	8.23 $\pm$ 4.73	11.99 $\pm$ 8.23	8.41 $\pm$ 5.77	3.43 $\pm$ 0.83
Fractalkine	90.68 $\pm$ 48.57	232.78 $\pm$ 90.97	127.41 $\pm$ 46.11	104.56 $\pm$ 20.31
G-CSF	609.66 $\pm$ 447.43	218.96 $\pm$ 128.73	72.41 $\pm$ 30.7	13.84 $\pm$ 2.77
GM-CSF	37.45 $\pm$ 43.65	220.01 $\pm$ 314.8	53.27 $\pm$ 58.83	4.65 $\pm$ 3.05
GRO α	312.56 $\pm$ 233.6	25.38 $\pm$ 24.93	31.58 $\pm$ 31.86	40.27 $\pm$ 36.41
IFN α 2	8.93 $\pm$ 6.12	49.36 $\pm$ 42.42	126.8 $\pm$ 97.89	76.8 $\pm$ 13.15
IFN γ	3.4 $\pm$ 4.11	12.46 $\pm$ 20.86	13.43 $\pm$ 17.55	0.72 $\pm$ 0.57
IL-1 α	182.76 $\pm$ 151.35	59.14 $\pm$ 109.52	88.85 $\pm$ 96.36	1.1 $\pm$ 0.6
IL-1 β	7.27 $\pm$ 6.93	3.4 $\pm$ 4.29	40.46 $\pm$ 35.45	37.76 $\pm$ 9.95
IL-1RA	859.56 $\pm$ 511.15	29.76 $\pm$ 72.83	14.12 $\pm$ 11.63	3.03 $\pm$ 1.4
IL-2	1.43 $\pm$ 2.26	0.64 $\pm$ 0.58	6.48 $\pm$ 8.69	0.46 $\pm$ 0.31
IL-3	0.36 $\pm$ 0.37	1.49 $\pm$ 1.01	1.99 $\pm$ 1.5	1.45 $\pm$ 0.86
IL-4	0.26 $\pm$ 0.17	2.45 $\pm$ 2.52	2.04 $\pm$ 1.22	0.42 $\pm$ 0.26
IL-5	0.55 $\pm$ 0.42	8.66 $\pm$ 8.77	2.84 $\pm$ 2.99	0.05 $\pm$ 0.01
IL-6	475.61 $\pm$ 470.52	8.79 $\pm$ 7.49	2.44 $\pm$ 1.87	14.5 $\pm$ 4.93
IL-7	1.03 $\pm$ 2.32	5.13 $\pm$ 3.99	7.08 $\pm$ 17.96	1.01 $\pm$ 0.18
IL-8	724.31 $\pm$ 1093.59	6.37 $\pm$ 3.81	3.72 $\pm$ 3.99	1.67 $\pm$ 1.04
IL-9	5.67 $\pm$ 2.46	15.74 $\pm$ 5.7	18.24 $\pm$ 8.43	19.97 $\pm$ 3.84
IL-10	4.19 $\pm$ 2.74	6.23 $\pm$ 3.28	4.03 $\pm$ 3.35	1.58 $\pm$ 0.88
IL-12p40	25.12 $\pm$ 13.37	716.98 $\pm$ 401.01	110.37 $\pm$ 88.89	111.77 $\pm$ 23.93
IL-12p70	2.41 $\pm$ 1.58	3.42 $\pm$ 2.56	5.76 $\pm$ 3.7	0.52 $\pm$ 0.33
IL-13	11.72 $\pm$ 7.09	49.76 $\pm$ 28.86	68.56 $\pm$ 33.6	16.42 $\pm$ 3.87

Cytokine	Amniotic Fluid (N = 32) pg/mL	Umbilical Cord Serum (N = 33) pg/mL	Maternal Serum (N = 33) pg/mL	Placenta Tissue (N = 36) pg/mL
IL-15	40.58 ± 22.11	8.66 ± 4.88	12.43 ± 9.65	18.04 ± 4.47
IL-17A	2 ± 4.13	2.15 ± 2.57	16.23 ± 19.85	0.47 ± 0.51
IL-17E/IL-25	22.37 ± 18.32	480.08 ± 400.01	1690.49 ± 1384.48	4.96 ± 2.87
IL-17F	5.78 ± 6.98	4.62 ± 9.04	21.77 ± 25.09	3.55 ± 1.18
IL-18	7.25 ± 5.04	89.58 ± 61.95	98.58 ± 73.98	1.97 ± 0.31
IL-22	19.67 ± 15.36	105.86 ± 73.51	138.57 ± 103.28	43.95 ± 34.58
IL-27	1186.64 ± 1160.39	720.85 ± 518.76	243154.92 ± 316858.09	1294.94 ± 628.74
IP-10	1474.82 ± 3303.54	199.98 ± 222.49	216.04 ± 153.83	40.92 ± 32.82
M-CSF	240.59 ± 114.86	353.23 ± 185.57	91.85 ± 62.17	321.34 ± 85.73
MCP-1	540.63 ± 440.81	343.76 ± 182.27	161.3 ± 54.85	27.35 ± 6.77
MCP-3	6.1 ± 3.02	12.92 ± 6.13	19.85 ± 6.44	13.63 ± 2.08
MDC	47.68 ± 116.79	3126.44 ± 5174.55	447.28 ± 260.81	4.55 ± 1.09
MIG/CXCL9	313.1 ± 380.23	1524.71 ± 898.87	899.84 ± 501.38	138.06 ± 144.45
MIP-1 α	17.75 ± 19.81	29.27 ± 16.02	32.61 ± 16.65	38.64 ± 12.72
MIP-1 β	15.22 ± 25.17	73.99 ± 48.83	22.32 ± 10.67	3.44 ± 2.95
PDGF-AA	368.41 ± 234.49	2280.52 ± 2145.16	1401.48 ± 1118.27	95.12 ± 14.17
PDGF-AB/BB	865.74 ± 999.12	11727.34 ± 14492.56	8823.32 ± 6532.88	497.36 ± 114.27
RANTES	766.52 ± 581.48	6783.31 ± 6390.38	4086.1 ± 4143.15	517.91 ± 228.32
sCD40L	210.1 ± 219.94	4859.81 ± 6496.61	2068.27 ± 1913.15	9.96 ± 5.06
TGF α	5.8 ± 4.78	4.18 ± 3.7	7.98 ± 6.69	1.54 ± 0.74
TNF α	7.19 ± 5.88	93.72 ± 43.22	35.6 ± 20.72	1.42 ± 0.57
TNF β	3.47 ± 2.16	4.12 ± 4.77	6.11 ± 4.95	1.23 ± 1.25
VEGF-A	0.24 ± 0.14	122 ± 171.99	14.22 ± 14.45	0.42 ± 0.54
6CKine	340.14 ± 103.89	1103.79 ± 625.88	887.14 ± 491.42	190.04 ± 22.13
APRIL	2163.52 ± 2109.77	2485.35 ± 2442.87	1497.69 ± 1269.6	821.39 ± 205.16

Cytokine	Amniotic Fluid (N = 32) pg/mL	Umbilical Cord Serum (N = 33) pg/mL	Maternal Serum (N = 33) pg/mL	Placenta Tissue (N = 36) pg/mL
BAFF	228.46 ± 73.37	1900.04 ± 856.1	749.93 ± 349.87	19.81 ± 4.16
BCA-1	30.45 ± 27.92	89.53 ± 31.72	295.09 ± 215.41	1.1 ± 1.01
CCL28	304.12 ± 166.02	176.67 ± 157.83	575.94 ± 671.16	119.41 ± 44.77
CTACK	54.36 ± 32.43	490.49 ± 186.68	499.1 ± 162.27	4 ± 0.75
CXCL16	84.47 ± 24.58	4669.41 ± 3518.08	988.7 ± 721.37	14.72 ± 9.36
ENA-78	850.42 ± 589.87	3608.12 ± 2620.56	882.67 ± 1051.69	11.36 ± 5.51
Eotaxin-2	43.24 ± 59.34	275.47 ± 169.69	208.09 ± 166.39	20.92 ± 6.58
Eotaxin-3	4.57 ± 3.72	22.82 ± 8.89	26.76 ± 9.77	1.22 ± 1.75
GCP-2	139.28 ± 101.58	98.94 ± 63.69	94.34 ± 72.85	34.38 ± 19.56
Granzyme A	15.76 ± 12.67	230.05 ± 101.58	48.19 ± 36.4	44.71 ± 18.39
Granzyme B	29.93 ± 51.27	13.92 ± 19.73	26.48 ± 88.96	50.57 ± 30.76
HMGB1	1920.4 ± 1745.94	1567.39 ± 678.47	1220.91 ± 412.47	1990.96 ± 660.32
I-309	16.05 ± 9.28	9.2 ± 3.07	12.72 ± 18.72	0.38 ± 0.21
I-TAC	13.1 ± 11.21	215.46 ± 378.23	128.59 ± 105.37	10.01 ± 4.08
IFN β	1.03 ± 1.02	24.09 ± 8.1	36.73 ± 20.13	2.21 ± 0.32
IFN ω	2.38 ± 3.23	13.12 ± 3.78	17.05 ± 9.99	1.56 ± 0.69
IL-11	12.19 ± 11.44	5.7 ± 2.05	6.55 ± 2.76	7.08 ± 1.9
IL-16	146.04 ± 151.94	384.53 ± 203.88	281.43 ± 266.21	163.46 ± 43.31
IL-20	44.88 ± 32.77	133.25 ± 43.27	164.53 ± 173.65	1.86 ± 0.65
IL-21	9.56 ± 19.79	4.97 ± 2.51	10.65 ± 15.85	17.87 ± 3.76
IL-23	232.57 ± 325.4	43.48 ± 35.51	5786.9 ± 8614.38	11.74 ± 8.43
IL-24	38.88 ± 42.15	49.59 ± 19.77	190.05 ± 118.02	51.81 ± 15.54
IL-28A	3.42 ± 2.69	10.27 ± 3.43	13.66 ± 5.24	15.73 ± 1.98
IL-29	11.74 ± 12.25	23.6 ± 4.68	21 ± 6.79	2.13 ± 1.63
IL-31	7.05 ± 7.66	8.25 ± 7.66	24.77 ± 26.99	12.28 ± 2.9

Cytokine	Amniotic Fluid (N = 32) pg/mL	Umbilical Cord Serum (N = 33) pg/mL	Maternal Serum (N = 33) pg/mL	Placenta Tissue (N = 36) pg/mL
IL-33	27.74 ± 65.87	34.49 ± 11.19	45.7 ± 90.79	3385.3 ± 1119.57
IL-34	10.82 ± 8.47	54.02 ± 27.36	32 ± 11.9	32.15 ± 12.53
IL-35	40.59 ± 42.94	55.85 ± 20.8	359.38 ± 521.42	104.86 ± 23.64
LIF	13.54 ± 20.08	3.8 ± 1.26	6.85 ± 9.33	0.96 ± 0.48
Lymphotactin	51.38 ± 21.07	35.55 ± 7.74	61.19 ± 51.98	17.34 ± 5.13
MCP-2	13.09 ± 6.32	12.52 ± 5.09	10.46 ± 4.2	9.35 ± 3.15
MCP-4	44.05 ± 24.84	70.42 ± 40.19	33.6 ± 14.59	25 ± 10.74
MIP 1δ	1970.49 ± 931.96	162381.06 ± 247905.87	19498.8 ± 57636.97	64.18 ± 10.57
MIP-3α	15.92 ± 11.82	38.61 ± 22.56	16.68 ± 19.68	0.53 ± 0.47
MIP-3β	4.59 ± 2.43	254.76 ± 144.47	88.33 ± 37.92	0.9 ± 0.25
MPIF-1	454.4 ± 480.42	420.15 ± 242.83	211.56 ± 123.23	10.03 ± 1.56
Perforin	332.33 ± 527.79	9030.61 ± 4325.56	8211.59 ± 2732.78	42.83 ± 12.52
sCD137	28.43 ± 14.18	25.78 ± 5.97	11.11 ± 4.66	0.71 ± 0.29
SCF	573.89 ± 219.78	2486.95 ± 484.59	887.74 ± 291.39	17.08 ± 4.36
SDF-1	1251.75 ± 404.06	5228.13 ± 4996.28	3299.14 ± 1593.96	43.39 ± 9.03
sFas	7.2 ± 14.37	34.62 ± 90.82	46.75 ± 172.11	69.32 ± 18.95
sFasL	29.65 ± 16.59	634.35 ± 357.72	178.02 ± 122.12	6.66 ± 2.1
TARC	11.88 ± 16.71	359.95 ± 819.19	52.08 ± 192.87 ^a	2.07 ± 0.95
TPO	53.77 ± 59.54	1731.75 ± 1100.93	406.61 ± 274.55	4.09 ± 4.9
TRAIL	140.76 ± 69.74	214.44 ± 112.26	24.85 ± 12.99	118.33 ± 28.03
TSLP	1 ± 0.85	1.23 ± 0.3	1.43 ± 0.85	0.28 ± 0.11
TGF-β1	895.36 ± 753.58	16639.44 ± 13027.09	13910.31 ± 7585.82	64.61 ± 9.54
TGF-β2	549.96 ± 473.93	600.35 ± 405.73	525.91 ± 366.38	153.23 ± 251.53
TGF-β3	13.83 ± 12.58	16.73 ± 16.55	21.5 ± 20.68	5.73 ± 2.36

^aFor maternal serum, TARC mean and standard deviation calculated from n = 32 due to low bead count for 1 sample.

Appendix D

Table A 4. Abundances of cytokines and chemokines comparing cotinine-positive and cotinine-negative samples.

Cytokine	Cotinine-Positive (N = 7) pg/mL	Cotinine-Negative (N = 25) pg/mL	<i>p</i> Value
MIG/CXCL9	761.11 ± 586.28	187.65 ± 160.86	0.0002
IP-10	5167.28 ± 5871.21	440.94 ± 623.31	0.0017
CTACK	36.81 ± 13.5	59.27 ± 34.61	0.0074
CCL28	256.52 ± 217.11	317.44 ± 151.56	0.0084
IL-10	7 ± 3.58	3.4 ± 1.87	0.0139
IFN γ	5.82 ± 4.01	2.72 ± 3.95	0.0196
Eotaxin-2	24.72 ± 26.86	48.43 ± 65.12	0.0454
IL-6	836.66 ± 572.74	374.52 ± 394.07	0.0488
I-TAC	19.13 ± 11.05	11.41 ± 10.87	0.0694
FLT-3L	12.63 ± 5.94	7 ± 3.58	0.0748
MIP-1 β	8.55 ± 2.25	17.08 ± 28.29	0.0757
FGF-2	127.53 ± 99.4	162.5 ± 108.09	0.0894
IL-27	861.19 ± 1147.03	1277.77 ± 1170.8	0.0949
APRIL	1448.38 ± 364.91	2363.76 ± 2350.56	0.1055
TARC	5.88 ± 3.92	13.56 ± 18.53	0.106
TGF- β 1	905.99 ± 1141.57	892.39 ± 638.5	0.1166
MIP-3 β	4 ± 1.6	4.75 ± 2.62	0.1318
VEGF-A	0.17 ± 0.12	0.27 ± 0.14	0.1323
MCP-3	5.22 ± 3.32	6.35 ± 2.96	0.133
IL-35	30.15 ± 21.77	43.51 ± 47.14	0.1671
PDGF-AB/BB	732.5 ± 1019.46	903.05 ± 1011.41	0.1674
IL-33	15.85 ± 7.85	31.06 ± 74.41	0.1766
SDF-1	1206.82 ± 401.57	1264.33 ± 412.08	0.1902
MIP-3 α	11.26 ± 6.8	17.23 ± 12.68	0.1958
Perforin	196.89 ± 245.55	370.26 ± 581.28	0.2161
IL-11	8.37 ± 3.76	13.26 ± 12.65	0.2315
TNF α	9.98 ± 7	6.41 ± 5.43	0.2362
6CKine	332.91 ± 157.15	342.16 ± 88.02	0.253
IL-1 β	5.34 ± 4.38	7.81 ± 7.48	0.2737
GCP-2	116.16 ± 60.98	145.75 ± 110.45	0.2902
MDC	24.03 ± 13.82	54.31 ± 131.77	0.3015
GM-CSF	17.94 ± 14.22	42.91 ± 47.63	0.3027
HMGB1	1410.77 ± 753.82	2063.1 ± 1923.11	0.3146
Eotaxin	9.75 ± 2.64	10.48 ± 2.8	0.3168
IL-18	6.42 ± 2.94	7.48 ± 5.51	0.3245

Cytokine	Cotinine-Positive (N = 7) pg/mL	Cotinine-Negative (N = 25) pg/mL	<i>p</i> Value
TNFβ	4.46 ± 1.81	3.2 ± 2.2	0.3248
IL-17A	1.35 ± 2.05	2.18 ± 4.56	0.3326
MPIF-1	622.82 ± 608.58	407.24 ± 441.51	0.3475
RANTES	707.05 ± 571.14	783.17 ± 594.87	0.3562
IL-9	5.34 ± 1.96	5.77 ± 2.61	0.362
sFasL	35.96 ± 14.97	27.89 ± 16.87	0.3739
MCP-2	13 ± 5.94	13.12 ± 6.54	0.3922
Eotaxin-3	4.34 ± 3.04	4.63 ± 3.94	0.4016
IL-1RA	804.93 ± 487.05	874.86 ± 526.37	0.4183
IL-15	35.91 ± 12.77	41.89 ± 24.13	0.4423
IL-23	143.32 ± 101.31	257.57 ± 362.26	0.445
TGF-β2	845.03 ± 752.91	467.33 ± 340.43	0.4539
IL-13	11.54 ± 7.49	11.76 ± 7.13	0.4578
IL-21	6.48 ± 5.53	10.42 ± 22.24	0.4592
IL-20	57.44 ± 36.96	41.36 ± 31.41	0.4607
IL-22	24 ± 15.91	18.46 ± 15.31	0.4722
IFNβ	1.41 ± 1.05	0.93 ± 1.01	0.4801
MCP-4	40.84 ± 15.86	44.95 ± 27.02	0.5257
IL-17E/IL-25	28.06 ± 21.36	20.78 ± 17.53	0.5323
ENA-78	1064.89 ± 642.83	790.37 ± 573.54	0.5369
IL-28A	2.76 ± 1.81	3.61 ± 2.89	0.5658
IL-31	6.78 ± 6.19	7.13 ± 8.14	0.5689
IL-12p40	30.8 ± 16.55	23.53 ± 12.27	0.571
IL-4	0.29 ± 0.16	0.25 ± 0.18	0.5727
IL-5	0.53 ± 0.2	0.55 ± 0.47	0.5944
TGF-β3	20.28 ± 20.4	12.02 ± 9.21	0.5994
G-CSF	718.26 ± 473.08	579.25 ± 445.22	0.6081
Lymphotactin	52.47 ± 19.25	51.07 ± 21.92	0.6126
CXCL16	99.34 ± 22.14	80.31 ± 23.99	0.6318
IFNα2	8.88 ± 5.44	8.94 ± 6.41	0.669
Granzyme B	34.45 ± 53.03	28.66 ± 51.82	0.6769
sFas	5.26 ± 1.81	7.74 ± 16.26	0.6848
IL-34	10.62 ± 8.34	10.88 ± 8.67	0.6932
IL-16	129.49 ± 71.1	150.67 ± 168.69	0.6954
BAFF	230.52 ± 52.44	227.89 ± 79.15	0.7027

Cytokine	Cotinine-Positive (N = 7) pg/mL	Cotinine-Negative (N = 25) pg/mL	<i>p</i> Value
TPO	52.31 ± 53.32	54.18 ± 62.19	0.7302
MIP-1 α	15.66 ± 13.37	18.33 ± 21.46	0.7327
TRAIL	160.89 ± 66.45	135.12 ± 70.9	0.7613
IL-12p70	2.72 ± 2.11	2.33 ± 1.43	0.7694
MCP-1	547.85 ± 268.86	538.61 ± 482.59	0.7723
PDGF-AA	365.04 ± 147.38	369.35 ± 256.1	0.7855
I-309	18.08 ± 6.81	15.48 ± 9.9	0.791
sCD40L	267.95 ± 326.09	193.91 ± 186.15	0.7969
M-CSF	251.95 ± 104.23	237.41 ± 119.48	0.7979
EGF	185.36 ± 72.81	192.04 ± 99.73	0.7986
IFN ω	2.66 ± 3.14	2.29 ± 3.32	0.8161
TSLP	1.28 ± 1.09	0.92 ± 0.77	0.8353
IL-8	453.76 ± 294.34	800.06 ± 1223.01	0.8363
LIF	15.7 ± 18.39	12.93 ± 20.84	0.8365
Fractalkine	95.22 ± 44.96	89.41 ± 50.34	0.8367
IL-1 α	188.29 ± 194.58	181.21 ± 141.82	0.8382
IL-17F	6.4 ± 6.85	5.6 ± 7.14	0.8653
Granzyme A	15.53 ± 8.51	15.83 ± 13.75	0.8656
GRO α	312.5 ± 144.28	312.58 ± 255.5	0.8878
SCF	625.72 ± 185.53	559.38 ± 229.75	0.8974
IL-7	0.82 ± 0.52	1.09 ± 2.62	0.9
IL-24	31.59 ± 19.71	40.93 ± 46.67	0.9281
IL-29	12.85 ± 11.86	11.42 ± 12.58	0.9291
TGF α	6.31 ± 4.18	5.65 ± 5.01	0.9512
BCA-1	30.42 ± 18.15	30.46 ± 30.41	0.9518
IL-2	1.14 ± 0.59	1.5 ± 2.54	0.9686
sCD137	31.37 ± 13.12	27.6 ± 14.61	0.9753
MIP 1 δ	2018.7 ± 447.93	1956.99 ± 1034.82	0.9756
IL-3	0.43 ± 0.29	0.34 ± 0.39	0.9919

References

References

1. Barker DJ. The foetal and infant origins of inequalities in health in Britain. *J Public Health Med.* 1991 May;13(2):64-8. PMID: 1854527.
2. Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ. Weight in infancy and death from ischaemic heart disease. *Lancet.* 1989 Sep 9;2(8663):577-80.
3. Lacagnina S. The Developmental Origins of Health and Disease (DOHaD). *American Journal of Lifestyle Medicine.* 2020 Jan;14(1):47-50.
4. Tarasi B, Cornuz J, Clair C, Baud D. Cigarette smoking during pregnancy and adverse perinatal outcomes: a cross-sectional study over 10 years. *BMC Public Health.* 2022 Dec 21;22(1):2403.
5. Ewesesan R, Chartier MJ, Nickel NC, Wall-Wieler E, Urquia ML. Psychosocial and behavioral health indicators among immigrant and non-immigrant recent mothers. *BMC Pregnancy Childbirth.* 2022 Aug 26;22(1):612.
6. Ware JH, Dockery DW, Louis TA, Xu X, Ferris BG, Speizer FE. LONGITUDINAL AND CROSS-SECTIONAL ESTIMATES OF PULMONARY FUNCTION DECLINE IN NEVER-SMOKING ADULTS. *American Journal of Epidemiology.* 1990 Oct;132(4):685-700.
7. McGeachie MJ, Yates KP, Zhou X, Guo F, Sternberg AL, Van Natta ML, et al. Patterns of Growth and Decline in Lung Function in Persistent Childhood Asthma. *N Engl J Med.* 2016 May 12;374(19):1842-52.
8. Okyere DO, Bui DS, Washko GR, Lodge CJ, Lowe AJ, Cassim R, et al. Predictors of lung function trajectories in population-based studies: A systematic review. *Respirology.* 2021 Oct;26(10):938-59.
9. Allinson JP, Hardy R, Donaldson GC, Shaheen SO, Kuh D, Wedzicha JA. Combined Impact of Smoking and Early-Life Exposures on Adult Lung Function Trajectories. *Am J Respir Crit Care Med.* 2017 Oct 15;196(8):1021-30.
10. Berry CE, Billheimer D, Jenkins IC, Lu ZJ, Stern DA, Gerald LB, et al. A Distinct Low Lung Function Trajectory from Childhood to the Fourth Decade of Life. *Am J Respir Crit Care Med.* 2016 Sep 1;194(5):607-12.
11. Mathew AR, Bhatt SP, Colangelo LA, Allen NB, Jacobs DR, Auer R, et al. Life-Course Smoking Trajectories and Risk for Emphysema in Middle Age: The CARDIA Lung Study. *Am J Respir Crit Care Med.* 2019 Jan 15;199(2):237-40.
12. Rosewich M, Schulze J, Eickmeier O, Adler S, Rose MA, Schubert R, et al. Early impact of smoking on lung function, health, and well-being in adolescents. *Pediatric Pulmonology.* 2012 Jul;47(7):692-9.

13. Ware JH, Dockery DW, Spiro A. Passive Smoking, Gas Cooking, and Respiratory Health of Children Living in Six Cities.
14. Stocks J, Dezaux C. The effect of parental smoking on lung function and development during infancy. *Respirology*. 2003 Sep;8(3):266–85.
15. Weber P, Menezes AMB, Gonçalves H, De Oliveira PD, Wendt A, Perez-Padilla R, et al. Smoking exposure trajectories and pulmonary function in early adulthood in a Brazilian cohort. *Pulmonology*. 2023 Oct;S253104372300168X.
16. Martinez FD, Cline M, Burrows B. Increased Incidence of Asthma in Children of Smoking Mothers. *Pediatrics*. 1992 Jan 1;89(1):21–6.
17. Wang B, Chen H, Chan YL, Wang G, Oliver BG. Why Do Intrauterine Exposure to Air Pollution and Cigarette Smoke Increase the Risk of Asthma? *Front Cell Dev Biol*. 2020 Feb 5;8:38.
18. Leon Hsu HH, Mathilda Chiu YH, Coull BA, Kloog I, Schwartz J, Lee A, et al. Prenatal Particulate Air Pollution and Asthma Onset in Urban Children. Identifying Sensitive Windows and Sex Differences. *Am J Respir Crit Care Med*. 2015 Nov 1;192(9):1052–9.
19. Tager IB, Ngo L, Hanrahan JP. Maternal smoking during pregnancy. Effects on lung function during the first 18 months of life. *Am J Respir Crit Care Med*. 1995 Sep;152(3):977–83.
20. Han VX, Patel S, Jones HF, Nielsen TC, Mohammad SS, Hofer MJ, et al. Maternal acute and chronic inflammation in pregnancy is associated with common neurodevelopmental disorders: a systematic review. *Transl Psychiatry*. 2021 Jan 21;11(1):71.
21. Hayatbakhsh MR, Sadasivam S, Mamun AA, Najman JM, Williams GM, O’Callaghan MJ. Maternal smoking during and after pregnancy and lung function in early adulthood: a prospective study. *Thorax*. 2009 Sep 1;64(9):810–4.
22. Lodrup Carlsen K, Jaakkola J, Nafstad P, Carlsen K. In utero exposure to cigarette smoking influences lung function at birth. *Eur Respir J*. 1997 Aug 1;10(8):1774–9.
23. Gilliland FD. Maternal smoking during pregnancy, environmental tobacco smoke exposure and childhood lung function. *Thorax*. 2000 Apr 1;55(4):271–6.
24. Sekhon HS, Jia Y, Raab R, Kuryatov A, Pankow JF, Whitsett JA, et al. Prenatal nicotine increases pulmonary $\alpha 7$ nicotinic receptor expression and alters fetal lung development in monkeys. *J Clin Invest*. 1999 Mar 1;103(5):637–47.
25. Wongtrakool C, Roser-Page S, Rivera HN, Roman J. Nicotine alters lung branching morphogenesis through the $\alpha 7$ nicotinic acetylcholine receptor. *American Journal of Physiology-Lung Cellular and Molecular Physiology*. 2007 Sep;293(3):L611–8.

26. Werhmeister FC, Nunes BP, Loret De Mola C, Gómez-Cofré N, De Oliveira PD, Marco PL, et al. Intrauterine exposure to smoking and wheezing in adolescence: the 1993 Pelotas Birth Cohort. *J Dev Orig Health Dis*. 2015 Jun;6(3):217–24.
27. Li YF, Langholz B, Salam MT, Gilliland FD. Maternal and Grandmaternal Smoking Patterns Are Associated With Early Childhood Asthma. *Chest*. 2005 Apr;127(4):1232–41.
28. Mahon GM, Koppelman GH, Vonk JM. Grandmaternal smoking, asthma and lung function in the offspring: the Lifelines cohort study. *Thorax*. 2021 May;76(5):441–7.
29. Bråbäck L, Lodge CJ, Lowe AJ, Dharmage SC, Olsson D, Forsberg B. Childhood asthma and smoking exposures before conception—A three-generational cohort study. *Pediatric Allergy Immunology*. 2018 Jun;29(4):361–8.
30. Svanes C, Koplein J, Skulstad SM, Johannessen A, Bertelsen RJ, Benediktsdottir B, et al. Father's environment before conception and asthma risk in his children: a multi-generation analysis of the Respiratory Health In Northern Europe study. *Int J Epidemiol*. 2016 Aug 25;dyw151.
31. Toth A, Steinmeyer S, Kannan P, Gray J, Jackson CM, Mukherjee S, Demmert M, Sheak JR, Benson D, Kitzmiller J, Wayman JA, Presicce P, Cates C, Rubin R, Chetal K, Du Y, Miao Y, Gu M, Guo M, Kalinichenko VV, Kallapur SG, Miraldi ER, Xu Y, Swarr D, Lewkowich I, Salomonis N, Miller L, Sucre JS, Whitsett JA, Chougnet CA, Jobe AH, Deshmukh H, Zacharias WJ. Inflammatory blockade prevents injury to the developing pulmonary gas exchange surface in preterm primates. *Sci Transl Med*. 2022 Mar 30;14(638):eabl8574. doi: 10.1126/scitranslmed.abl8574. Epub 2022 Mar 30. PMID: 35353543; PMCID: PMC9082785.
32. Kallapur SG, Nitsos I, Moss TJM, Polglase GR, Pillow JJ, Cheah FC, et al. IL-1 Mediates Pulmonary and Systemic Inflammatory Responses to Chorioamnionitis Induced by Lipopolysaccharide. *Am J Respir Crit Care Med*. 2009 May 15;179(10):955–61.
33. Jackson CM, Mukherjee S, Wilburn AN, Cates C, Lewkowich IP, Deshmukh H, et al. Pulmonary Consequences of Prenatal Inflammatory Exposures: Clinical Perspective and Review of Basic Immunological Mechanisms. *Front Immunol*. 2020 Jun 19;11:1285.
34. Jain VG, Willis KA, Jobe A, Ambalavanan N. Chorioamnionitis and neonatal outcomes. *Pediatr Res*. 2022 Jan;91(2):289–96.
35. Li J, Wang Z, Chu Q, Jiang K, Li J, Tang N. The Strength of Mechanical Forces Determines the Differentiation of Alveolar Epithelial Cells. *Developmental Cell*. 2018 Feb;44(3):297-312.e5.
36. Hooper SB, Harding R. FETAL LUNG LIQUID: A MAJOR DETERMINANT OF THE GROWTH AND FUNCTIONAL DEVELOPMENT OF THE FETAL LUNG. *Clin Exp Pharma Physio*. 1995 Apr;22(4):235–41.

37. Miller AA, Hooper SB, Harding R. Role of fetal breathing movements in control of fetal lung distension. *Journal of Applied Physiology*. 1993 Dec 1;75(6):2711–7.
38. Alapati D, Zacharias WJ, Hartman HA, Rossidis AC, Stratigis JD, Ahn NJ, et al. In utero gene editing for monogenic lung disease. *Sci Transl Med*. 2019 Apr 17;11(488):eaav8375.
39. Raby KL, Michaeloudes C, Tonkin J, Chung KF, Bhavsar PK. Mechanisms of airway epithelial injury and abnormal repair in asthma and COPD. *Front Immunol*. 2023 Jul 13;14:1201658.
40. Underwood MA, Gilbert WM, Sherman MP. Amniotic Fluid: Not Just Fetal Urine Anymore. *J Perinatol*. 2005 May 1;25(5):341–8.
41. Beall MH, Van Den Wijngaard JPHM, Van Gemert MJC, Ross MG. Amniotic Fluid Water Dynamics. *Placenta*. 2007 Aug;28(8–9):816–23.
42. Tong XL, Wang L, Gao TB, Qin YG, Qi YQ, Xu YP. Potential Function of Amniotic Fluid in Fetal Development—Novel Insights by Comparing the Composition of Human Amniotic Fluid with Umbilical Cord and Maternal Serum at Mid and Late Gestation. *Journal of the Chinese Medical Association*. 2009 Jul;72(7):368–73.
43. Campbell J, Wathen N, Macintosh M, Cass P, Chard T, Mainwaring-Burton R. Biochemical composition of amniotic fluid and extraembryonic coelomic fluid in the first trimester of pregnancy. *BJOG:An international journal of O&G*. 1992 Jul;99(7):563–5.
44. Rüetschi U, Rosén Å, Karlsson G, Zetterberg H, Rymo L, Hagberg H, et al. Proteomic Analysis Using Protein Chips to Detect Biomarkers in Cervical and Amniotic Fluid in Women with Intra-Amniotic Inflammation. *J Proteome Res*. 2005 Dec 1;4(6):2236–42.
45. Fang H, Wang W, Wang L, Zhu J, Lin W, Deng H, et al. Lipidomic profiling of amniotic fluid reveals aberrant fetal lung development and fetal growth disrupted by lipid disorders during gestational asthma. *Journal of Pharmaceutical and Biomedical Analysis*. 2025 Jan;252:116475.
46. Saito S, Kasahara T, Kato Y, Ishihara Y, Ichijo M. Elevation of amniotic fluid interleukin 6 (IL-6), IL-8 and granulocyte colony stimulating factor (G-CSF) in term and preterm parturition. *Cytokine*. 1993 Jan;5(1):81–8.
47. Shobokshi A, Shaarawy M. Maternal serum and amniotic fluid cytokines in patients with preterm premature rupture of membranes with and without intrauterine infection. *Intl J Gynecology & Obste*. 2002 Dec;79(3):209–15.
48. Ghezzi F, Gomez R, Romero R, Yoon BH, Edwin SS, David C, et al. Elevated interleukin-8 concentrations in amniotic fluid of mothers whose neonates subsequently develop bronchopulmonary dysplasia. *European Journal of Obstetrics & Gynecology and Reproductive Biology*. 1998 May;78(1):5–10.

49. Sano M, Nagura H, Ueno S, Nakashima A. Amino Acid Composition of Amniotic Fluid during the Perinatal Period Reflects Mother's Fat and Carbohydrate Intake. *Nutrients*. 2021 Jun 22;13(7):2136.
50. Xue J, Xu Z, Hu X, Lu Y, Zhao Y, Zhang H. Microplastics in maternal amniotic fluid and their associations with gestational age. *Science of The Total Environment*. 2024 Apr;920:171044.
51. Olver RE, Strang LB. Ion fluxes across the pulmonary epithelium and the secretion of lung liquid in the foetal lamb. *The Journal of Physiology*. 1974 Sep;241(2):327–57.
52. Kalache KD, Chaoui R, Bollmann R. Doppler assessment of tracheal and nasal fluid flow during fetal breathing movements: preliminary observations. *Ultrasound in Obstet & Gyne*. 1997 Apr;9(4):257–61.
53. Rypens F, Metens T, Rocourt N, Sonigo P, Brunelle F, Quere MP, et al. Fetal Lung Volume: Estimation at MR Imaging—Initial Results. *Radiology*. 2001 Apr;219(1):236–41.
54. De Vries JIP, Fong BF. Normal fetal motility: an overview. *Ultrasound in Obstet & Gyne*. 2006 Jun;27(6):701–11.
55. Lutfi MF. The physiological basis and clinical significance of lung volume measurements. *Multidiscip Respir Med*. 2017 Dec;12(1):3.
56. Wigglesworth JS, Desai R. Effects on lung growth of cervical cord section in the rabbit fetus. *Early Human Development*. 1979 Mar;3(1):51–65.
57. Collins MH, Blanc WA, Rey HR, James LS. Oligohydramnios-Induced Lung Hypoplasia: The Influence of Timing and Duration in Gestation.
58. Kizilcan F, Tanyel FC, Çakar N, Büyükpamukçu N, Hiçsönmez A. The effect of low amniotic pressure without oligohydramnios on fetal lung development in a rabbit model. *American Journal of Obstetrics and Gynecology*. 1995 Jul;173(1):36–41.
59. Liu W li, Zhou Y, Zhang C, Chen J, Yin X feng, Zhou F xia, et al. Relationship between chorioamnionitis or funisitis and lung injury among preterm infants: meta-analysis involved 16 observational studies with 68,397 participants. *BMC Pediatr*. 2024 Mar 5;24(1):157.
60. Romero R, Gotsch F, Pineles B, Kusanovic JP. Inflammation in Pregnancy: Its Roles in Reproductive Physiology, Obstetrical Complications, and Fetal Injury. *Nutrition Reviews*. 2008 Jun 28;65:S194–202.
61. Dutta S, Sengupta P, Liew FF. Cytokine landscapes of pregnancy: mapping gestational immune phases. *gocm*. 2024 Apr;4(1):e000011.
62. Jarmund AH, Giskeødegård GF, Ryssdal M, Steinkjer B, Stokkeland LMT, Madssen TS, et al. Cytokine Patterns in Maternal Serum From First Trimester to Term and Beyond. *Front Immunol*. 2021 Oct 14;12:752660.

63. Meyyazhagan A, Kuchi Bhotla H, Pappuswamy M, Tsibizova V, Al Qasem M, Di Renzo GC. Cytokine see-saw across pregnancy, its related complexities and consequences. *Intl J Gynecology & Obste.* 2023 Feb;160(2):516–25.
64. Mor G, Cardenas I. REVIEW ARTICLE: The Immune System in Pregnancy: A Unique Complexity. *American J Rep Immunol.* 2010 Jun;63(6):425–33.
65. Goldstein JA, Gallagher K, Beck C, Kumar R, Gernand AD. Maternal-Fetal Inflammation in the Placenta and the Developmental Origins of Health and Disease. *Front Immunol.* 2020 Nov 13;11:531543.
66. Megli CJ, Coyne CB. Infections at the maternal–fetal interface: an overview of pathogenesis and defence. *Nat Rev Microbiol.* 2022 Feb;20(2):67–82.
67. Cao L, Wang J, Tseu I, Luo D, Post M. Maternal exposure to endotoxin delays alveolarization during postnatal rat lung development. *American Journal of Physiology-Lung Cellular and Molecular Physiology.* 2009 May;296(5):L726–37.
68. Willet KE, Kramer BW, Kallapur SG, Ikegami M, Newnham JP, Moss TJ, et al. Intra-amniotic injection of IL-1 induces inflammation and maturation in fetal sheep lung. *American Journal of Physiology-Lung Cellular and Molecular Physiology.* 2002 Mar 1;282(3):L411–20.
69. Kallapur SG, Jobe AH, Ball MK, Nitsos I, Moss TJM, Hillman NH, et al. Intra-Amniotic IL-1b Induces Fetal Inflammation in Rhesus Monkeys and Alters the Regulatory T Cell/IL-17 Balance. *The Journal of Immunology.* 2007 Dec 15;179(12):8491–9.
70. Hansbro PM, Starkey MR, Kim RY, Stevens RL, Foster PS, Horvat JC. Programming of the lung by early-life infection. *J Devel Orig Health Dis.* 2012 Jun;3(03):153–8.
71. Yu H, Li D, Zhao X, Fu J. Fetal origin of bronchopulmonary dysplasia: contribution of intrauterine inflammation. *Mol Med.* 2024 Sep 3;30(1):135.
72. McCartney SA, Kapur R, Liggitt HD, Baldessari A, Coleman M, Orvis A, et al. Amniotic fluid interleukin 6 and interleukin 8 are superior predictors of fetal lung injury compared with maternal or fetal plasma cytokines or placental histopathology in a nonhuman primate model. *American Journal of Obstetrics and Gynecology.* 2021 Jul;225(1):89.e1-89.e16.
73. Weinblatt ME, Keystone EC, Furst DE, Moreland LW, Weisman MH, Birbara CA, et al. Adalimumab, a fully human anti–tumor necrosis factor α monoclonal antibody, for the treatment of rheumatoid arthritis in patients taking concomitant methotrexate: The ARMADA trial. *Arthritis & Rheumatism.* 2003 Jan;48(1):35–45.
74. Lebold KM, Drake MG, Hales-Beck LB, Fryer AD, Jacoby DB. IL-5 Exposure *In Utero* Increases Lung Nerve Density and Airway Reactivity in Adult Offspring. *Am J Respir Cell Mol Biol.* 2020 Apr;62(4):493–502.

75. Nachman RM, Mao G, Zhang X, Hong X, Chen Z, Soria CS, et al. Intrauterine Inflammation and Maternal Exposure to Ambient PM_{2.5} during Preconception and Specific Periods of Pregnancy: The Boston Birth Cohort. *Environ Health Perspect.* 2016 Oct;124(10):1608–15.
76. Mestan K, Yu Y, Matoba N, Cerda S, Demmin B, Pearson C, et al. Placental Inflammatory Response Is Associated With Poor Neonatal Growth: Preterm Birth Cohort Study. *Pediatrics.* 2010 Apr 1;125(4):e891–8.
77. Fischer ST, Lili LN, Li S, Tran VT, Stewart KB, Schwartz CE, et al. Low-level maternal exposure to nicotine associates with significant metabolic perturbations in second-trimester amniotic fluid. *Environment International.* 2017 Oct;107:227–34.
78. Noakes PS. Maternal smoking is associated with impaired neonatal toll-like-receptor-mediated immune responses. *European Respiratory Journal.* 2006 Oct 1;28(4):721–9.
79. Noakes PS, Holt PG, Prescott SL. Maternal smoking in pregnancy alters neonatal cytokine responses. *Allergy.* 2003 Oct;58(10):1053–8.
80. Chen GY, Nuñez G. Sterile inflammation: sensing and reacting to damage. *Nat Rev Immunol.* 2010 Dec;10(12):826–37.
81. Kim CJ, Romero R, Chaemsaitong P, Chaiyasit N, Yoon BH, Kim YM. Acute chorioamnionitis and funisitis: definition, pathologic features, and clinical significance. *American Journal of Obstetrics and Gynecology.* 2015 Oct;213(4):S29–52.
82. La Sala GB, Ardizzoni A, Capodanno F, Manca L, Baschieri MC, Soncini E, et al. Protein Microarrays and Midtrimester Amniotic Fluids: A Novel Approach for the Diagnosis of Early Intrauterine Inflammation Related to Preterm Delivery. *Int J Immunopathol Pharmacol.* 2012 Oct;25(4):1029–40.
83. Wang C, Chen Q, Wang Y. Correlation of amniotic fluid inflammatory markers with preterm birth: a meta-analysis. *Journal of Obstetrics and Gynaecology.* 2024 Dec 31;44(1):2368764.
84. Abdallah MW, Larsen N, Grove J, Nørgaard-Pedersen B, Thorsen P, Mortensen EL, et al. Amniotic fluid inflammatory cytokines: Potential markers of immunologic dysfunction in autism spectrum disorders. *The World Journal of Biological Psychiatry.* 2013 Sep;14(7):528–38.
85. Pala Ş, Atilgan R, İlhan N. High amniotic fluid fractalkine and MIP-1β levels are associated with intrauterine growth restriction: a prospective cohort study. *Turkish Journal of Medical Sciences.* 2024 Jan 1;54(1):280–90.
86. Yoon BH, Jun JK, Romero R, Gomez R. Amniotic fluid inflammatory cytokines (interleukin-6, interleukin-1[3, and tumor necrosis factor-s), neonatal brain white matter lesions, and cerebral palsy. *Am J Obstet Gynecol.* 1997;177(1).

87. Sosenko IRS, Kallapur SG, Nitsos I, Moss TJM, Newnham JP, Ikegami M, et al. IL-1 α Causes Lung Inflammation and Maturation by Direct Effects on Preterm Fetal Lamb Lungs. *Pediatr Res*. 2006 Sep;60(3):294–8.
88. Griffiths SK, Campbell JP. Placental structure, function and drug transfer. *Continuing Education in Anaesthesia Critical Care & Pain*. 2015 Apr;15(2):84–9.
89. Piedimonte G, Harford TJ. Effects of maternal–fetal transmission of viruses and other environmental agents on lung development. *Pediatr Res*. 2020 Jan;87(2):420–6.
90. Piedimonte G, Walton C, Samsell L. Vertical Transmission of Respiratory Syncytial Virus Modulates Pre- and Postnatal Innervation and Reactivity of Rat Airways. Tripp R, editor. *PLoS ONE*. 2013 Apr 18;8(4):e61309.
91. Brown PM, Harford TJ, Agrawal V, Yen-Lieberman B, Rezaee F, Piedimonte G. Prenatal Exposure to Respiratory Syncytial Virus Alters Postnatal Immunity and Airway Smooth Muscle Contractility during Early-Life Reinfections. Tregoning JS, editor. *PLoS ONE*. 2017 Feb 8;12(2):e0168786.
92. Paul E, Franco-Montoya ML, Paineau E, Angeletti B, Vibhushan S, Ridoux A, et al. Pulmonary exposure to metallic nanomaterials during pregnancy irreversibly impairs lung development of the offspring. *Nanotoxicology*. 2017 Apr 21;11(4):484–95.
93. Mielke G, Benda N. Cardiac Output and Central Distribution of Blood Flow in the Human Fetus. *Circulation*. 2001 Mar 27;103(12):1662–8.
94. Hellings PW, Steelant B. Epithelial barriers in allergy and asthma. *Journal of Allergy and Clinical Immunology*. 2020 Jun;145(6):1499–509.
95. Knight DA, Holgate ST. The airway epithelium: Structural and functional properties in health and disease. *Respirology*. 2003 Dec;8(4):432–46.
96. Cao Y, Chen M, Dong D, Xie S, Liu M. Environmental pollutants damage airway epithelial cell cilia: Implications for the prevention of obstructive lung diseases. *Thoracic Cancer*. 2020 Mar;11(3):505–10.
97. Xian M, Ma S, Wang K, Lou H, Wang Y, Zhang L, et al. Particulate Matter 2.5 Causes Deficiency in Barrier Integrity in Human Nasal Epithelial Cells. *Allergy Asthma Immunol Res*. 2020;12(1):56.
98. Rezaee F, Georas SN. Breaking Barriers. New Insights into Airway Epithelial Barrier Function in Health and Disease. *Am J Respir Cell Mol Biol*. 2014 May;50(5):857–69.
99. Varricchi G, Brightling CE, Grainge C, Lambrecht BN, Chanez P. Airway remodelling in asthma and the epithelium: on the edge of a new era. *Eur Respir J*. 2024 Apr;63(4):2301619.

100. AbuJabal R, Ramakrishnan RK, Bajbouj K, Hamid Q. Role of IL -5 in asthma and airway remodelling. *Clin Experimental Allergy*. 2024 Aug;54(8):538–49.
101. Russell RJ, Boulet LP, Brightling CE, Pavord ID, Porsbjerg C, Dorscheid D, et al. The airway epithelium: an orchestrator of inflammation, a key structural barrier and a therapeutic target in severe asthma. *Eur Respir J*. 2024 Apr;63(4):2301397.
102. Fu XW, Lindstrom J, Spindel ER. Nicotine Activates and Up-Regulates Nicotinic Acetylcholine Receptors in Bronchial Epithelial Cells. *Am J Respir Cell Mol Biol*. 2009 Jul;41(1):93–9.
103. Wongtrakool C, Wang N, Hyde DM, Roman J, Spindel ER. Prenatal Nicotine Exposure Alters Lung Function and Airway Geometry through $\alpha 7$ Nicotinic Receptors. *Am J Respir Cell Mol Biol*. 2012 May;46(5):695–702.
104. Maouche K, Polette M, Jolly T, Medjber K, Cloëz-Tayarani I, Changeux JP, et al. $\alpha 7$ Nicotinic Acetylcholine Receptor Regulates Airway Epithelium Differentiation by Controlling Basal Cell Proliferation. *The American Journal of Pathology*. 2009 Nov;175(5):1868–82.
105. Shaykhiev R, Zuo WL, Chao I, Fukui T, Witover B, Brekman A, et al. EGF shifts human airway basal cell fate toward a smoking-associated airway epithelial phenotype. *Proc Natl Acad Sci USA*. 2013 Jul 16;110(29):12102–7.
106. Tan X, Vrana K, Ding ZM. Cotinine: Pharmacologically Active Metabolite of Nicotine and Neural Mechanisms for Its Actions. *Front Behav Neurosci*. 2021 Oct 21;15:758252.
107. Jacob N, Golmard JL, Berlin I. Fetal exposure to tobacco: nicotine and cotinine concentration in amniotic fluid and maternal saliva. *The Journal of Maternal-Fetal & Neonatal Medicine*. 2017 Jan 17;30(2):233–9.
108. Porsbjerg C, Melén E, Lehtimäki L, Shaw D. Asthma. *The Lancet*. 2023 Mar;401(10379):858–73.
109. Rochman M, Klinger AM, Caldwell JM, Sadovsky Y, Rothenberg ME. Amniotic fluid modifies esophageal epithelium differentiation and inflammatory responses. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 2024 Nov 1;327(5):G629–39.
110. Lange P, Celli B, Agustí A, Boje Jensen G, Divo M, Faner R, et al. Lung-Function Trajectories Leading to Chronic Obstructive Pulmonary Disease. *N Engl J Med*. 2015 Jul 9;373(2):111–22.
111. Buchert M, Turksen K, Hollande F. Methods to Examine Tight Junction Physiology in Cancer Stem Cells: TEER, Paracellular Permeability, and Dilution Potential Measurements. *Stem Cell Rev and Rep*. 2012 Sep;8(3):1030–4.
112. Szklarczyk D, Kirsch R, Koutrouli M, Nastou K, Mehryary F, Hachilif R, et al. The STRING database in 2023: protein–protein association networks and functional enrichment

- analyses for any sequenced genome of interest. *Nucleic Acids Research*. 2023 Jan 6;51(D1):D638–46.
113. Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Meirelles GV, et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinformatics*. 2013 Dec;14(1):128.
 114. Zhang P, West NP, Chen PY, Thang MWC, Price G, Cripps AW, et al. Selection of microbial biomarkers with genetic algorithm and principal component analysis. *BMC Bioinformatics*. 2019 Dec;20(S6):413.
 115. Neuman Å, Hohmann C, Orsini N, Pershagen G, Eller E, Kjaer HF, et al. Maternal Smoking in Pregnancy and Asthma in Preschool Children: A Pooled Analysis of Eight Birth Cohorts. *Am J Respir Crit Care Med*. 2012 Nov 15;186(10):1037–43.
 116. Mullassery D, Smith NP. Lung development. *Seminars in Pediatric Surgery*. 2015 Aug;24(4):152–5.
 117. Burri PH. Fetal and postnatal development of the lung. *Annu Rev Physiol*. 1984;46:617-28. doi: 10.1146/annurev.ph.46.030184.003153. PMID: 6370120.
 118. Speizer FE, Tager IB. EPIDEMIOLOGY OF CHRONIC MUCUS HYPERSECRETION AND OBSTRUCTIVE AIRWAYS DISEASE. *Epidemiologic Reviews*. 1979;1(1):124–42.
 119. Turner S, Fielding S, Mullane D, Cox DW, Goldblatt J, Landau L, et al. A longitudinal study of lung function from 1 month to 18 years of age. *Thorax*. 2014 Nov;69(11):1015–20.
 120. Lima C, Souza VMO, Faquim-Mauro EL, Hoshida MS, Bevilacqua E, Macedo MS, et al. Modulation of the Induction of Lung and Airway Allergy in the Offspring of IFN- γ -Treated Mother Mice. *The Journal of Immunology*. 2005 Sep 15;175(6):3554–9.
 121. Macaubas C, de Klerk NH, Holt BJ, Wee C, Kendall G, Firth M, et al. Association between antenatal cytokine production and the development of atopy and asthma at age 6 years. *THE LANCET*. 2003;362.
 122. Rothers J, Stern DA, Lohman IC, Spangenberg A, Wright AL, DeVries A, et al. Maternal Cytokine Profiles during Pregnancy Predict Asthma in Children of Mothers without Asthma. *Am J Respir Cell Mol Biol*. 2018 Nov;59(5):592–600.
 123. Strandjord TP, Clark JG, Madtes DK. Expression of TGF- α , EGF, and EGF receptor in fetal rat lung. *American Journal of Physiology-Lung Cellular and Molecular Physiology*. 1994 Oct 1;267(4):L384–9.
 124. Chandrasekaran P, Negretti NM, Sivakumar A, Liberti DC, Wen H, Peers De Nieuwburgh M, et al. CXCL12 defines lung endothelial heterogeneity and promotes distal vascular growth. *Development*. 2022 Nov 1;149(21):dev200909.

125. Bartram U, Speer CP. The Role of Transforming Growth Factor β in Lung Development and Disease. *Chest*. 2004 Feb;125(2):754–65.
126. Wu S, Peng J, Duncan MR, Kasisomayajula K, Grotendorst G, Bancalari E. ALK-5 Mediates Endogenous and TGF- β 1–Induced Expression of Connective Tissue Growth Factor in Embryonic Lung. *Am J Respir Cell Mol Biol*. 2007 May;36(5):552–61.
127. Chow SSW, Craig ME, Jones CA, Hall B, Catteau J, Lloyd AR, et al. Differences in amniotic fluid and maternal serum cytokine levels in early midtrimester women without evidence of infection. *Cytokine*. 2008 Oct;44(1):78–84.
128. Hu GM, Mai TL, Chen CM. Visualizing the GPCR Network: Classification and Evolution. *Sci Rep*. 2017 Nov 14;7(1):15495.
129. Liu C, Chu D, Kalantar-Zadeh K, George J, Young HA, Liu G. Cytokines: From Clinical Significance to Quantification. *Advanced Science*. 2021 Aug;8(15):2004433.
130. Hammad H, Lambrecht BN. The basic immunology of asthma. *Cell*. 2021 Mar;184(6):1469–85.
131. Collinson RJ, Mazza-Parton A, Fuller KA, Linden MD, Erber WN, Guo BB. Gene Expression of *CXCL1* (GRO- α) and *EGF* by Platelets in Myeloproliferative Neoplasms. *HemaSphere*. 2020 Dec;4(6):e490.
132. Luck W, Nau H, Hansen R, Steldinger R. Extent of Nicotine and Cotinine Transfer to the Human Fetus, Placenta and Amniotic Fluid of Smoking Mothers. *Dev Pharmacol Ther*. 1985;8(6):384–95.
133. Beck AL, Uldbjerg CS, Lim Y, Coull BA, Sørensen KM, Utiko MM, et al. Cotinine concentrations in maternal serum and amniotic fluid during pregnancy and risk of testicular germ cell cancer in the offspring: A prospective nested case-control study. *Intl Journal of Cancer*. 2024 Jan;154(1):71–80.
134. Terzioglu F, Boztepe H, Erkekoglu P, Er Korucu A, Kocer-Gumusel B, Kandemir O. The effects of amniotic fluid and foetal cord blood cotinine concentrations on pregnancy complications and the anthropometric measurements of newborns. *Journal of Obstetrics and Gynaecology*. 2019 Oct 3;39(7):952–8.
135. Proskocil BJ, Sekhon HS, Jia Y, Savchenko V, Blakely RD, Lindstrom J, et al. Acetylcholine Is an Autocrine or Paracrine Hormone Synthesized and Secreted by Airway Bronchial Epithelial Cells. *Endocrinology*. 2004 May;145(5):2498–506.
136. Holgate ST, Davies DE, Puddicombe S, Richter A, Lackie P, Lordan J, et al. Mechanisms of airway epithelial damage: epithelial-mesenchymal interactions in the pathogenesis of asthma. *Eur Respir J*. 2003 Sep 20;22(44 suppl):24s–9s.
137. Parker JC, Thavagnanam S, Skibinski G, Lyons J, Bell J, Heaney LG, et al. Chronic IL9 and IL-13 Exposure Leads to an Altered Differentiation of Ciliated Cells in a Well-

Differentiated Paediatric Bronchial Epithelial Cell Model. Semple MG, editor. PLoS ONE. 2013 May 9;8(5):e61023.

138. Schmidt H, Braubach P, Schilpp C, Lochbaum R, Neuland K, Thompson K, et al. IL-13 Impairs Tight Junctions in Airway Epithelia. *IJMS*. 2019 Jun 30;20(13):3222.
139. Major J, Crotta S, Llorian M, McCabe TM, Gad HH, Priestnall SL, et al. Type I and III interferons disrupt lung epithelial repair during recovery from viral infection. 2020;
140. Loxham M, Smart DE, Bedke NJ, Smithers NP, Filippi I, Blume C, et al. Allergenic proteases cleave the chemokine CX3CL1 directly from the surface of airway epithelium and augment the effect of rhinovirus. *Mucosal Immunology*. 2018 Mar;11(2):404–14.
141. Fujimoto K, Imaizumi T, Yoshida H, Takanashi S, Okumura K, Satoh K. Interferon- γ Stimulates Fractalkine Expression in Human Bronchial Epithelial Cells and Regulates Mononuclear Cell Adherence. *Am J Respir Cell Mol Biol*. 2001 Aug 1;25(2):233–8.
142. Hodge S, Hodge G, Nairn J, Holmes M, Reynolds PN. Increased Airway Granzyme b and Perforin in Current and Ex-Smoking COPD Subjects. *COPD: Journal of Chronic Obstructive Pulmonary Disease*. 2006 Jan;3(4):179–87.
143. Xu P, Becker H, Elizalde M, Pierik M, Masclee A, Jonkers D. Interleukin-28A induces epithelial barrier dysfunction in CD patient-derived intestinal organoids. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 2021 May 1;320(5):G689–99.
144. Olszewska-Pazdrak B, Casola A, Saito T, Alam R, Crowe SE, Mei F, et al. Cell-Specific Expression of RANTES, MCP-1, and MIP-1 α by Lower Airway Epithelial Cells and Eosinophils Infected with Respiratory Syncytial Virus. *J Virol*. 1998 Jun;72(6):4756–64.
145. Jarczak D, Nierhaus A. Cytokine Storm—Definition, Causes, and Implications. *IJMS*. 2022 Oct 3;23(19):11740.
146. Nyman E, Huss F, Nyman T, Junker J, Kratz G. Hyaluronic acid, an important factor in the wound healing properties of amniotic fluid: *In vitro* studies of re-epithelialisation in human skin wounds. *Journal of Plastic Surgery and Hand Surgery*. 2013 Apr;47(2):89–92.
147. Sezen D, Perni U, Herway C, Bongiovanni AM, Skupski D, Witkin SS. Hyaluronan modulates pro-inflammatory immune activity in the mid-trimester amniotic cavity. *Journal of Reproductive Immunology*. 2009 Oct;82(1):89–93.
148. Zemet R, Maktabi MA, Tinfow A, Giordano JL, Heisler TM, Yan Q, et al. Amniocentesis in pregnancies at or beyond 24 weeks: an international multicenter study. *American Journal of Obstetrics and Gynecology*. 2025 Apr;232(4):402.e1-402.e16.
149. Kim YY, Kwak J, Kang BC, Ku SY. Non-human primate: the new frontier model of female reproductive engineering. *Front Bioeng Biotechnol*. 2025 Mar 28;13:1536750.

150. Wang H, Feng Q, Li C, Zhang H, Peng Y. Ultrasonographic study of hemodynamics and contrast-enhanced ultrasound in the rhesus monkey kidney. *Exp Anim.* 2022;71(2):116–22.
151. Lemke A, Castillo-Sánchez JC, Prodinger F, Ceranic A, Hennerbichler-Lugscheider S, Pérez-Gil J, et al. Human amniotic membrane as newly identified source of amniotic fluid pulmonary surfactant. *Sci Rep.* 2017 Jul 25;7(1):6406.
152. Liu Y, Wu P, Wang Y, Liu Y, Yang H, Zhou G, et al. Application of Precision-Cut Lung Slices as an In Vitro Model for Research of Inflammatory Respiratory Diseases. *Bioengineering.* 2022 Dec 4;9(12):767.
153. Koziol-White C, Gebiski E, Cao G, Panettieri RA. Precision cut lung slices: an integrated ex vivo model for studying lung physiology, pharmacology, disease pathogenesis and drug discovery. *Respir Res.* 2024 Jun 1;25(1):231.
154. Seal RL, Braschi B, Gray K, Jones TEM, Tweedie S, Haim-Vilmovsky L, et al. Genenames.org: the HGNC resources in 2023. *Nucleic Acids Research.* 2023 Jan 6;51(D1):D1003–9.