THIRD TRIMESTER AMNIOTIC FLUID CHARACTERIZATION OF CELLS AND EXTRACELLULAR VESICLES FOR THE PREVENTION OF NECROTIZING ENTEROCOLITIS

AN ABSTRACT SUBMITTED ON THE 15TH DAY OF DECEMBER 2023 TO THE BIOINNOVATION PROGRAM IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE SCHOOL OF SCIENCE AND ENGINEERING OF TULANE UNIVERSITY FOR THE DEGREE OF

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[Abstract]

Amniotic fluid plays a crucial role in fetal development, yet a comprehensive understanding of its composition, temporal changes, and mechanisms of action remains elusive. The increased accessibility and abundance of third-trimester amniotic fluid from Cesarean sections provide an opportune moment to explore its therapeutic potential. Necrotizing Enterocolitis (NEC) stands as the leading cause of gastrointestinal-related mortality in premature infants within the Neonatal Intensive Care Unit (NICU). Affecting 1-5% of neonatal intensive care admissions and 5-10% of very low birth weight infants, NEC's pathophysiology involves inflammation, bacterial invasion, cellular damage, and necrosis, often leading to fatal outcomes. Surgery is required in 20-30% of NEC cases, with high fatality rates, contributing to substantial NICU costs. Exclusive human milk-based diets (EHMD) have shown promise in reducing NEC risk, mortality, and hospitalization costs. However, the expense associated with EHMD, particularly using Prolacta Bioscience Inc.'s exclusive fortifier, poses a significant financial challenge for hospitals. Non-privately-owned hospitals, with limited budgets, may benefit from a lower-cost preventative therapy compatible with bovine fortifiers while still reducing the risk of NEC, mortality, and overall expenses.

The proposed research aims to explore the potential of amniotic fluid, a vital in utero growth medium containing cellular and non-cellular elements, as a therapeutic product to mitigate the risk of NEC in the NICU. After isolating amniotic fluid cells, their characterization included assessing mesenchymal stem cell qualities through plastic adherence, stem cell marker expression, and differentiation. Similarly, extracellular

vesicles isolated from amniotic fluid were characterized as exosomes based on size, morphology, and positive exosome protein identification. Subsequently, an in vitro NEC model utilizing T84 intestinal epithelial cells and Lipopolysaccharides (LPS) was employed to examine the impact of these components on proliferation, cell viability, intestinal barrier function, and tight junction gene regulation. Notably, isolated exosomes from amniotic fluid demonstrated a positive effect on intestinal epithelial cell proliferation and barrier function, effectively preventing LPS-induced intestinal barrier injury. Importantly, this preventative effect cannot be solely attributed to increased proliferation or changes in mRNA expression of tight junction proteins, necessitating further exploration of other mechanisms of action. Despite extensive research, NEC incidence rates and mortality have only marginally decreased by 5% over the past two decades. The potential of third trimester human amniotic exosome exposure to prevent NEC suggests a promising avenue for further therapeutic development.

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[Dedication]

To my grandparents and my goddaughters, without them none of this would have been possible.

[Acknowledgement]

Firstly, I would like to extend my sincerest thanks to Dr. Donald Gaver, my committee chair. Thank you for creating the BioInnovation program for dreamers like me. Thank you for seeing something in me and my project. Thank you for never letting me give up. Your support has meant everything to me.

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community building. I hope you are very happy and successful in your new position. I would also like to thank all the students that contributed to this work Ann Marie Barfield, Ann Byerly, Paulina Giacomelli, Elizabeth Bryan, and Lakia Williams. Mentoring you was one of the highlights of my time at Tulane University. From Dr. Hu's lab I would like to thank Dr. Lilli Zhang and Dr. Li Yang for patiently teaching me all the methods needed for my second aim.

As my project required expertise in different areas, I must thank Alan Tucker for running my flow analyses, Dr. Jibao He for his assistance in taking pictures on the TEM, and Dr. Jacob Bitoun for the intestinal epithelial cell lines. I would also like to thank Dr. Isabela Sledge and the whole Tides Medical group who saw potential in our partnership and worked hard to get me amniotic fluid donations. I would be remiss if I didn't express my most heartfelt gratitude for the women who agreed to donate their amniotic fluid for my studies, without you this project would not have been possible.

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1. INTRODUCTION

Amniotic fluid is a complex and dynamic biological fluid that is crucial to fetal development. Despite its role in fetal diagnostics, a comprehensive understanding of its biological composition, temporal changes, and mechanisms of action remains unknown. The newfound accessibility and abundance of amniotic fluid, from third trimester Cesarean sections, presents an opportune moment to better understand it's potential therapeutic applications.

1.1 Amniotic Fluid in Fetal Development

Amniotic fluid (AF) dynamically changes with gestation and significantly contributes to fetal wellbeing by being a protective surrounding and ingested growth media [1]. Fetal deformations would occur without the mechanical supportive cushioning provided by AF that allows for fetal movement of extremities and growth [1, 2]. Animal studies suggest that oral ingestion of amniotic fluid provides 10% - 15% of the nutritional intake of the fetus in later pregnancy as well as cytokines and growth factors similar to human milk including epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), insulin-like growth factor-1 (IGF-1), and transforming growth factor beta-1 (TGFβ-1) [1-3]. AF is a growth liquid for the developing fetus mainly composed of water, electrolytes, sugars, proteins, lipids, hormones, enzymes, cells, and extracellular vesicles [1, 4]. AF begins to appear at week 2 of gestation as only a small film of liquid; the water originally comes from maternal plasma and passes through the fetal membranes based on hydrostatic and osmotic forces [5, 6]. From weeks 10 to 20 the AF composition is similar to plasma [5, 6]. By week 8, the fetal kidneys

begin to make urine (600 to 1200 mL/day) and by week 11 fetal swallowing begins [5, 6]. AF volume is determined by the gestational age and the pathways of fluid exchange between the amniotic space, the growing neonate, and the surrounding tissues, seen in Figure 1A [1]. The volume of AF increases up to a peak of 800-1000 ml by week 28-34 in normal pregnancies, seen in Figure 1B [1, 7]. During the second half of pregnancy, up to term, the fetus swallows about 400-800 ml daily [1, 2, 8]. Human neonates with congenital obstructions of the digestive tract show reduced birth weight without AF exposure to the intestines [2]. Experimental studies of esophageal ligations in rabbits and lambs resulted in severe atrophy of the intestinal walls, decreased mucus production, and reduced epithelial cell migration, which was reversed when swallowing of AF was restored [9, 10].

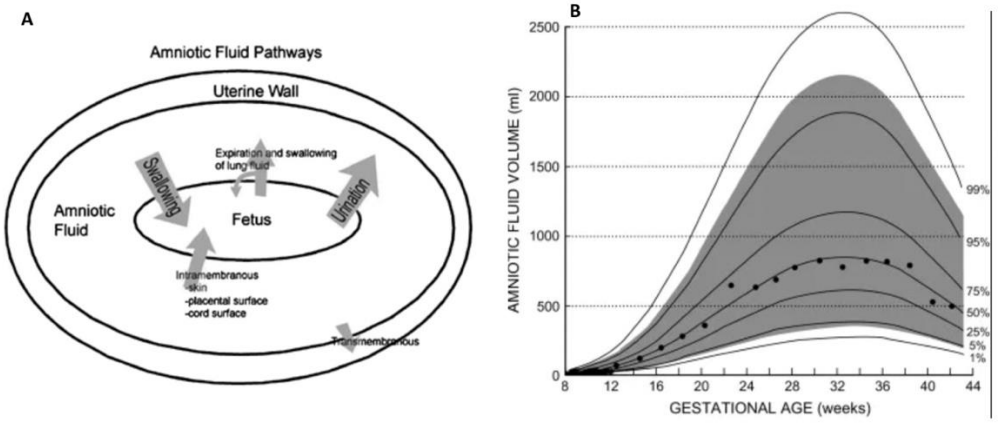

Figure 1 Amniotic fluid overview.

A) Amniotic fluid pathways [2]. B) Normal range of amniotic fluid volume in human gestation [11].

1.2 Preterm Birth's Interruption of Gastrointestinal Development

Human gastrointestinal (GI) development begins in the first trimester with embryonic organogenesis and the formation of the primitive gut tube. The second and third trimesters of pregnancy, weeks 13-40, are crucial for the anatomical,

functional, and digestive processes, seen in Figure 2. During this time, the intestines exhibit rapid growth that outpaces the fetal body as a whole [7]. For example, the intestinal length including villus and microvillus structures grows to 125 cm by week 20, 200 cm by week 30, and 275 cm at term [12]. Preterm babies who are removed from this rapid growth state do not perform functions of a healthy gut including the rapid proliferation of intestinal cells, functional tight junctions of enterocytes, differentiation of intestinal stem cells, production of a thick mucosal lining by goblet cells, and secreted antimicrobial peptides by Paneth cells [13-15]. Immature development of anatomical, functional, and digestive abilities like innervation and motility makes it difficult to transition preterm babies to enteral feedings while meeting nutritional needs, leading to poor extrauterine growth and critical GI complications, such as necrotizing enterocolitis (NEC) [12].

While it remains inconclusive whether amniotic fluid actively contributes to the colonization of the intestinal microbiome, evidence suggests that the fetus ingests substantial amounts of amniotic fluid throughout pregnancy. Researchers have detected microbial DNA in meconium, the initial fetal bowel movement, indicating potential exposure of the fetal gut to amniotic fluid microbes in utero [16] [17]. Post-birth, exposure to amniotic fluid ceases, and the gastrointestinal tract continues its development through interactions with external microbes. The first wave of gut bacterial colonization depends on the mode of childbirth and the second wave of colonization occurs with feeding type, breastfed or formula fed [3]. The gut microbiome, composed of microorganisms within the intestine, plays a crucial role in safeguarding against pathogens and fostering the development of both the

gastrointestinal and immune systems. Breastfeeding further contributes to mucosal differentiation and enhances intestinal development [16] [18]. Disruptions in the gut microbiome, influenced by factors like neonatal intensive care unit (NICU) stabilization techniques, gestational age, feeding practices, antibiotic usage, and maternal microbiome, have the potential to impact the structure and function of the neonatal intestine [19, 20].

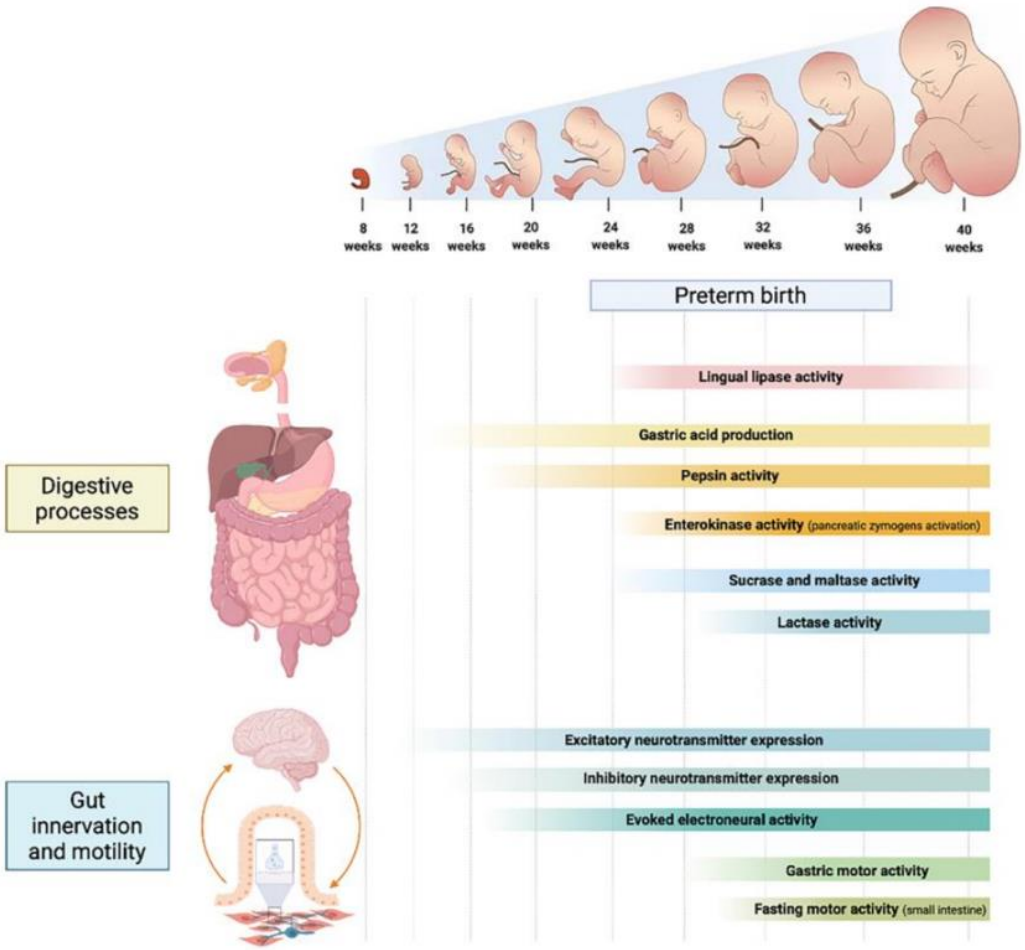

Figure 2: Gut Development.

Gut development phases of the main digestive processes through gestational weeks and impact of preterm birth [12].

1.3 Necrotizing Enterocolitis

Necrotizing Enterocolitis (NEC) is the leading cause of gastrointestinal-related mortality in premature infants within the Neonatal Intensive Care Unit (NICU) [21]. It manifests in 1-5% of all neonatal intensive care admissions and affects 5-10% of infants with very low birth weight (<1,500 g) [3]. NEC's pathophysiology involves inflammation in the intestine prompting a bacterial invasion and resulting in cellular damage and death, ultimately leading to necrosis of the colon or intestine. Advanced NEC can lead to intestinal perforation causing sepsis and death. The full understanding of NEC pathogenesis remains elusive, with risk factors including prematurity, feeding type (especially formula feedings), intestinal ischemia, bacterial presence, lower gestational age, lower birth weight, small for gestational age, prolonged rupture of membranes, lower oxygen saturation targets, and severe anemia [20, 22-24]. Despite the growing body of research and publications on NEC over the past two decades, there has been only a marginal 5% decrease in the incidence rates and mortality from NEC [25, 26]. Addressing NEC is a complex challenge, and finding solutions may also be intricate.

1.3.1 NEC Etiology

The mechanisms underlying NEC development remain incompletely understood, but associations with intestinal prematurity, underdeveloped immune defense, abnormal bacterial colonization, formula feeding, and hypoxia have been established [27, 28]. As well as, conditions causing placental insufficiency, such as hypertension and preeclampsia, and postnatal conditions causing decreased intestinal blood flow, such as cardiac diseases [29].

NEC is triggered in part by the invasion of bacteria into the intestinal wall, leading to inflammation and damage to the cells. Toll-like receptor 4 (TLR4) activation in the intestinal epithelium plays a pivotal role, triggering enterocyte and intestinal stem cell apoptosis, reduced proliferation, impaired mucosal healing, and barrier injury. This sequence leads to luminal bacteria translocation, elevating the risk of systemic sepsis (Figure 3) [21]. Additionally, translocated bacteria interacting with TLR4 on mesenteric blood vessel linings can result in vasoconstriction, intestinal ischemia, and more severe NEC presentations [30].

TLR4, expressed on intestinal stem cells, exhibits higher expression in preterm infants and even more elevated levels in infants with NEC. This could explain why NEC is primarily observed, 70% of cases, in preterm infants [21, 29, 31]. Activation of TLR4 by bacterial lipopolysaccharides (LPS) initiates a pro-inflammatory cascade, involving T helper 17 cell recruitment and the release of pro-inflammatory cytokines [3, 31]. Research suggests increased circulating levels of LPS in infants diagnosed with NEC [3]. Positive blood cultures for enteric organisms, LPS, and an imbalance of gram-negative bacteria have been identified in patients even prior to the onset of NEC [21, 32]. Therapies targeting TLR4 inhibition, such as probiotic factors, amniotic fluid products, and human milk oligosaccharides, are currently under investigation [30].

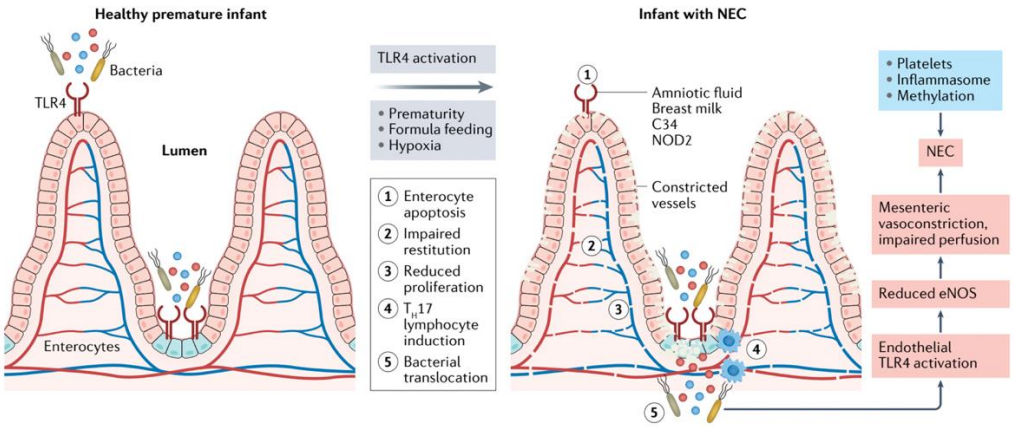

Figure 3: A model of the pathogenesis of NEC.

A model of the pathogenesis of NEC in the healthy intestines (left) bacterial colonization does not induce an inflammatory response. In a premature intestines (right) Toll-like receptor 4 (TLR4) is expressed more and induces more signaling in response to bacteria [31].

1.3.2 NEC Clinical Manifestations

The indications of NEC lack specificity, necessitating healthcare professionals to be vigilant when encountering them. These signs include reduced activity, fatigue, diminished appetite, vomiting, diarrhea, and the presence of blood in stool. Physical examinations may reveal abdominal distension (Figure 4B), abdominal tenderness, observable intestinal loops, and reduced bowel sounds [29].

Histological analysis of the intestinal wall tissue in NEC patients reveals inflammation, bacterial invasion, ischemia, perforations, and necrosis (Figure 4A) [33]. Perforations can result in pneumatosis intestinalis, where air accumulates within the intestinal wall, extending into the peritoneal cavity—the membrane lining the interior walls of the abdomen—resulting in contamination with stool and further abdominal inflammation [29].

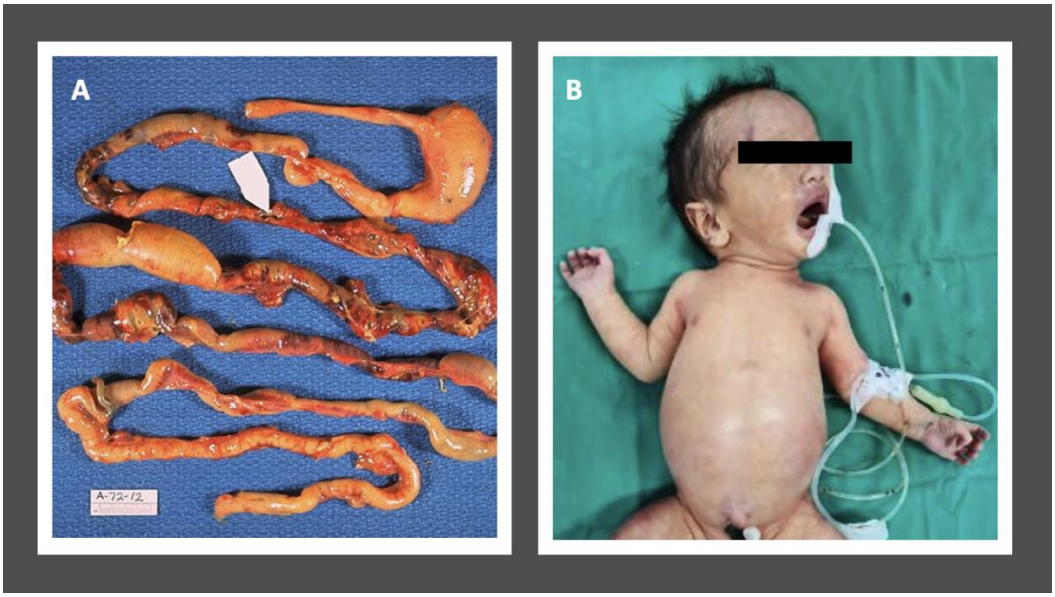

Figure 4: NEC presentations.

(A) Intestinal tract of infant with NEC; including intestinal necrosis, pneumatosis intestinalis, and perforation shown by white arrow [29]. (B) Clinical presentation of NEC with abdominal distension [34].

1.3.3 NEC Evaluation and Treatment Implications

The primary diagnostic tool for NEC used by physicians is an abdominal plain film abdominal radiographs. These films can reveal dilated loops of bowel, pneumatosis intestinalis (small amounts of air within the bowel), portal venous air (accumulation of gas in the portal vein), or free air resulting from a perforation [29]. Plain film abdominal radiographs are performed every 6 hours due to the rapid evolution that can occur in the patient's clinical condition. Laboratory tests are non-specific for NEC but a white blood count below 1500 per microliter can indicate sepsis [29]. There are currently no approved diagnosis tests for NEC.

Current clinical preventative treatments involve enteral feedings of human milk, enriched with TLR4 inhibitors, nitrate, epidermal growth factor (EGF), and human milk oligosaccharides (HMO). Mother’s own milk (MOM) is preferable to

pasteurized donor milk due to its higher microbe concentration and distinct bacterial genera abundance [35]. Also feeding premature infants an exclusively human milk diet (EHMD), using Prolacta's human milk fortifier instead of bovine milk fortifier, reduces the incidence of NEC associated with enteral feeding [36].

The use of probiotics in NEC prevention is a subject of mixed research. Observational studies suggest potential benefits, including reduced morbidity, sepsis, and mortality, but the current American Academy of Pediatrics does not endorse probiotics due to a lack of FDA-regulated pharmaceutical-grade products and insufficient strain-specific data [37, 38].

The first intervention when NEC is suspected is to stop enteral feedings and start intravenous antibiotics [3]. A broad spectrum of antibiotics is recommended covering both aerobic and anaerobic bacteria because no single organism has proven to consistently cause NEC. The most frequently used antibiotics include ampicillin, gentamycin, clindamycin, metronidazole, and gentamycin [39]. However, antibiotics pose risks if used for a prolonged amount of time (>5 days), including a higher risk of NEC and altered microbiome composition [40, 41].

While the infant has been taken off enteral feeds, total parenteral nutrition will be provided. If the bowel rest and antibiotics therapy is effective, infants will resume enteral feedings once signs of infection are resolved. The presence of a normal bowel movement means the return of bowel function. This can take several days to a week.

In cases of worsening conditions in infants or critical situations, such as intestinal perforation, surgical intervention becomes necessary. These surgeries aim

to remove only necrotic or perforated tissue. However, due to the fragile state of preterm infants, the mortality rate associated with surgery related to NEC can be as high as 50% [42].

The prognosis for NEC depends on the severity of the condition and the need for surgery. The overall mortality ranges from 10%-50% however for infants with advance NEC, including perforations and peritonitis, mortality can approach 100% [29]. Morbidities associated with NEC include intraventricular hemorrhage, chronic lung disease, neurodevelopmental impairment, short bowel syndrome, inadequate digestion, poor growth and development, total parenteral nutrition-associated cholestasis, post operative adhesions and scarring leading to stricture formation and obstruction, and recurrent NEC [3, 29, 43, 44].

1.4 Amniotic Fluid Based Experimental Treatments for NEC

The soluble fraction of amniotic fluid includes carbohydrates, proteins, lipids, electrolytes, enzymes, hormones, growth factors, and cytokines, which contributes to its anti-inflammatory and antimicrobial properties [45]. In contrast, the insoluble fraction consists of various cell types and extracellular vesicles, originating from embryonic and extraembryonic tissues, whose roles are actively being researched [6]. Both the soluble and insoluble fractions have been used in regenerative medicine (Figure 5).

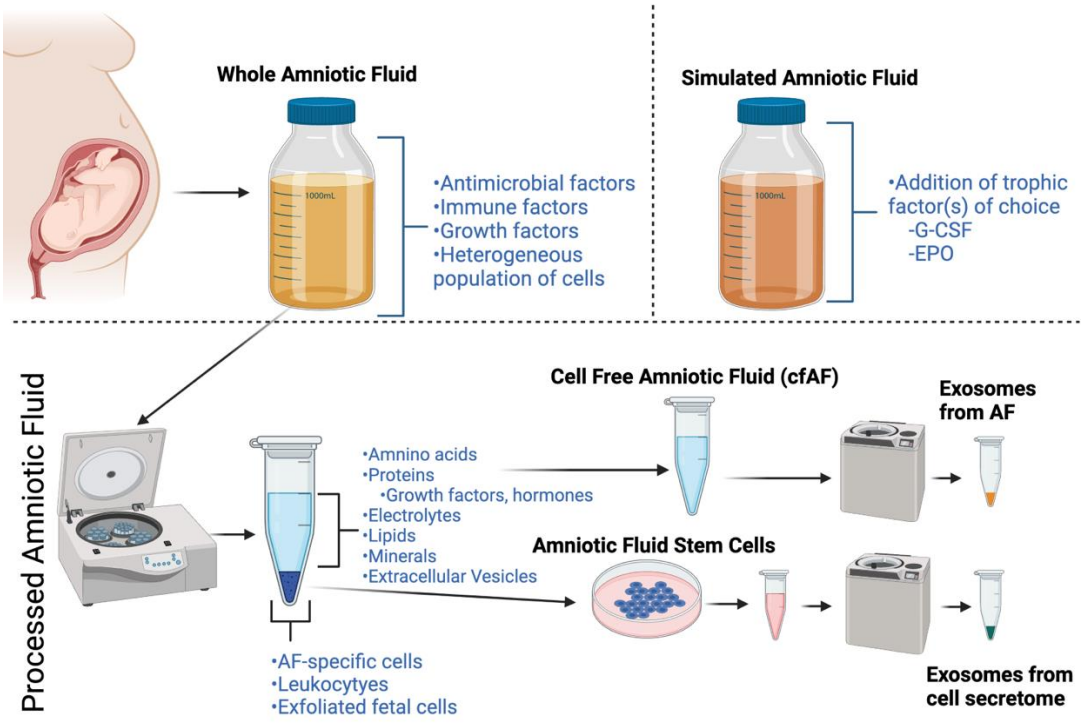

Figure 5: Amniotic fluid products.

Schematic overview and comparison of human amniotic fluid, simulated amniotic fluid, cell free amniotic fluid, amniotic fluid stem cells, and exosomes from amniotic fluid and cell secretome. Granulocyte colony-stimulating factor (G-CSF), Erythropoeitin (EPO). Created with BioRender.com.

1.4.1 Postnatal Enteral Amniotic Fluid Administration

Multiple researchers have suggested the administration of amniotic fluid for the prevention of NEC because it is the enteral diet when in utero and contains similar components to breast milk which has been shown to reduce NEC incidence and severity. However, the administration of amniotic fluid to premature infants is a technical hurdle not many have overcome due to difficulties in collection, preparation, time of administration, and optimal dosing. Certain researchers have employed entire amniotic fluid for experimentation. For instance, in a preterm pig

model, Siggers et al. administered porcine amniotic fluid as parenteral nutrition before enteral feedings. This approach led to notable outcomes, including increased weight, decreased intestinal bacterial populations, decreased inflammatory gene expression, and lower NEC scores [46]. It's worth noting that there was no observed effect when amniotic fluid was administered solely during enteral feedings [46]. Ostergaard et al. showed in a preterm pig model that including amniotic fluid in enteral feeds could reduce intestinal inflammation and increase body weight, but could not protect against NEC development or severity [47]. Good et al. showed that daily enteral amniotic fluid administration to C57BL/6 mice decreased NEC severity and in vitro experiments with intestinal epithelial cells showed amniotic fluid's ability to inhibit TLR4 signaling through epidermal growth factor (EGF) via peroxisome proliferator-activated receptors and EGF receptors [48]. Jain et al. showed reduced NEC frequency and severity when 30% late-gestation rat amniotic fluid was supplemented into formula feedings, claiming that cytokine, hepatocyte growth factor, as partly responsible for the protective effect [49].

1.4.2 Administration of Cell-Free Amniotic Fluid and Simulated Amniotic Fluid

Simulated amniotic fluid is designed to replicate the composition of natural amniotic fluid. Typically, it is a sterile isotonic solution that mirrors the electrolyte composition of human amniotic fluid. Additionally, it contains extra bioactive factors, such as granulocyte-colony stimulating factor (G-CSF) and erythropoietin (EPO), both of which can be synthetically produced through recombinant methods. These factors, naturally present in amniotic fluid, colostrum, and human milk, play a role in promoting the growth and development of gut villi by binding to their

intestinal receptors [50]. Soltani et al. demonstrated that enteral administration of G-CSF in very low birth weight infants (<1,500 g) increased feeding tolerance and significantly reduced the incidence of NEC [50]. Wang et al. demonstrated that administering repeated low doses of recombinant human EPO to preterm infants (<32 weeks gestation) significantly reduced the incidence of NEC. However, the long-term outcomes of NEC patients following this treatment are yet to be determined [51]. Other studies looking at the soluble components of amniotic fluid include Jain et al. who showed that amniotic fluid cytokine, hepatocyte growth factor, increased cell migration, proliferation, and NEC survival in IEC-6 cells [49].

Simulated amniotic fluid may also encompass the cell secretome derived from amniotic fluid or amnion cells. O'Connell et al. demonstrated that intraperitoneal injection of conditioned media from amniotic fluid stem cells in C57BL/6 mice resulted in restored intestinal regeneration and recovery, as evidenced by reduced intestinal damage, decreased mucosal inflammation, diminished neutrophil infiltration, decreased epithelial apoptosis, and improved intestinal angiogenesis [52]. Proteomic analysis revealed that proteins in the cell secretome play roles in immune regulation, cell cycle regulation, and stem cell regulation [52]. An alternative approach involves the use of synthetic amniotic fluid products, such as ST266, derived from the secretome of multipotent cells found in the amnion. This product has demonstrated efficacy in preventing and treating NEC in mice and piglets by exerting an anti-inflammatory effect and upregulating genes associated with enhanced gut remodeling, intestinal immunity, and gut

differentiation [53]. Significantly, the protective effects of ST266 were attributed to TLR4 inhibition, operating independently of epidermal growth factor [53].

1.4.3 Administration of Amniotic Fluid Cells and Their Extracellular Vesicles

Zani et al. examined the insoluble elements of amniotic fluid, demonstrating that amniotic fluid stem cells integrated into the bowel wall. This integration resulted in improved NEC survival, decreased NEC incidence, reduced gut damage, and enhanced intestinal function in Sprague-Dawley rats [54]. The positive outcomes were attributed to the modulation of stromal cells expressing COX-2 in the lamina propria of the intestines [54]. Li et al. showed in a C57BL/6 mouse model that both the amniotic fluid stem cells and their extracellular vesicles attenuated NEC intestinal injury by activating the Wnt signaling pathway, restoring epithelial regeneration, and stimulating stem cells [55]. McCulloh et al. demonstrated that when multiple stem cell sources, including amniotic fluid-derived Mesenchymal stem cells (MSC) and amniotic fluid-derived neural stem cells, were intraperitoneally injected into Sprague-Dawley rats it reduced experimental NEC incidence and severity [56]. McCulloh et al. subsequently demonstrated that extracellular vesicles from the same stem cell sources were equally effective in reducing NEC incidence and severity in Lewis rats when administered at a concentration of at least 4.0×10^{8} exosomes/50 μL [57]. O'Connell et al. demonstrated that intraperitoneal injection of extracellular vesicles derived from human amniotic fluid stem cells into C57BL/6 mice resulted in a reduction in NEC incidence, alleviated intestinal injury, mitigated inflammation, enhanced stem cell expression, and promoted cellular proliferation [52]. Li et al.

demonstrated that the administration of amniotic fluid stem cells prevented epithelial permeability and tight junction disruption in intestinal organoids. Furthermore, the study showed that amniotic fluid stem cells had the ability to restore tight junction activity and rescue intestinal permeability in C57BL/6 pups with NEC [58]. These protective effects on tight junction activity and permeability restoration were attributed to the endoplasmic reticulum stress response [58]. In a subsequent study, Li et al. demonstrated that pretreating C57BL/6 mice with intraperitoneal injections of amniotic fluid stem cells and MSCs before NEC induction resulted in the unique capability of amniotic fluid stem cells to increase intestinal growth and decrease NEC-induced injury [59]. Proteomic analysis revealed that proteins secreted by amniotic fluid stem cells were involved in biological adhesion, cellular processes, development, growth, metabolism, and reproduction [59].

1.5 Bench to Bedside

1.5.1 Amniotic Fluid Stem Cell and Exosome Isolation

Although various formulations of amniotic fluid have been talked about for their therapeutic properties against NEC, the purpose of this work is to explore the therapeutic potential of third trimester amniotic fluid stem cells and exosomes. There are three main protocols for isolating amniotic fluid stem cells from human amniotic fluid. The first method is a one-stage method which has mostly been used with samples from second trimester amniocentesis samples. The samples are centrifuged and the cells are resuspended in complete media usually including Dulbecco's Modified Eagles Medium (DMEM) with Fetal Bovine Serum (FBS).

The cells are allowed to adhere to plastic culture plates or flasks overnight at 37°C under 5% CO_2. The complete culture media is changes after 3-5 days to remove non-adherent cells and primary cells reach confluence in 4-5 days [60]. The second approach was also created for second trimester amniocentesis samples involves a two-stage culture protocol developed by Tsai et al., where non-adherent amniotic fluid cells obtained from the primary culture (stage one) are then plated for the second stage [61]. This protocol was originally designed to not interrupt with the amniocentesis fetal karyotyping process and revealed that amniotic fluid stem cells with MSC characteristics were found in the non-adherent cells from the first culture. The third method is an immunoselection based on surface antigens using a cell sorter and fluorescent stains. Ditadi et al. was the first to show that the c-Kit (CD117+) population of second trimester amniotic fluid cells have hematopoietic potential [62]. While a consensus on the optimal method for third-trimester amniotic fluid cell isolation is lacking, similar one-stage, two-stage, and C-kit methods have been employed. Researchers have utilized different media formulations, including AmnioMAX-C100 basal with AmnioMAX-C100 supplement [63], Chang Medium C media [64], and minimum essential medium alpha media [64].

Various methods have been proposed for the isolation of extracellular vesicles, including the smaller exosomes, each with its specific advantages and limitations. Differential ultracentrifugation yields well but is susceptible to protein contamination. Density gradient ultracentrifugation achieves high extracellular vesicle purity but introduces contamination from the gradient solution.

Polyethylene glycol provides a rapid procedure without the need for specialized equipment but poses a high risk of protein contamination. Size exclusion chromatography ensures high purity with minimal protein contamination but involves the use of expensive reagents. Anion-exchange chromatography is effective for achieving low protein contamination but requires specific equipment [42].

For all amniotic products, such as amniotic fluid stem cells and extracellular vesicles, there is a strong need to define proper standardization of their isolation, purification, quantification, and characterization. So that in the future these products can be created following good manufacturing practices (GMP), conforming to the guidelines recommended by the regulating agencies.

1.5.2 FDA Regulations of the Commercialization of Amniotic Fluid

Before May 31, 2021, the commercialization of amniotic fluid fell under the regulation of 21 CFR 1271 and Section 361 of the Public Health Services (PHS) act, governing the manufacturing of human cells, tissues, and cellular and tissue-based products (HCT/Ps). This framework allowed amniotic fluid products to be marketed and utilized for the treatment of ailments based on "homologous use", indicating their intended performance of the same basic functions as the donor tissue in the recipient. The ambiguous language and limited understanding of amniotic fluid's role during pregnancy led to the marketing of these products for various applications, ranging from "barrier use" in wound care and orthopedics to "immunomodulatory and anti-inflammatory" applications for treating COVID-19 long haulers [65, 66]. Notably, these applications did not require supporting

evidence. In November 2017, the FDA expressed its intent to classify amniotic fluid as a drug under Section 351 of the PHS act because amniotic fluid is considered to be a secreted or extracted human product, and therefore it does not meet the definition of an HCT/P. Starting from June 1, 2021, the FDA mandated the removal of amniotic fluid injectables from the market unless the company held an approved Biological License Application (BLA) [67]. Some companies are now submitting Investigational New Drug (IND) applications to conduct clinical trials using amniotic fluid for specific indications [6].

Amniotic fluid stem cells and extracellular vesicles are regulated under Section 351 of PHS as most are allogeneic applications and require an IND application and clinical trials to prove safety and efficacy. Critical aspects, including donor and recipient safety, release criteria encompassing stem cell and extracellular characterization, hypothesized mechanism of action, and microbiological controls, will undergo thorough evaluation before clinical trials commence [68]. Notably, there are no standardized protocols for the isolation or storage of stem cells or extracellular vesicles, necessitating the development of tailor-made protocols. There are currently no approved amniotic fluid stem cell or amniotic fluid extracellular vesicle treatments available.

1.6 Challenges of Clinical Applications

Addressing challenges in the clinical application of amniotic fluid-based therapies for NEC involves navigating several ethical and practical questions. The collection of amniotic fluid poses ethical dilemmas, raising questions about the appropriate methods and timing for collection. Should it be exclusive to cesarean

section deliveries to prevent contamination from vaginal deliveries? Is there a preference for autologous or allogeneic use of amniotic fluid products, and what are the potential long-term effects of postnatal administration? Additionally, there is uncertainty about the necessity of sterilization and the possible detrimental effects of using amniotic fluid or fluid-derived products.

Cell-free amniotic fluid faces challenges due to limited supply during cesarean section and natural variations in its composition between donors, impacting the therapeutic potential. The mechanisms of cell-free amniotic fluid also require further understanding. Simulated amniotic fluid presents challenges as it may not fully represent all essential molecules found in amniotic fluid. Processed amniotic fluid, which filters out insoluble components, may lose the beneficial effects of cells and cellular vesicles.

Amniotic fluid cell-based therapy encounters multifaceted challenges related to manufacturing practices, including cryopreservation, storage, and distribution. Current cryopreservation methods involve toxic substances, making direct human application problematic. Understanding the impact of these techniques on target cells and tissues is crucial. Recent research suggests that amniotic fluid stem cells from third-trimester collections may be less therapeutic than those from the second trimester, adding complexity to the collection process [16]. Moreover, the existing FDA gap for stem cell therapies, attributed to the risk of tumors, prompts exploration of alternative solutions for amniotic fluid-based therapies.

1.7 Summary

Amniotic fluid provides vital biological and physical support for the developing fetus. Babies born prematurely, before reaching 37 weeks of gestation, face an elevated risk of health issues, including NEC. NEC is the leading cause of death from gastrointestinal disease in premature infants. Patients who develop NEC have a very high mortality rate sometimes within 48 hours of diagnosis, illustrating the importance of a preventative treatment [69]. NEC in part arises from exaggerated signaling via the bacterial receptor toll like receptor 4 (TLR4) on the intestinal epithelium, leading to widespread intestinal inflammation and intestinal ischemia. Strategies that limit the extent of TLR4 signaling, reduce inflammation, reduce apoptosis, and increase proliferation have been investigated. These include amniotic fluid and its soluble and insoluble components. A number of studies using whole amniotic fluid, stimulated amniotic fluid, cell-free amniotic fluid, amniotic fluid stem cells, and amniotic fluid stem cell extracellular vesicles were discussed. The findings, of reducing NEC incidence, collectively show the potential of amniotic fluid in the prevention and treatment of NEC.

Standardization of isolation, purification, quantification, and characterization processes for amniotic products, including stem cells and extracellular vesicles, is crucial for further research and translational products. Currently, there are three main protocols for isolating amniotic fluid stem cells originally catered to second trimester samples but that have now been adapted for third trimester samples. For isolating extracellular vesicles there are also various techniques, such as ultracentrifugation, density gradient ultracentrifugation,

ultrafiltration, polyethylene glycol, size exclusion chromatography, and anion-exchange chromatography each with distinct advantages and limitations.

Additionally, with the FDA's regulatory changes post-May 31, 2021, amniotic fluid products will require a Biological License Applications (BLA), meaning an increased burden of proof that the products are made in a repeatable way and that they are safe and effective. Challenges in clinical applications of amniotic fluid-based therapies for NEC involve collection and processing decisions, stability and storage, and defining the mechanism of action for the therapeutic effects seen. Yet, even a minor decrease in NEC rates could be the difference between an infant facing a life-altering, potentially life-threatening illness and an infant thriving at home with their family. Therefore, it is vital for the research community to work together and share the most promising ideas for prevention and therapy.

1.8 Significance

This study emphasizes the need for a better understanding of amniotic fluid; a complex biological fluid with applications in fetal diagnostics and regenerative medicine. Despite its potential, aspects such as its mechanism of action, complete biological composition, and changes over time, remain unclear. While the majority of studies have concentrated on amniotic fluid from the second trimester, the recent ability to collect it during the third trimester introduces new prospects for potential therapies. This research holds importance as it specifically aims to characterize the components of amniotic fluid from the third trimester, exploring their therapeutic potential.

Necrotizing enterocolitis, a significant cause of mortality linked to the immaturity of the GI tract and immune system, lacks preventive therapies or cures. While research has explored the therapeutic potential of human amniotic fluid MSCs and their extracellular vesicles, the role of amniotic fluid extracellular vesicles (AF-EVs) and their impact on GI development have not been fully investigated. This study aims to contribute to developmental biology by examining intestinal epithelium responses to third-trimester amniotic fluid exosome exposure, providing insights for potential regenerative medicine applications, particularly in reducing NEC risk in preterm infants.

1.9 Statement of Hypothesis

Our central hypothesis is that amniotic fluid, a growth medium in utero, contains cellular and non-cellular components that could aid in GI development and can be processed as therapeutic products for reducing risk of NEC in the NICU.

1.10 Specific Aims

1.10.1 Aim 1

Isolate and characterize third trimester human amniotic fluid (T3 hAF) cells to determine if they have properties of Mesenchymal Stem Cells (MSC) according to requirements of the International Society for Cellular Therapy (ISCT). We hypothesize that we can isolate the live cells from T3 hAF samples and further select stem cells through culturing techniques. We further hypothesize that the cultured stem cells are MSCs based on literature characterizing cells in second trimester amniotic fluid (T2 AF) samples as MSC. MSC characterization will be accomplished by studying proliferation, colony forming ability, cell surface protein

markers, and MSC differentiation potential. T2 AF-derived MSCs have been shown to decrease incidence and severity of NEC in animal models but their restricted availability limits their translational potential and therefore more accessible T3 hAF needs to be evaluated [57, 70].

1.10.2 Aim 2

Isolate third trimester human amniotic fluid extracellular vesicles (T3 hAF-EVs) and characterize them as exosomes (T3 hAF-EXOs) according to requirements of the International Society for Extracellular Vesicles (ISEV). We hypothesize that we can isolate extracellular vesicles from T3 hAF samples and further select exosomes through purification protocols. We further hypothesize that the T3 hAF-EXOs will meet the ISEV definition of "exosome". This will be accomplished by evaluating their morphology, size profiles, and membrane proteins. Cell-derived biologic therapies such as exosomes can complete paracrine signaling of cells without cellular logistical issues, but the optimal sources of exosomes are still under investigation. T3 hAF-EVs have shown potential in preventing pulmonary disease, but their potential as a therapy for NEC has not been evaluated [71].

1.10.3 Aim 3

Investigate T3 hAF-MSCs or T3 hAF-EXOs therapeutic effect in an in vitro model of NEC. We hypothesize that exposure of T3 hAF-MSCs or T3 hAF-EXOs to intestinal epithelial cells (IEC) will prevent apoptosis and tight junction injury caused by pathogen-associated surface molecules. We will accomplish this by evaluating IECs in an in vitro model of NEC. Specifically, proliferation and tight

junction function and quality through gene regulation and transepithelial electrical resistance (TEER). NEC is characterized by intestinal tissue necrosis and bacterial translocation leading to systemic sepsis, if T3 hAF-MSCs or T3 hAF-EXOs exposure to IEC prevents cell death or maintains tight junctions integrity it would warrant further therapeutic development.

2. CHARACTERIZATION OF AMNIOTIC FLUID CELLS

2.1 Study Design

Amniotic fluid, a nutrient-rich medium crucial for fetal development, contains essential components such as nutrients, growth factors, and cells [1]. The viability of amniotic fluid from the second trimester as a stem cell source for regenerative medicine has been explored since 2003 [72]. However, the decline in second trimester amniocentesis procedures, attributed to advancements in blood tests and the associated 2% risk of miscarriage, has limited the availability of this source [73]. In response to this challenge, researchers have turned their attention to evaluating the regenerative potential of third trimester amniotic fluid, first recognized for its stem cell content in 2004 [74]. The collection of amniotic fluid during third trimester elective Cesarean sections is advantageous as it is readily available and considered a byproduct of delivery minimizing ethical concerns. Through established culture protocols, this fluid can be processed to isolate plastic adherent cells, providing a valuable resource for regenerative medicine.

The purpose of this study is to isolate and characterize third trimester human amniotic fluid cells to determine if they have properties of Mesenchymal Stem Cells (MSC) according to requirements of the International Society for Cellular Therapy (ISCT) [75]. At a minimum this includes being plastic adherent, expressing CD105, CD73 and CD90 surface markers while lacking expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR, and differentiating into osteoblasts, adipocytes or chondroblasts in vitro [75]. In

addition, we tested cell proliferation over 21 days and colony forming unit (CFU) ability.

2.2 Materials and Methods

2.2.1 Amniotic Fluid Collection

Third trimester human amniotic fluid samples were donated by Tides Medical after collection by Louisiana Organization for Transplant (LOFT). LOFT completed all donation paperwork including informed consent with mothers and followed all state regulations and guidelines of the American Association of Tissue Banks. Third trimester samples were collected from elected Cesarean sections, as opposed to post labor collections, to limit influence from labor induced changes in the inflammatory environment [76].

Fluid samples were collected between gestational age 32-39 weeks of the third trimester. Physicians used a suction catheter and hose to evacuate amniotic fluid from the Cesarean incision into a collection vessel before delivering the baby. After collection, the de-identified fluid samples were shipped on ice overnight to the Tulane Center for Stem Cell Research and Regenerative Medicine. Once received, the fluid samples were stored at 4°C and processed for cell isolation within 48 hours.

Since this research used secondary de-identified samples only the Tulane University Human Research Protection Office determined that the activities were not “human subjects research” as defined by the Common Federal Rule and as such did not require an Institutional Review Board review and approval.

2.2.2 Cell Isolation and Cell Culture

A two-stage protocol from Tsai et. al. was utilized for amniotic fluid stem cell isolation, seen in Figure 6 [61]. The amniotic fluid samples were first centrifuged at 300xg for 5 minutes to pellet the suspended cells, the supernatant was stored in –80°C for future studies. The cells were suspended in 1X phosphate-buffered saline (PBS) and filtered through 70-100 μm cell strainer (Corning, Corning, NY, cat # CLS431751) to remove vernix and hair. If the samples had blood contamination, an additional red blood cell (RBC) lyse buffer step was added. This step included incubating the cell pellet in RBC lyse buffer (Thermo Fisher Scientific, Waltham, MA, cat # 00-4333-57) for 10 minutes, washing with 1X PBS, then centrifuging at 300xg for 5 minutes to collect the cell pellet again. The cell pellet was then resuspended in complete growth media made from Dulbecco's Modified Eagles Medium (DMEM) F12 (Hyclone, Logan, UT, cat # SH30272.01), 20% fetal bovine serum (FBS, Hyclone, Logan, UT, cat # SH30070.03IH25-40), and 1% antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA, cat # 15240062). Cells were plated in T175 flasks in complete growth media and incubated at 37°C with 5% CO_2.

In the first stage, the amniotic fluid cell pellet was seeded into a T175 flask, as previously described, and incubated for 5 days. At day 5, the supernatant of the flask was obtained, centrifuged at 300xg for 5 minutes, and the cell pellet was seeded into a second flask (stage 2). The first flask was then discarded. The second flask was kept in culture for 20 days, and at day 20 the complete growth media was exchanged. When the cell colonies reached confluency, they were passaged into a

new flask. To passage the cells, the adherent cells were lifted by adding 0.25% trypsin/1 mM EDTA (Thermo Fisher Scientific, Waltham, MA, cat # 25200056) to the flask then incubated for 5-8 minutes. Cells were then seeded into new flasks at $2x10^3/cm^2$ and maintained in complete growth media. All experiments were completed using passage 3 to passage 5 cells.

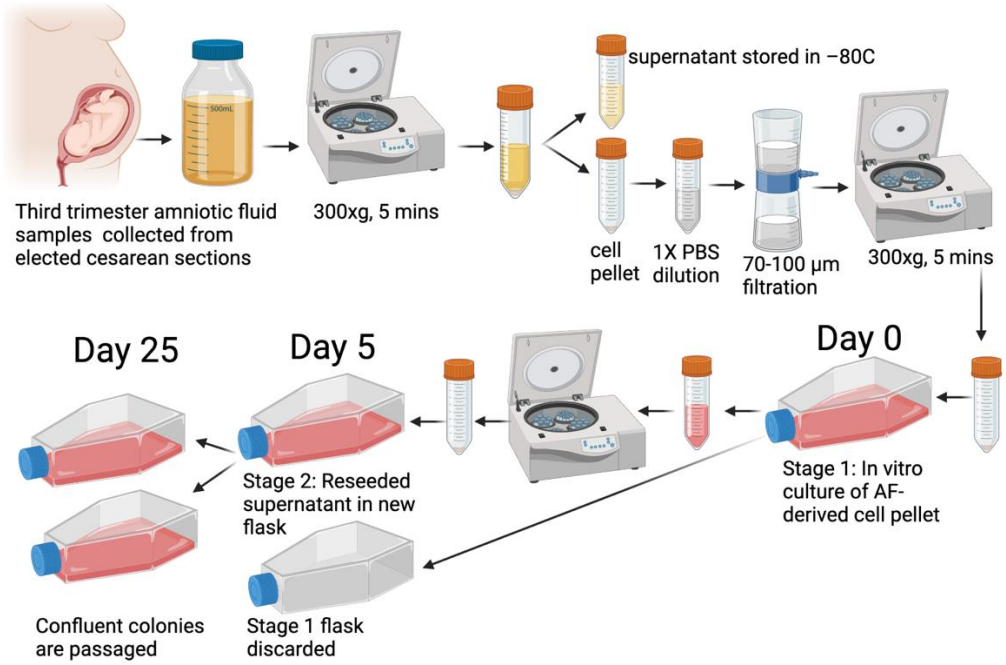

Figure 6: Amniotic fluid collection and cell isolation.

Amniotic fluid collection, cell isolation, and two-stage culture protocol. Created with BioRender.com.

2.2.3 Alamar Blue Cell Proliferation Assay

A proliferation assay was adapted from Al-Ghadban et al. to determine the rate of amniotic fluid cell growth [77]. Cells were seeded in 96-well plates in two concentrations of 3×10^3 cells/cm^2 and 15.6×10^3 cells/cm^2 with complete growth media made from Dulbecco's Modified Eagles Medium (DMEM) F12 (Hyclone, Logan, UT, cat # SH30272.01), 20% fetal bovine serum (FBS, Hyclone, Logan,

UT, cat # SH30070.03IH25-40), and 1% antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA, cat # 15240062). The experimental setups were performed in triplicate and maintained under controlled conditions at 37°C with 5% CO_2, with regular bi-weekly media replacement. Quantitative assessment of cell proliferation was conducted using the Alamar Blue reagent (Thermo Fisher Scientific, Waltham, MA, cat # DAL1025). Alamar Blue operates on an oxidation-reduction mechanism, manifesting changes in color and fluorescence in direct proportion to the quantity of viable cells engaged in metabolic processes, thus enabling precise measurement of cell growth dynamics [78]. Cell proliferation was measured on days 1, 7,14, and 21. The cells were incubated for 2 hours with 20uL of Alamar Blue at 37°C with 5% CO_2. After 2 hours, the fluorescence intensity was measured using excitation and emission at 570nm with a Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Winooski, VT, cat # BTS1A).

2.2.4 Colony-Forming Unit Assay

A colony forming unit (CFU) assay was adapted from Al-Ghadban et al. to determine the self-renewal properties of amniotic fluid cells [77]. Amniotic fluid cells were seeded at a density of 0.5×10^2 cells/cm^2 in a 100cm petri dish and were cultured for 14 days. The experiment was seeded in triplicates. The cells were cultured in complete growth media made from Dulbecco's Modified Eagles Medium (DMEM) F12 (Hyclone, Logan, UT, cat # SH30272.01), 20% fetal bovine serum (FBS, Hyclone, Logan, UT, cat # SH30070.03IH25-40), and 1% antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA, cat # 15240062) for 14 days, without media exchange. After 14 days, the wells were washed with

1X PBS and then stained with 3% (w/v) crystal violet (Sigma, St. Louis, MO, cat # 548-62-9) for 30 min at room temperature. The stained dishes were washed with deionized water and dried overnight. The number of colonies was manually quantified, with only CFUs greater than 2 mm^2 in diameter being recorded in the CFU total.

2.2.5 Imaging

All cells were imaged using a Nikon Eclipse TE200 with Nikon Digital Camera DXM1200F and Nikon ACT-1 software version 2.7 (Nikon, Melville, NY) at magnification of 4X, 10X, or 20X.

2.2.6 Flow Cytometry

Flow cytometry was employed using a protocol adapted from Al-Ghadban et al. for the phenotypic analysis of amniotic fluid cells [77]. Amniotic fluid cells were washed in 1X PBS before being blocked with 1% Bovine Serum Albumin (BSA, Thermo Fisher, Waltham, MA, cat # B14) and stained with the following antibodies at room temperature for 15 min: CD2 (Thermo Fisher, Waltham, MA, cat # 13-0029-82), CD3 (BD Biosciences, San Jose, CA, cat # 562406), CD4 (Invitrogen, Waltham, MA, cat # 11-0041-82), CD5 (Invitrogen, Waltham, MA, cat # 45-0051-82), CD8 (Invitrogen, Waltham, MA cat # 12-0088-42), CD14 (Beckman-Coulter, Brea, CA, cat # IM2640U), CD19 (Invitrogen, Waltham, MA cat # 12-0199-42), CD29 (Invitrogen, Waltham, MA cat #14-0299-82), CD31 (BD Biosciences, San Jose, CA, cat # 563651), CD36 (Invitrogen, Waltham, MA cat # PA1-16813), CD44 (Invitrogen, Waltham, MA cat # 12-0441-82), CD45 (Beckman-Coulter Brea, CA, cat # A71117), CD73 (BD Biosciences, San Jose,

CA, cat # 550257), CD90 (Invitrogen, Waltham, MA, cat # 11-0909-42), CD105 (Invitrogen, Waltham, MA, cat # 17-1057-42), CD117 (Invitrogen, Waltham, MA, cat # 12-1172-82), CD146 (Invitrogen, Waltham, MA, cat # 35-7800), and MAC-1 (Invitrogen, Waltham, MA, cat # 3684-MSM10-P1). The cells were then fixed with 1% paraformaldehyde (PFA, Thermo Scientific, Waltham, MA, cat # J19943.K2) and a total of 10,000 events were captured and analyzed with a Gallios Flow Cytometer using Kaluza software (Beckman Coulter, Brea, CA).

2.2.7 Differentiation of Isolated Amniotic Fluid Cells into Osteocytes and Adipocytes

Cell populations were differentiated along adipocyte and osteocyte lineages using a protocol adapted from Al-Ghadban et al. [77]. Briefly, cells were seeded in 12-well plates at 1.25×10^4 cells/cm^2 in complete growth media made from Dulbecco's Modified Eagles Medium (DMEM) F12 (Hyclone, Logan, UT, cat # SH30272.01), 20% fetal bovine serum (FBS, Hyclone, Logan, UT, cat # SH30070.03IH25-40), and 1% antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA, cat # 15240062). The experiment was seeded in duplicate. The cells were allowed to reach 100% confluency in complete growth media before being replaced with differentiation media. The osteogenic differentiation media was made using complete culture media with 10 nM dexamethasone (Sigma, St. Louis, MO, cat # D4902), 20 mM β-glycerophosphate (STEMCELL Technologies, Cambridge, MA), and 50 μM L-ascorbic acid-2-phosphate (Sigma, St. Louis, MO, cat # A8960). Commercially available adipogenic media, AdipoQual (AQ), was purchased from Obatala Sciences (Obatala Sciences Inc, New Orleans, LA).

Differentiation media was replaced every 3-4 days for 28 days. Images were acquired every seven days, starting at day 1, at 20X magnification on a Nikon Eclipse TE200 microscope equipped with Nikon Digital Camera DXM1200F and Nikon ACT-1 software version 2.7 (Nikon, Melville, NY).

2.2.8 Data and Statistical Analysis

Data were analyzed using Microsoft Excel and PRISM 9 software (GraphPad, Inc., Boston, MA). Data are presented as mean ± standard deviation from the mean and all experiments were performed in triplicate. Statistical analysis was conducted using either a one-way analysis of variance (ANOVA) for a single independent variable or a two-way ANOVA for two independent variables to identify statistical differences. Asterisks indicates statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

2.3 Results

2.3.1 Amniotic Fluid Collection, Isolation, and Culture

The average age of the donors was 28 ± 4.87 years with an average body weight of 96.9 ± 26.1 kg, data available in the appendix. A total of 51 amniotic fluid samples were collected averaging 222 ± 113 mL volume per sample, data available in the appendix. The appearance of the fluid varied from very clean collections resulting in a pale-yellow straw color to shades of light pink to dark red depending on the amount of blood contamination. Severe blood contamination resulted in the sample being discarded. The amount of blood contamination was controlled by the physician's collection process. Some physicians had techniques for cleaner collections but did not disclose their protocol. Small amounts of blood

contamination could be removed with RBC lysis buffer, seen in Figure 7, but the effects of the contamination and lysis treatment were not investigated in this study. The examination of cells from the amniotic fluid cell pellet, stained with Trypan blue and depicted in Figure 8, showed approximately 90% of the cells were non-viable.

The two-stage isolation protocol, as illustrated in Figure 6, allowed more time for the delayed adherent cells—those that didn't attach to the plastic culture flask within the first five days—to become plastic-adherent after being extracted from the stage one supernatant. The initial stage one flask displayed minimal cell colony growth before being discarded. In contrast, the stage two flask, seeded on day 5, exhibited cell adherence and diverse mature colony formation after 15 days.

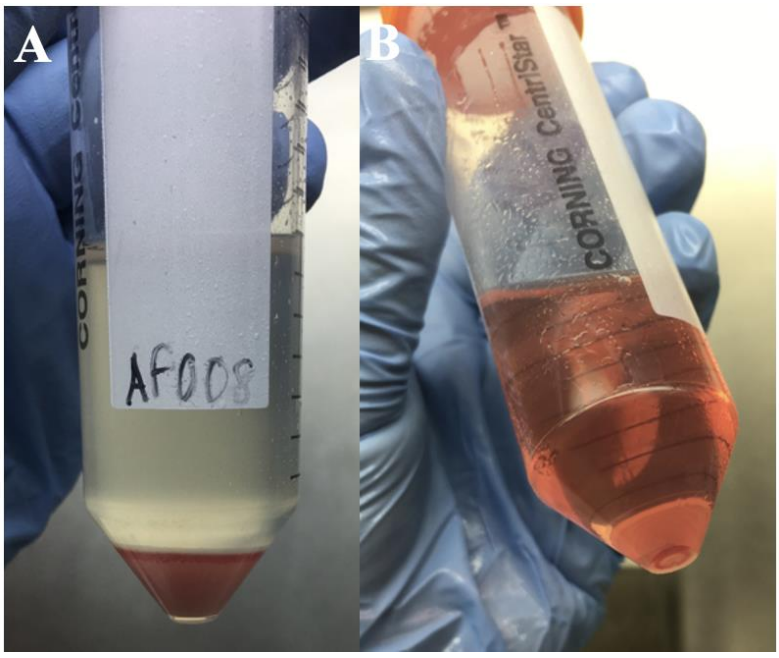

Figure 7: Removing RBC from cell culture pellet.

(A) Pelleted cells with RBC results in a reddish pellet color (B) Pelleted cells after RBC lysis buffer results in a white pellet color.

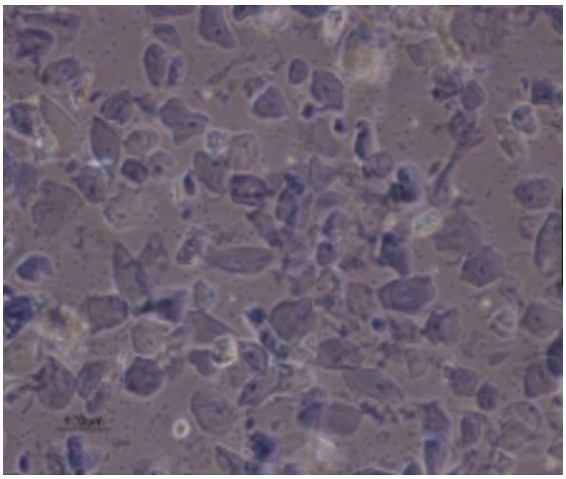

Figure 8: Amniotic fluid cell pellet.

Cells from the amniotic fluid cell pellet stained with Trypan blue. Blue cells are dead while white cells are viable.

2.3.2 Morphology

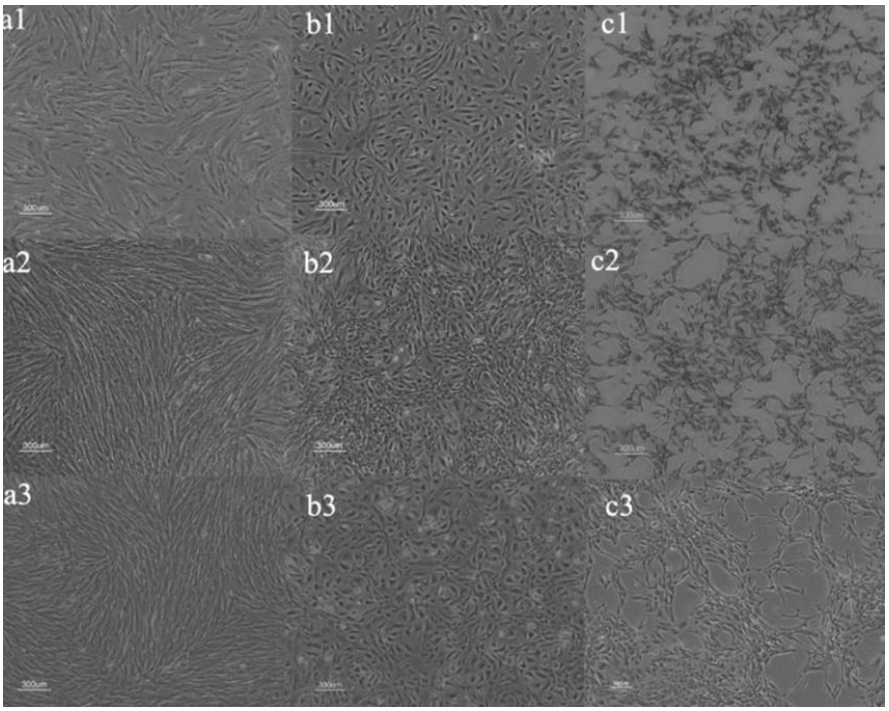

Figure 9: Cell Morphology.

Representation third trimester expanded cell culture major morphologies; (A1, A2, A3) fibroblast-type cells, (B1, B2, B3) round epithelial-type cells, (C1, C2, C3) spindle-polygonal-type cells. Scale bar is 300 nm.

Figure 9 presents the three major cell morphologies identified in amniotic fluid cell culture: fibroblast, round epithelial, and spindle-polygonal. This shows that fibroblast-type cells have the traditional fibroblast shape and grew into large confluent colonies. Epithelial-type cells were round with more cytoplasm per cell then other identifications. Spindle-polygonial-type cells had a spindle polygonal shape and grew confluent with lots of space between cells. Figure 10 presents a multiple passaging experiment with four sample amniotic fluid cell lines (AF011, AF012, AF013, AF014). This shows cell morphologies were maintained over eight passages.

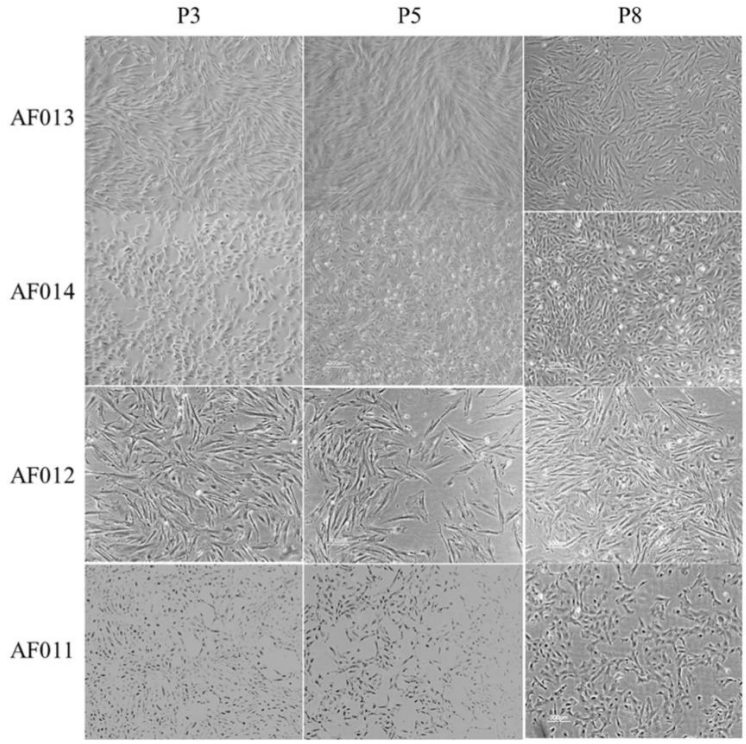

Figure 10: Morphological changes of amniotic fluid cells.

Morphological changes of amniotic fluid cells from passage 3 to passage 8. Displays morphologies from four amniotic fluid cell lines at p3, p5, and p8. There are morphology differences between cells lines, but morphologies remained consistent as a cell line increased in passaging. Images were taken at 4X objective, scale bar is equal to 300µm.

2.3.3 Flow Cytometry

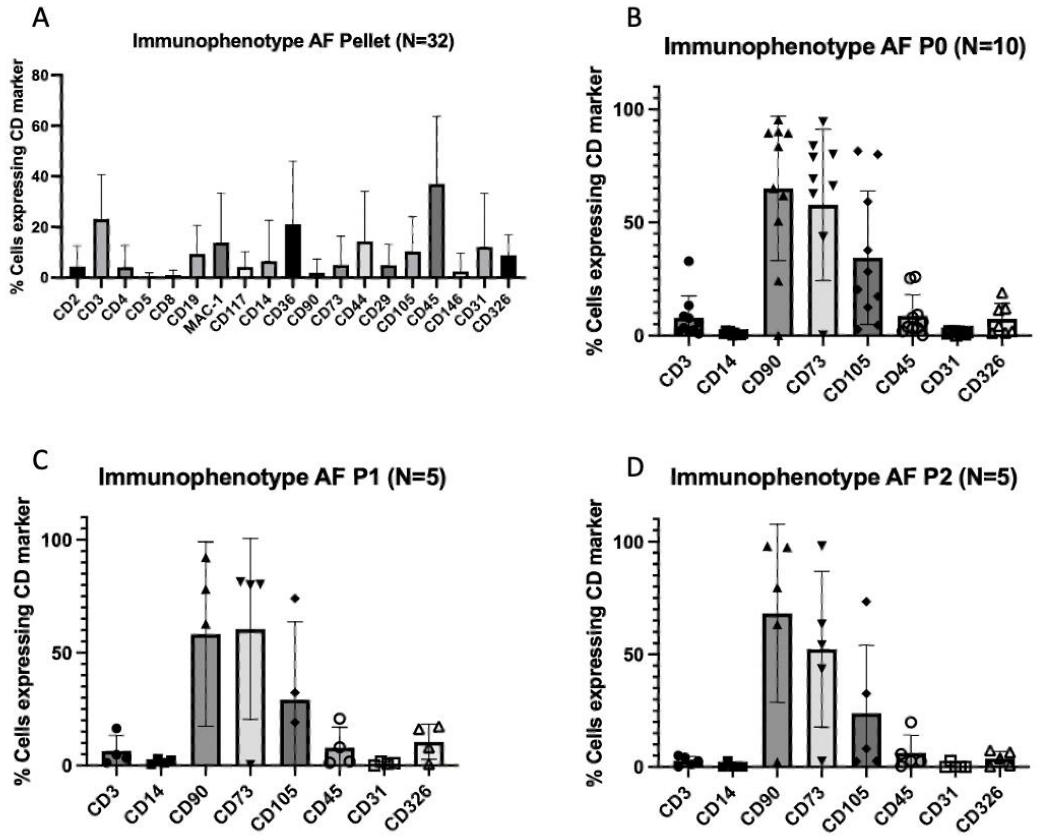

Figure 11: Flow cytometry.

Flow cytometry analysis of cells from amniotic fluid and passaged cells (A) Immunophenotype of amniotic fluid pellet N=32 (B) Immunophenotype of amniotic fluid passage 0 N=10 (C) Immunophenotype of amniotic fluid passage 1 N=5 (D) Immunophenotype of amniotic fluid passage 2 N=5.

Figure 11 illustrates the percentage of amniotic fluid cells expressing cell markers detailed in Table 1. In Figure 11A, flow cytometry of the initial amniotic fluid cell pellet reveals a diverse cell population with notable variability among samples. The primary components in the original pellet include immune cells marked with CD45 (36.9% ± 26.5%), CD3 (23.05% ± 17.52%), and CD36 (21.02% ± 27.06%).

Figures 11B, C, D depict the flow cytometry results of plastic adherent cells across passages 0 to 2. The results indicate a decline in the immune cell population from the initial amniotic fluid cell pellet to subsequent passages, as evidenced by reduced CD45, CD3, and CD36 positive populations. Additionally, there is an increase in the cell population expressing stem cell markers (CD73, CD90, CD105) from the initial pellet to sequential passages 0-2. These stem cell markers remain detectable up to at least P8, data not shown. However, the average CD105 cell population decreases from P0 (34.45% ± 29.48%) to P1 (14.24% ± 20.95%) to P2 (4.46% ± 3.14%). Lastly, an amniotic fluid epithelial cell population is identified in both the original cell pellet and passaged cell lines, with a small population of cells expressing the epithelial marker CD326. There was an observed decrease in the epithelial cell population over time in culture. This reduction averaged from 14% in passage 1 to just 7% in passage 2.

Table 1: Antibodies used for flow cytometry

CD Marker	Cell Type
CD2	B Cell, T Cell
CD3	T Cell
CD4	T Cell
CD5	T Cell
CD8	T Cell
CD19	B Cell
CD14	Macrophage

CD36	Macrophage
CD117	Macrophage
MAC-1	Macrophage
CD29	Stem Cell Precursor
CD44	Stem Cell Precursor
CD73	Stem Cell Precursor
CD90	Stem Cell Precursor
CD105	Stem Cell Precursor
CD45	Leukocyte
CD146	Endothelial
CD31	Endothelial

2.3.4 Proliferation Assay

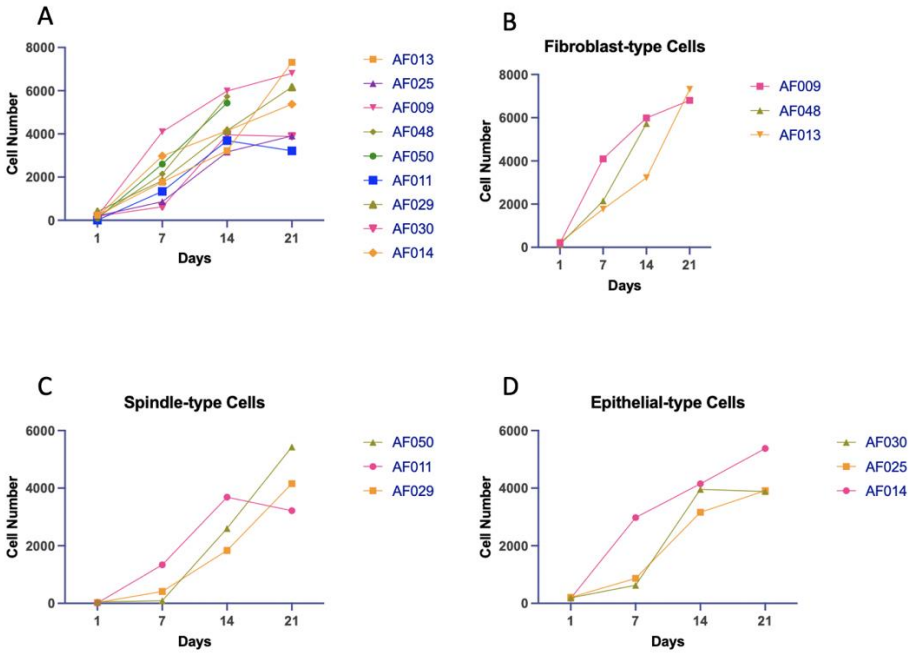

Figure 12: Proliferation.

Proliferation analysis of cells from amniotic fluid passaged cells, passage 3-5, over 21 days. (A) Combined proliferation of samples will all cell types N=9 (B) proliferation of samples with fibroblast-type cells N=3 (C) proliferation of samples with spindle-type cells N=3 (D) proliferation of samples with epithelial-type cells N=3.

Figure 12 illustrates the proliferation of cells from amniotic fluid observed over a span of 21 days. The results indicate that amniotic fluid cell lines exhibited either linear or exponential growth during the initial fourteen days of the proliferation experiments. Beyond this period, the cell lines demonstrated either sustained growth or entered a plateau phase. Figures 12B-D provide insights when the cell lines are categorized into their predominant cell types—fibroblast-type, spindle-type, and epithelial-type—while measuring proliferation over 21 days. Notably, the fibroblast-type cell lines exhibited greater cell growth during the 21-day observation period compared to spindle-type or epithelial-type cell lines.

Additionally, the proliferation of epithelial-type cell lines tended to plateau at 14 days.

2.3.5 Colony Forming Unit Assay

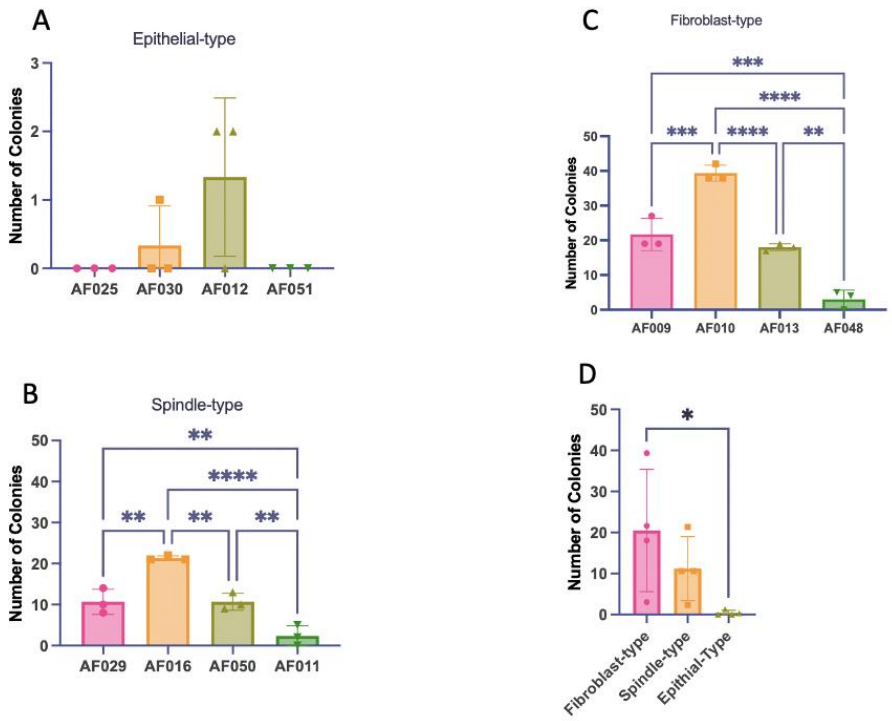

Figure 13: Colony forming unit.

Colony forming unit analysis of cells from amniotic fluid passaged cells, passage 3-5, seeded at 100 cells per plate and grown for 14 days before colony analysis (A) CFU of epithelial-type cells N=4 (B) CFU of fibroblast-type cells N=4 (C) CFU of spindle-type cells N=4 (D) CFU comparison between cell types, N=12.

Figure 13 displays amniotic fluid cell samples and the corresponding colony-forming units (CFU) when seeded at a concentration of 100 cells per plate and incubated for two weeks. The results reveal that amniotic fluid cell samples successfully formed colonies after 14 days at the specified cell concentration per petri dish. Figure 13D indicates that fibroblast-type samples exhibited increased CFU compared to spindle-type and significantly higher CFU compared to

epithelial-type samples. Figure 13A highlights that epithelial-type samples, specifically AF025 and AF051, did not produce colonies large enough to be counted as CFU within the two-week timeframe. Figure 13B demonstrates that fibroblast-type samples showed the highest colony growth, with significant variability between samples. Figure 13C indicates that spindle-type samples grew fewer colonies than fibroblast-type samples and exhibited high variability among samples.

2.3.6 Differentiation of Isolated Amniotic Fluid Cells into Osteocytes and Adipocytes

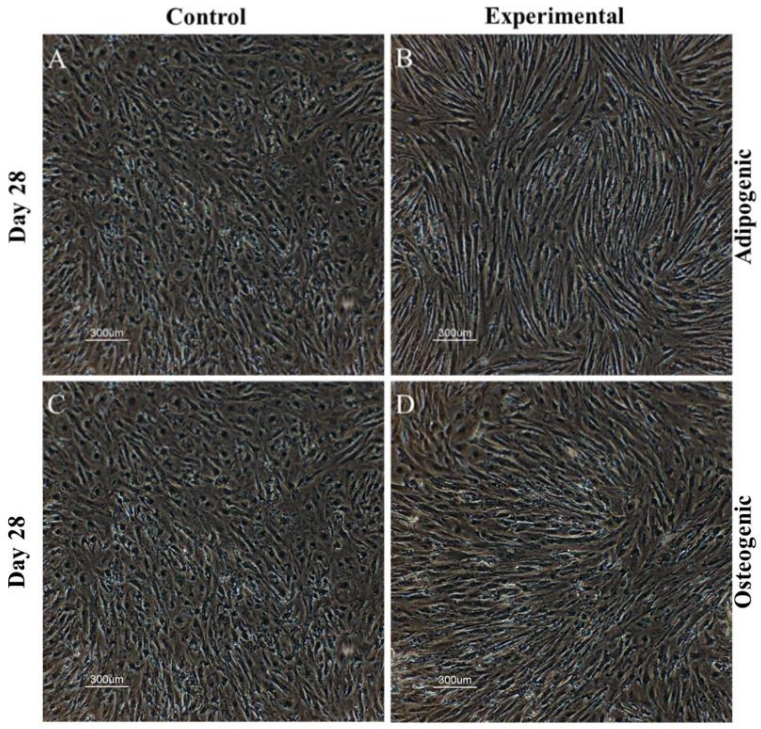

Figure 14: Differentiation.

Representative images of amniotic fluid cells at day 28 of adipogenic and osteogenic differentiation. (A) Control cells. (B) Cells induced with AdipoQual adipogenic differentiation media showing pre-adipocyte formation. (C) Control cells. (D) Cells induced with osteogenic differentiation media showing elongation of the cells.

Figure 14 illustrates the adipogenic and osteogenic differentiation potential of amniotic fluid cells in their respective differentiation media over a 28-day period. Figure 14B presents the morphological changes of amniotic fluid cells into pre-adipocytes observed by day 14. However, these cells remained in the pre-adipocyte stage throughout the 28-day period without fully maturing into adipocytes with lipid globules. Figure 14D demonstrates that amniotic fluid cells transformed into pre-osteogenic differentiated cells, evidenced by the change in cell orientation. However, these cells did not mature into mature osteocyte cells with the ability to produce calcium deposits.

2.4Discussion

Third-trimester amniotic fluid samples were successfully collected and processed for cellular analysis. An evaluation of the original amniotic fluid cell sample stained with Trypan blue indicated that approximately 90% of the cells were non-viable. This aligns with existing literature suggesting that the majority of nucleated cells in term amniotic fluid are non-viable squamous epithelial cells shed from the fetus [79, 80]. Next, we examined various cellular isolation methods (single-stage, two-stage, and c-kit immunoselection) previously employed for both second and third trimester amniotic fluid cells. Attempting a single-stage culture resulted in single-cell attachment, typically failing to form colonies or proliferate, leading to the loss of cell lines AF001-AF006. Similar challenges with proliferation using the single-stage culture have been reported in the literature [72]. We also tried the c-kit immunoselection protocols from second and third trimester which should select 1-5% of CD117+ cells from the initial cellular fraction of whole amniotic

fluid, but when we did our cell sorting the populations were <1% of our total population and did not form colonies when expanded [64, 81, 82]. Using the two-stage isolation protocol we were able to show that amniotic fluid cells can be isolated, demonstrate plastic adherence, form colonies after 15-20 days of culture, and be expanded for multiple passages. The amniotic fluid cell's ability to be plastic adherent meets the first of the ISCT requirements for MSCs.

The origin of amniotic fluid cells is primarily unknown, but it is reasonable to assume that they are predominantly of fetal origin, encompassing various tissues such as skin, respiratory, intestinal, and urinary tract, as well as amniotic membranes or other extraembryonic connective tissues [4]. The overall volume of amniotic fluid increases up to a peak of approximately 800-1000 mL by week 28-34, with a cell count ranging between 1 cell/mL to $1x10^6$ cells/mL [45, 72, 83]. Third-trimester samples can exhibit heterogeneous morphologies in comparison to second-trimester samples [64]. While second trimester samples typically contain two morphologies, epithelial-type and fibroblast-type, the third trimester may have two to four reported cell types, including fibroblast-type, spindle-type, epithelial-type, and "amniotic fluid type" [72, 79, 83, 84]. Our cell isolation process resulted in three distinct cell morphologies: fibroblast-type, spindle-type, and epithelial-type.

Fibroblast cells exhibited a fusiform shape, forming tightly confluent colonies, while spindle cells, smaller in size, maintained spaces between cells in their confluent colonies. Epithelial cells, on the other hand, appeared round or polygonal in shape, with a larger cytoplasm-to-nucleus ratio compared to spindle

or fibroblast types. Notably, there was an observed decrease in the epithelial cell population over time in culture, both visually and through the flow cytometry marker CD326. This reduction averaged from 14% in passage 1 to just 7% in passage 2. Similar declines in epithelial cells have been documented in literature for both second and third trimester cell cultures [72, 83].

Through flow cytometry analysis, we examined the heterogeneous cell population in the initial amniotic fluid samples and subsequent passages, revealing donor variability even at similar collection time points. Notably, cell lines predominantly composed of epithelial-type cells tended to exhibit lower expression of the stem cell marker CD90 and were positive for the epithelial marker CD326. In contrast, cell lines primarily composed of fibroblast-type cells showed high expression of the stem cell marker CD90 and were negative for the epithelial marker CD326. This higher expression of the stem cell marker CD90 in fibroblast cells aligns with observations in both second and third trimester isolated fibroblast-type cells [84]. While the initial pellet had higher populations of immune cells, subsequent passages were positive (5-80%) for stem cell markers CD90, CD73, and CD105 and negative (<5%) for the immune cell marker CD14, meeting the second of the ISCT requirements for MSCs.

In the CFU assay, fibroblast-type and spindle-type cells demonstrated extensive self-renewal, a characteristic property of stem cells [82]. On the other hand, epithelial-type cells exhibited the ability to self-renew and form colonies, but these colonies rarely exceeded $2mm^2$ in the 14-day duration of the experiment. While there isn't a universal rule dictating the number of colonies each cell type

should generate per 100 seeded cells, the statistically higher number of colonies formed by fibroblast-type cells compared to epithelial-type cells is noteworthy. This difference may also contribute to the observed decrease in epithelial cell populations in passaged cell lines, as the flasks became predominantly composed of fibroblast-type and spindle-type cells when reaching confluency. The CFU assay serves as an in vitro quantitative method to assess a single cell's capacity to grow into a larger colony through clonal expansion, also shedding light on the tumorigenic potential of stem cells. Alongside immune response considerations, the issue of tumorigenicity has impeded the widespread adoption of stem cell therapies. Multiple case reports have documented tumors in patients following clinical trials, including instances involving human amniotic membrane stem cells (hAMSCs) and human amniotic fluid MSCs (hAFMSCs) [85].

In addition to CFU analysis, we conducted an investigation into proliferation, revealing that all cell lines demonstrated the ability to proliferate over a 21-day period. Notably, epithelial-like cells exhibited a markedly slower proliferation rate when compared to fibroblast-type cells, suggesting an initial growth advantage of fibroblast-type cells over other cell types present during isolation. The observation of fibroblast-type cells outpacing epithelial cells in terms of proliferation aligns with findings in existing literature [84, 86]. The growth rate within cell lines of similar cell-types was also statistically different but similar trends were also seen in literature [87]. While we would expect MSCs to grow exponentially, we saw a slower linear increase with a plateau around 14 days for some cell lines. It has been reported that there is slower growth in late gestational

age such as the third trimester [63]. This could be due to the changes in the cell niche of these suspended cells in the third trimester and suggests that the cells of third trimester amniotic fluid might be related, but distinct populations from the first- and second trimester [63].

Before commencing differentiation experiments, we ensured the presence of stem cell markers in the cell lines at passage 3. Despite retaining these markers, the cells did not undergo adipogenic or osteogenic differentiation after 28 days in the respective differentiation media. The differentiation protocols, well-established in our laboratory and proven successful with adipose-derived stem cells in numerous studies, did not yield the same success with amniotic fluid cells [77, 88, 89].

While this research confirmed two of the three requirements of the ISCT MSCs, it does not definitively establish these third-trimester amniotic fluid cells as MSCs. Existing literature has suggested limited or no capacity for amniotic fluid cells from the third trimester to differentiate into adipogenic and osteogenic lineages [63, 74, 79, 87, 90].

Although these amniotic cells did not undergo differentiation, their response to adipogenic and osteogenic differentiation media was evident through altered cell orientation, indicating cellular reactions, even though mature differentiated cells did not form. It is conceivable that a second induction step using an additional reagent or employing a different differentiation medium for another cell type lineage may be necessary. Literature has referenced the ability of amniotic fluid cells to differentiate into myogenic, endothelial, neurogenic, and hepatic lineages,

encompassing all embryonic germ layers [82]. Therefore, these amniotic fluid cells could represent a distinct type of progenitor cell with therapeutic value beyond the scope of MSC lineages.

This research has certain limitations, notably the absence of a standardized protocol for amniotic fluid collection. Consequently, samples with varying degrees of blood contamination were utilized, and the impact of such contamination on downstream processing and amniotic fluid cell viability remains unknown. Additionally, our sample pool is restricted to women undergoing elective Cesarean sections. Unfortunately, information regarding the reasons for these Cesarean sections was not provided, and some may be associated with potential fetal issues, potentially influencing amniotic fluid cell populations. The variability among donors underscores the need to comprehensively characterize amniotic fluid, establish norms, and define release criteria for specific clinical applications. Furthermore, it emphasizes the importance of setting minimum guidelines for obtaining high-quality amniotic fluid samples [80].

Currently, there is a lack of consensus on a standardized method for isolating and culturing stem cells from amniotic fluid, leading to the generation of heterogeneous cell populations with varied phenotypes. This heterogeneity poses challenges in accurately assessing MSC traits, as homogenous populations would provide clearer flow cytometry, CFU, and proliferation data. The presence of heterogeneous populations may also have influenced each other through paracrine factors during culture, potentially hindering or impeding the differentiation process. To address these issues, there is a pressing need for improved isolation and cell

culture techniques that can yield more homogenous populations for subsequent testing. Furthermore, establishing guidance for the scientific community working with these stem cells is essential to foster the development of international standards. Similar initiatives, such as those by the International Federation for Adipose Therapeutics and Science and the International Society for Cellular Therapy, could serve as models for establishing reproducible parameters in this context.

2.5 Conclusion

In summary, the research on third-trimester amniotic fluid cells revealed donor variability and heterogeneity within cell populations. While these cells did not meet the criteria for true MSCs because they lacked differentiation potential, it is plausible that these cells represent another class of progenitor cells that do not align with the established criteria for adult MSCs [75, 79]. The study highlighted the need for standardized collection protocols and improved isolation techniques for homogenous cell populations. Overall, additional characterization, manufacturing, and medical investigations are necessary to fully understand and harness the potential of cultured amniotic fluid cells for clinical applications.

3. CHARACTERIZATION OF AMNIOTIC FLUID EXTRACELLULAR VESICLES

3.1 Study Design

Extracellular vesicles, distinguished by their size, biogenesis, and cargo, are released by every cell type into various biological fluids. Exosomes, the smallest type of extracellular vesicle, typically range from 50 to 150 nm [91]. They are formed either by direct budding from the plasma membrane or through the inward budding of vesicles (endocytosis) at the plasma membrane [92, 93]. The field of exosome research has experienced significant growth due to their pivotal role in cell-to-cell communication, both locally between neighboring cells and over long distances via biofluids like amniotic fluid. Despite the surge in interest, the comprehensive characterization of amniotic fluid exosomes remains incomplete. Such characterization is crucial to ascribe functional changes specifically to exosomes and not to co-isolated proteins or other vesicle types.

This study aims to develop an isolation protocol for third-trimester human amniotic fluid extracellular vesicles and assess whether these vesicles exhibit exosomal properties in accordance with the International Society for Extracellular Vesicles (ISEV) guidelines. To achieve this, we adapted and optimized a protocol originally designed for isolating extracellular vesicles from cell culture media. Following the ISEV's 2018 guidelines on Minimal Information for Studies of Extracellular Vesicles (MISEV2018), we conducted a comprehensive characterization, employing techniques such as nanoparticle tracking analysis

(NTA) for quantification, Western blot for positive protein identification, and transmission electron microscopy (TEM) for morphology characterization [94].

Additionally, we explored the functional impact of exosomes derived from amniotic fluid, amniotic fluid cell culture, and amniotic membrane cell culture. This involved exposing these exosomes to intestinal epithelial cells and measuring differences in cell proliferation. By integrating isolation protocol development, comprehensive characterization, and functional testing, this study aims to contribute valuable insights into the properties and potential therapeutic applications of third-trimester human amniotic fluid exosomes.

3.2 Methods

3.2.1 Ultracentrifugation

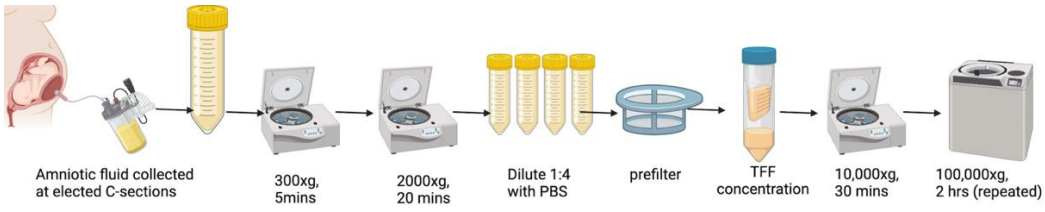

Figure 15: Extracellular vesicles isolation.

Schematic for the isolation of amniotic fluid extracellular vesicles. Amniotic fluid derived extracellular vesicles were isolated using differential centrifugation, dilution, membrane filtration, and size exclusion methods. Created with BioRender.com.

The noncellular supernatant of third trimester human amniotic fluid samples donated by Tides Medical were used for the isolation of extracellular vesicles using a protocol developed with Dr. Lili Zhang in Dr. Tony Hu's Lab, at Tulane Center for Cellular and Molecular Diagnosis (Fig. 15). First, cells were removed by centrifugations at 300xg for 5 minutes using a Megafuge 8 centrifuge (Thermo Fisher Scientific, Hampton, NH, cat # 75007210). A second centrifugation step at

2,000xg for 20 minutes removed remaining cellular debris. Next, the supernatant was diluted with phosphate-buffered saline (PBS) four-fold by volume and filtered using a 0.45µm prefilter (Corning, Corning, NY, cat # 431750). The supernatant was then concentrated on a 30-100 kDa tangential flow filtration (TFF) filter unit (Millipore, Burlington, MA, cat # UFC9030) at 3214xg for 20-30 minutes. We washed the concentrated medium from the upper TFF filter with PBS to remove remaining exosomes. The concentrated supernatant was then centrifuged at 10,000xg using an accuspin Micro 17 centrifuge (Thermo Fisher Scientific, Hampton, NH, cat # 75002461) to remove more cellular contaminants. We ultracentrifuged the samples at 100,000xg for 2 hours on a himac Preparative Ultracentrifuge (Hitachi, Tokyo, JP, cat # CP100WX, with cat # P50A3-0519 rotar) and resuspend the isolated pellet with PBS overnight. The ultracentrifuge step was repeated at 100,000xg for 2 hours. Isolated extracellular vesicles were resuspended in PBS and stored in –80˚C.

3.2.2 Exoquick-TC™ Isolation Kit

Exoquick-TC™ Isolation Kit for Tissue Culture Media (System Biosciences, Palo Alto CA, cat # EQULTRA-20TC-1) was adapted to isolate extracellular vesicles from amniotic fluid. The total volume of the fluid sample was centrifuged at 300xg for 5 minutes to remove cells. It was then centrifuged for 3,000xg for 15 minutes to remove cellular debris. Then 1mL of ExoQuick-TC volume was added to every 5mL of amniotic fluid sample. The sample was mixed and then incubated on ice for at least 12 hours at 4°C. Next, we centrifuged the ExoQuick-TC/amniotic fluid mixture at 3,000×g for 10 minutes to pellet

extracellular vesicles. The pellet was resuspended in 200 µl of Buffer B. Additional purification was completed using the included purification column. First 200µl of Buffer A was added to the sample, the column was rinsed with 500 µl of Buffer B, and then the sample was added to the purification column and mixed at room temperature on a rotating shaker for 5 minutes. The purified sample was eluted by centrifuging the column into an Eppendorf tube at 1,000xg for 30 seconds. Isolated extracellular vesicles were stored in –80˚C.

3.2.3 Amniotic Membrane Cell Isolation

Third trimester human amniotic membrane samples were donated by Tides Medical after collection by Louisiana Organization for Transplant (LOFT). LOFT completed all donation paperwork including informed consent with mothers and followed all state regulations and guidelines of the American Association of Tissue Banks. Third trimester samples were collected from elected cesarean sections and shipped on ice overnight to the Tulane Center for Stem Cell Research and Regenerative Medicine. Once received, the tissue samples were stored at 4°C and processed for cell isolation within 48 hours.

To obtain the amnion mesenchymal stomal cells we followed an adapted protocol from Alviano et al. [95]. The tissue section was cleaned by washing twice with 1X PBS to remove residual blood. It was then minced using a scalpel into small pieces (about 2x2 cm). The pieces were then digested for 15 minutes at 37°C in Trypsin-EDTA 0.25% (Thermo Fisher Scientific, Waltham, MA, USA). A second digestion was completed for 60 minutes at 37°C with 5% CO_2 using Trypsin-EDTA 0.25% and 0.1% Collagenase I in Dulbecco's Modified Eagles

Medium (DMEM)/F12 (Gibco, New York, NY, cat # 12634-010) to digest the collagen fibers. The tissue and fluid were then filtered through a 100 μm cell strainer (Corning, New York, NY, cat # CLS431752) and the supernatant was neutralized with fetal bovine serum (FBS, Hyclone, Logan, UT, cat # SH30070.03IH25-40). The cell suspension was centrifuged at 200xg for 10 minutes and resuspended in complete media, DMEM (Gibco, New York, NY, cat # 12634-010) + 20% FBS (Hyclone, Logan, UT, cat # SH30070.03IH25-40) + 1% antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA, cat # 15240062). The cells were seeded in 25 cm^2 flasks with complete media and cultured. All experiments were completed using passage 3 to passage 5 cells.

3.2.4 Exosome Isolation from Cell Culture Media

Amniotic fluid cells isolated per Chapter 2 and amnion mesenchymal stomal cells isolated per 3.2.2 were cultured to collect exosomes. First, $1x10^6$ cells were seeded into 75 cm^2 flasks and cells were grown to 80% confluency, determined by visual inspection, in complete growth media made from Dulbecco's Modified Eagles Medium (DMEM)/F12 (Gibco, New York, NY, cat # 12634-010), 5% fetal bovine serum (FBS, Hyclone, Logan, UT, cat # SH30070.03IH25-40), and 1% antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA, cat # 15240062). Once 80% confluent, the media was exchanged to complete growth media made with exosome-stripped FBS. Exosomes were removed from FBS using Exoquick-TC™ Isolation Kit (System Biosciences, Palo Alto, CA, cat # EQULTRA-20TC-1). After 48 hours in culture with exosome stripped complete growth media, the supernatant was collected for future exosome isolation.

3.2.5 Nanoparticle Tracking Analysis

Quantification of particle size in amniotic fluid was completed using nanoparticle tracking analysis (NTA) per the protocol described in Sheller-Miller et al. [91]. First the NS300 Nanosight (Malvern Instruments, Westborough, MA) was started up and quality controls were checked. Then we diluted exosome samples in 0.03-0.1μm filtered distilled water between 1:100 and 1:1000 depending on the sample. The total volume should be 1.0mL and the concentration should be less than $2x10^9$ particles/mL, or at least 50 particles per frame. We carefully aspirated the sample to ensure there were no bubbles and loaded the exosome sample onto the instrument by gently pushing the plunger until particles were visualized on the screen. We set the camera level, focus, and gain so that particles appeared in focus as sharp dots. We selected and ran a protocol that recorded five captures for 30 seconds each, after each capture we pressed the plunger to move the sample. After the protocol was complete, we set the gain and detection threshold so particles were identified with a red cross. The same thresholding was used for all five recordings. Finally, we analyzed the samples and exported the data. Results are shown as individual runs and as the average of all runs captured within one protocol.

3.2.6 Western Blot

Positive protein identification was completed by Western blot using the protocol described in Sheller-Miller et al. [91]. First, isolated amniotic fluid extracellular vesicles were lysed on ice for 50 minutes using 10X RIPA lysis buffer (Thermo Scientific, Waltham, MA, cat # 89900). We quantified the total protein

using the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, cat # 23225) following manufacturer's instructions. The target was 5-10ug of total protein in each well of the gel. Gels were run using Mini-Protean TGX Precast Gel (BIO-RAD, Hercules, CA, cat # 4561023) and transferred to PVDF membrane using an Invitrogen iBlot 2 transfer system (Thermo Scientific, Waltham, MA, cat # IB21001) and transfer stacks before being blocked in 5% bovine serum albumin (BSA, Sigma Aldrich, Burlington, MA, cat # A5611-106) blocking buffer in Tris-buffered saline with 1% Tween 20 (TBST, 10X TBS - Thermo Scientific, Waltham, MA, cat # BP24711, Tween 20 – Sigma Aldrich, Burlington, MA, cat # P9416) for one hour at room temperature. Washes were preformed using TBST, and all blots were incubated overnight with primary antibodies at 4°C in 5% milk TBST. The following primary antibodies were used 1:1000; biotin anti-human CD63 antibody (BioLegend, San Diego, CA, cat # 353017) and biotin anti-human CD81 antibody (BioLegend, San Diego, CA, cat # 349514). Blots were washed with TBST and incubated with 1:2000 secondary antibody HRP-Conjugated Streptavidin (Thermo Scientific, Waltham, MA, cat # N100) for one hour at room temperature. Blots were developed using the Clarity Western ECL substrate (BioRad, Hercules, CA, cat # 1705061) & ChemiDoc Imaging System (Thermo Scientific, Waltham, MA, cat # 12003153).

3.2.7 Silver Stain

ProtoSilver Plus Silver Stain Kit (Sigma Aldrich, Burlington, MA, cat # SLCD4365) was used to stain SDS-PAGE gels. Gels were fixed in the included fixing solution for 20 minutes before washing with 30% ethanol (Thermo

Scientific, Waltham, MA, AC615095000) for 10 minutes. Gels were then washed with deionized water for 10 minutes. Sensitization was completed using the included sensitizer solution and the gel was incubated for 10 minutes. The gel was then washed with deionized water twice for 10 minutes each. Next, we equilibrated the gel for 10 minutes in the included silver stain solution and washed with deionized water for 1 minute. The gel was developed with the included developer solution for 3 to 7 minutes until bands were visible. Then the included Proteosilver Stop solution was added for 5 minutes until bubbles developed. The final stained gel was stored in ultrapure water.

3.2.8 Transmission Electron Microscopy (TEM)

Morphology characterization was completed by evaluating TEM images. To fix amniotic fluid exosomes on an electron-microscope grid, methods from Sheller- Miller et al. and Jung et al. were used [91, 96]. Briefly, the exosome samples were resuspended in 0.2µm filtered deionized (DI) water and ultracentrifuged using himac Preparative Ultracentrifuge (Hitachi, Tokyo, JP, cat # CP100WX, with cat # P50A3-0519 rotar) for 2 hours at 100,000xg. We then fixed the purified exosome pellet for 5 minutes with 4% Paraformaldehyde (PFA, Thermo Scientific, Waltham, MA, cat # J19943.K2). To absorb the exosomes on the formvar-carbon coated 300-mesh copper grids (Electron Microscopy Sciences, Hatfield, PA, cat # FCF200-CU-50) we loaded 5-10µL of 300 ug/mL exosome suspension on top of the grid and incubated for 10 minutes at room temperature. To stain the exosomes, 20 drops of filtered 2% phosphotungstic acid (PTA, Thermo Scientific, Waltham, MA, cat # 040116.22) solution was placed on the surface of

the grid by syringe and allowed to dry for 60 minutes. Excess PTA solution was removed by contacting the grid edge with filter paper. The grids were then rinsed in a drop of filtered DI water on parafilm and excess water was removed by contacting the grid edge with filter paper, two rinses were completed. The grids were then allowed to dry for 10 minutes at room temperature before storing or capturing representative images on a 120keV JEM 1400 electron microscope (Jeol, Taiwan, CN, cat # JEM-1400Flash).

3.2.9 Proliferation

To determine if exosomes were taken up by target intestinal epithelial cells, a proliferation assay was adapted from Matsumoto et al. [97]. T84 or CaCo2 cells were seeded in 96-well plates at 1×10^3 cells per well with complete growth media made from Dulbecco's Modified Eagles Medium (DMEM)/F12 (Gibco, New York, NY, cat # 12634-010), 5% fetal bovine serum (FBS, Hyclone, Logan, UT, cat # SH30070.03IH25-40), and 1% antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA, cat # 15240062). The experiments were performed in triplicate and maintained under controlled conditions at 37°C with 5% CO_2. Quantitative assessment of cell proliferation was conducted using cell counting or a cell counting kit, CCK-8 (Sigma-Aldrich, Burlington, MA, cat # 96992). CCK-8 uses a tetrazolium salt, WST-8, that is reduced by cellular dehydrogenases to produce a water-soluble orange formazan dye. The amount of formazan dye produced is directly proportional to the number of living cells. Cell proliferation was measured either after 24 hours or after 48 hours. The cells were incubated for 1-2 hours with 10uL of CCK-8 at 37°C with 5% CO_2. After 1-2 hours, the fluorescence intensity

was measured using excitation and emission at 450nm with a SpectraMax i3X plate reader (Molecular Devices, Silicon Valley, CA, cat # i3x).

3.2.10 Statistical Analysis

Data were analyzed using Microsoft Excel and PRISM 9 software (GraphPad, Inc.). Data are presented as mean ± standard deviation from the mean and all experiments were performed in triplicate. Statistical analysis was conducted using either a one-way analysis of variance (ANOVA) for a single independent variable or a two-way ANOVA for two independent variables to identify statistical differences. Asterisks indicates statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

3.3 Results

3.3.1 Nanoparticle Tracking Analysis

Figure 16 presents particle size distributions and concentrations for samples AF024 and AF027 using Nanoparticle Tracking Analysis (NTA). Figures 16A and 16C display exosome concentrations (particles/mL) for each dimension (nm) of samples AF024 and AF027 over five recordings, each represented by a different color. Figures 16B and 16D show the averaged exosome concentration (particles/mL) for each dimension (nm). These results reveal heterogeneous vesicle populations by size in the individual runs, with both samples indicating low levels of particles >200 nm. The mean particle diameter for AF024 is 130.7 ± 2.4 nm, with two main peaks at 100 nm and 166 nm. For AF027, the mean particle diameter is 160.7 ± 3.8 nm, with two main peaks at 126 nm and 195 nm.

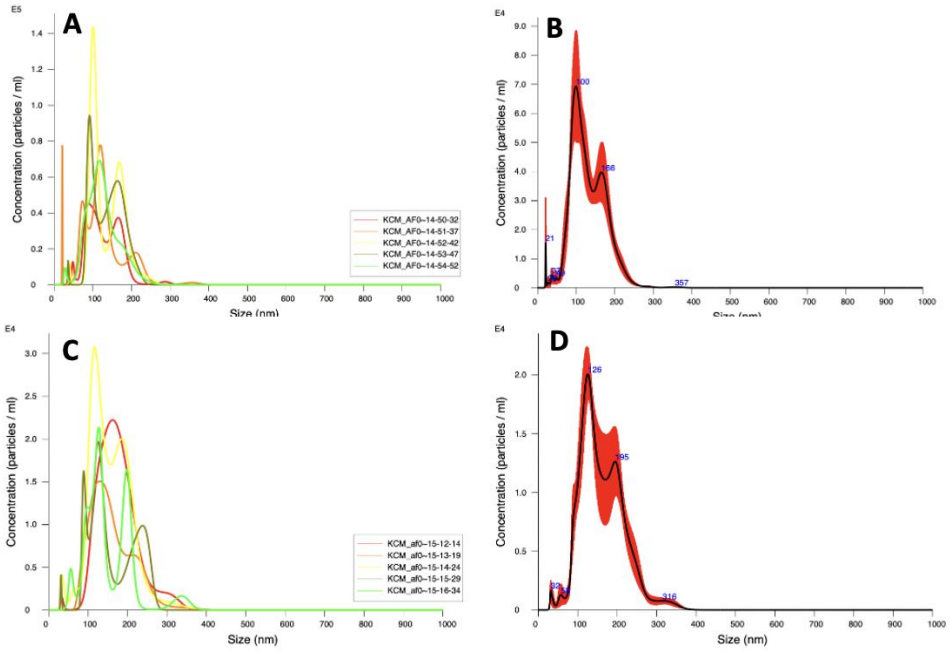

Figure 16: Nanosight Data.

Output of exosome analysis by Nanosight. (A) AF024 Exosome concentration (particles/mL) for each dimension (nm) for the five recordings represented with different colors. (B) AF024 averaged exosome concentration (particles/mL) for each dimension (nm). (C) AF027 Exosome concentration (particles/mL) for each dimension (nm) for the five recordings represented with different colors. (D) AF027 averaged exosome concentration (particles/mL) for each dimension (nm).

3.3.2 Western Blot

Figure 17 displays a Western blot that shows human amniotic fluid extracellular vesicles samples AF024 and AF027 isolated by ultracentrifuge without the second purification step, loaded at either 5µg or 10µg per lane, and tested for antibody CD63. The membrane was probed with biotin anti-human CD63 antibody (BioLegend, San Diego, CA, cat # 353017) followed by HRP-Conjugated Streptavidin secondary antibody (Thermo Scientific, Waltham, MA, cat # N100). A specific band was detected for CD63 at approximately 25 kDa (as indicated). Additionally, there is an unidentified protein present in all samples (37Kda) or non-specific binding from the secondary antibody.

Figure 18 displays a Western blot featuring human amniotic fluid extracellular vesicle samples (AF024, AF027, and AF030) and their extracellular vesicle-free supernatant, each loaded at 5µg. These samples underwent isolation by ultracentrifuge (with and without an additional ultracentrifuge purification step). The membrane underwent probing with a biotin anti-human CD81 antibody (BioLegend, San Diego, CA, cat # 349514), followed by an HRP-Conjugated Streptavidin secondary antibody (Thermo Scientific, Waltham, MA, cat # N100). A specific CD81 band, around 25 kDa, was detected (as indicated). Conversely, the extracellular vesicle-free supernatant showed no CD81 positivity, affirming the efficacy of the isolation method in extracting exosomes from amniotic fluid. Additionally, samples subjected to double ultracentrifugation displayed stronger CD81 bands, signifying a higher concentration of pure exosomes due to the supplementary wash step.

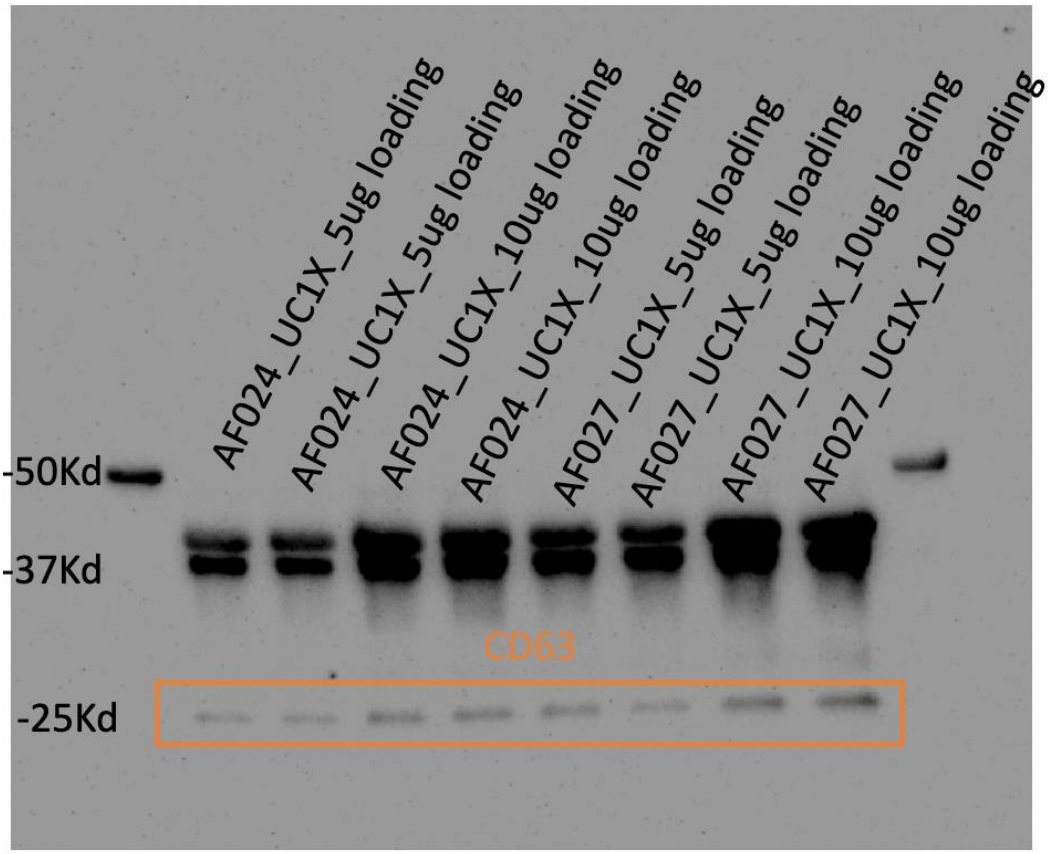

Figure 17: CD63 Western Blot.

Western blot analysis shows amniotic fluid exosomes are positive for tetraspanin CD63. UC1X: ultracentrifuged one time.

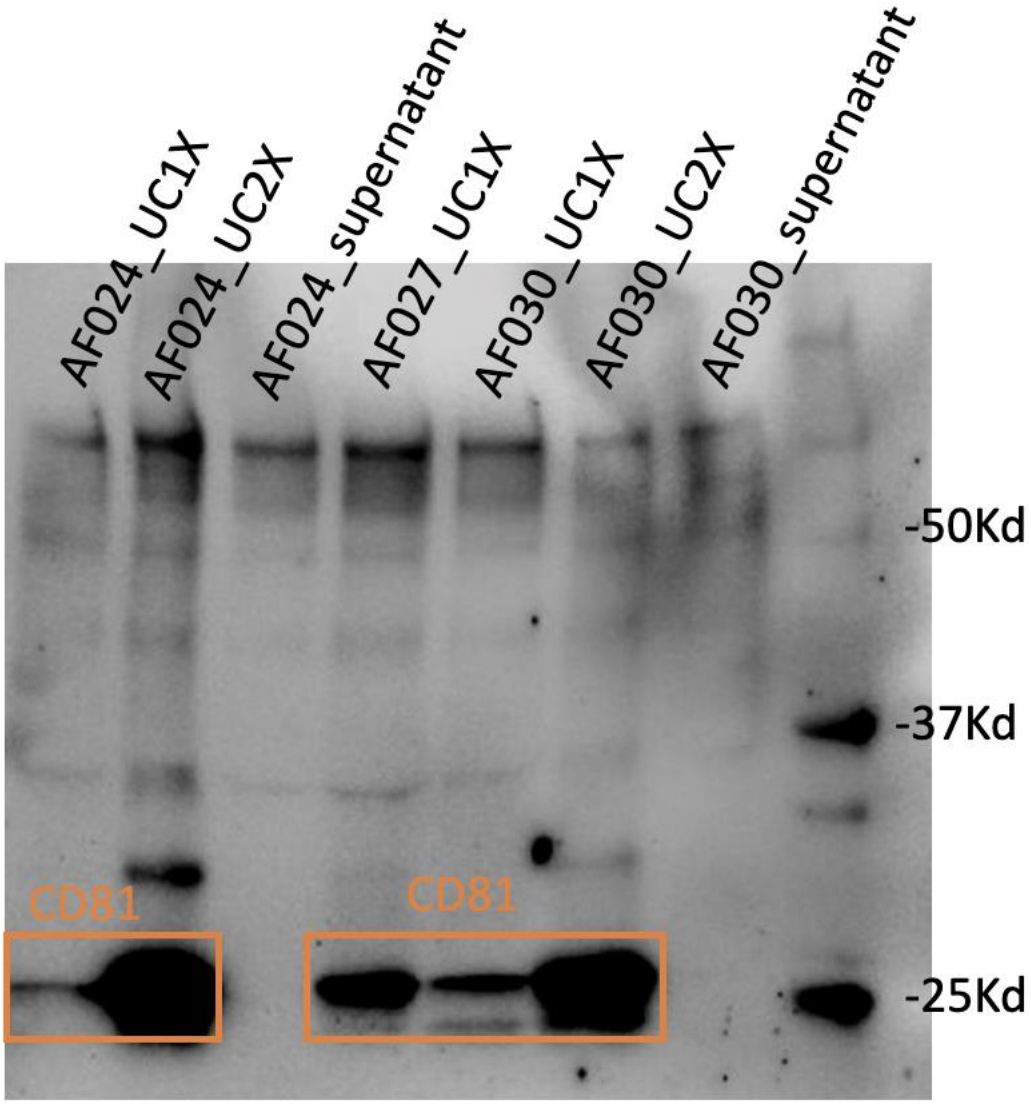

Figure 18: CD81 Western Blot.

Western blot analysis shows amniotic fluid exosomes are positive for tetraspanin CD81. UC1X: ultracentrifuge one time, UC2X: ultracentrifuge two times.

3.3.3 Transmission Electron Microscope (TEM) and Silver Stain

Negative staining is a qualitative method that uses deposition of heavy atom stains for examining the structure of isolated exosomes at the electron microscope level. For our preparations three amniotic fluid samples were combined (AF024,

AF027, AF0030). Figure 19A presents electron microscopy of negatively stained exosomes which shows round cup-shape morphologies of <200nm. Contaminating lipids and cellular debris are also observed within the same preparations.

Figure 19B displays a silver-stained SDS polyacrylamide gel featuring the separation of 5 µg of total exosome lysates per lane from second trimester and third trimester amniotic fluid, with each sample run in duplicate. This gel shows the banding patterns between the second trimester amniotic fluid sample (AS034115) and third trimester amniotic fluid sample (AF025) and reveals different protein expression. This suggests that the protein cargo and surface markers of amniotic fluid exosomes may change with gestational age.

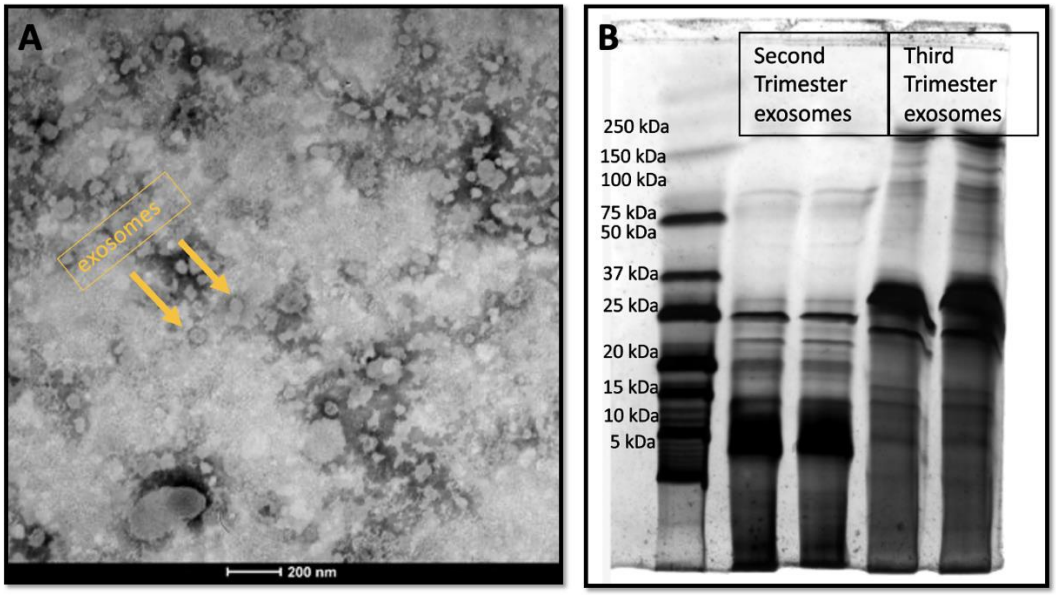

Figure 19: TEM and Silver Stain.

Typical characteristics of exosomes. (A) Electron-microscope observation of whole mounted exosomes purified from amniotic fluid. Arrows indicate exosomes. Scale bar = 200nm. (B) Silver-stained SDS polyacrylamide gel run with second trimester and third trimester amniotic fluid. Molecular weight markers were loaded in the first lane (kDa).

3.3.4 Proliferation

Figure 20 illustrates the outcomes of a 24-hour exposure of intestinal epithelial T84 cells to 25µg of amniotic fluid exosomes (AF049) and exosome-free supernatant. The results indicate that the presence of 25µg of amniotic fluid exosomes significantly enhances proliferation, measured by optical density (OD), compared to the control group. In contrast, exposing intestinal epithelial cells to exosome-free supernatant led to increased proliferation, but this change did not reach statistical significance (p=0.0983).

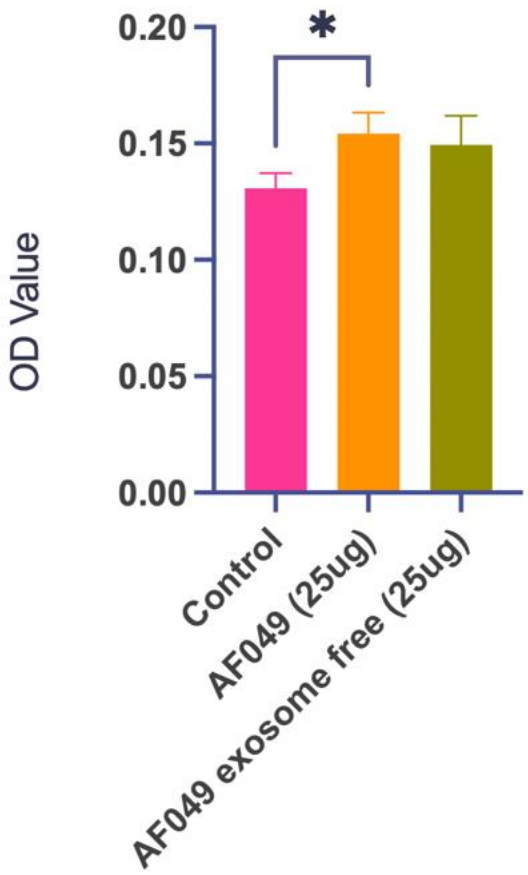

Figure 20: Proliferation with AF exosomes.

Proliferation of intestinal epithelial cells after 24 hours when exposed to 25µg of amniotic fluid exosomes and exosome free supernatant.

Figure 21 presents the outcome of a 24-hour exposure of intestinal epithelial T84 cells to 50µg of amniotic fluid exosomes. This shows that following a 24-hour incubation, only one of the tested amniotic fluid exosome samples (AF051 out of AF024, AF050, AF051, AF029) exhibited a slight increase in proliferation, albeit not reaching statistical significance.

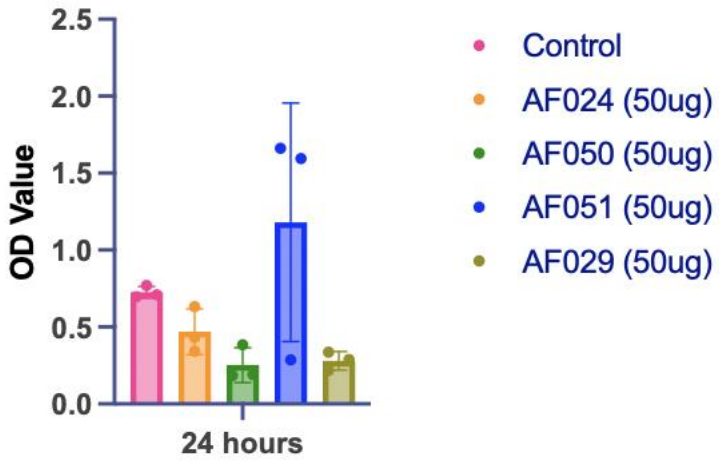

Figure 21: Proliferation with AF exosomes.

Proliferation of intestinal epithelial cells after 24 hours when exposed to 50µg of amniotic fluid exosomes.

Figure 22 presents the outcome of a 48-hour exposure of intestinal epithelial T84 cells to 200µg of amniotic fluid exosomes (AF050, AF051). This shows that following this extended exposure, amniotic fluid sample AF051 induced a statistically significant increase in the proliferation of intestinal epithelial cells when compared to the control group. In contrast, the application of sample AF050 did not elicit a significant increase in proliferation relative to the control, highlighting the inherent variability present among different amniotic fluid samples.

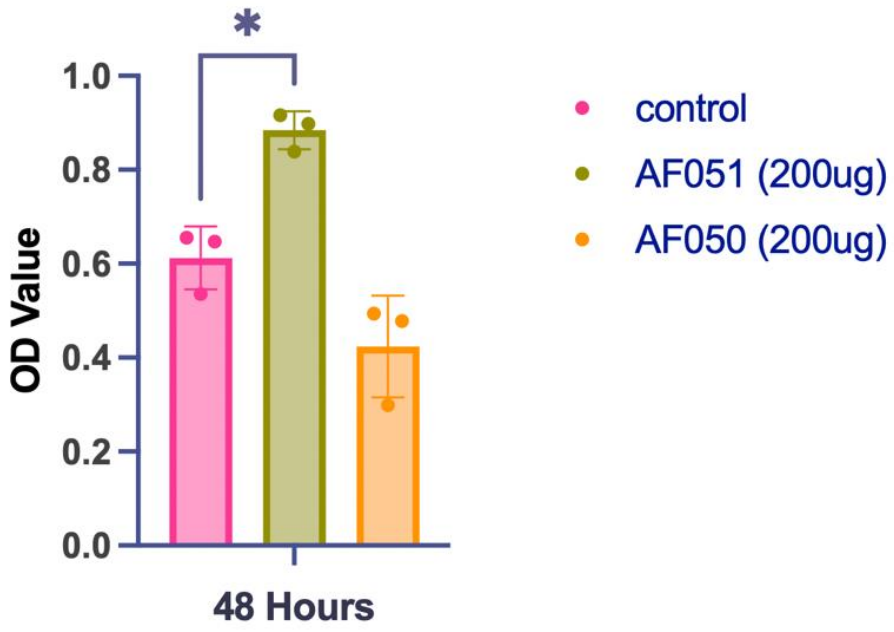

Figure 22: Proliferation with AF exosomes.

Proliferation of intestinal epithelial cells after 48 hours when exposed to 200µg of amniotic fluid exosomes.

In addition to assessing the impact of amniotic fluid exosomes on cellular proliferation, our investigation extended to exosomes from cells derived from both amniotic fluid and the amniotic membrane. Supernatant collected from these cultures allowed us to isolate exosomes for further analysis. Figure 23 shows the outcome of a 48-hour exposure of intestinal epithelial CaCo2 cells exposed to 156µg total protein from either amniotic membrane cell-cultured exosomes (AMcell-exo) or amniotic fluid cell-cultured derived exosomes (AFcell-exo). This shows that at both 12 and 24 hours, the cellular response to AFcell-exo treatment did not exhibit a significant difference when compared to the control group. Meanwhile, at the 12-hour mark, AMcell-exo treated samples did not significantly

differ from the control, but at 24 hours, there was an observable increase in cell proliferation, although statistical significance was not reached (p=0.0537).

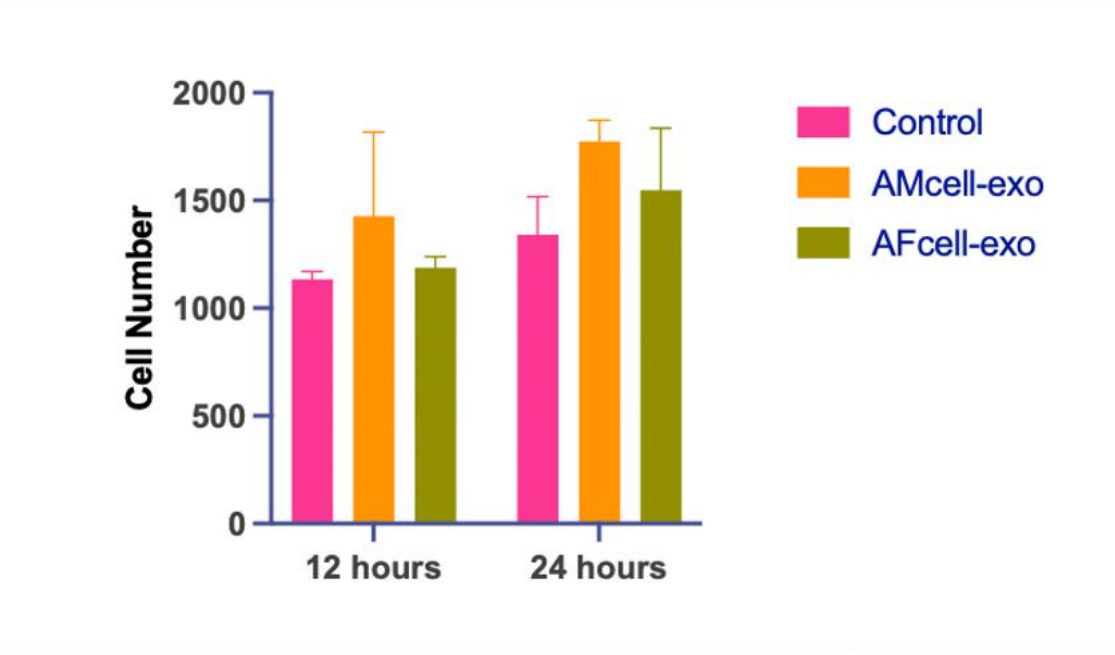

Figure 23: Proliferation with AFcell-exo and AMcell-exo.

Proliferation of intestinal epithelial cells after 12 and 48 hours when exposed to 156µg of amniotic fluid cell cultured exosomes (AFcell-exo) and amniotic membrane cultured exosomes (AMcell-exo).

3.4 Discussion

The isolation of human amniotic fluid exosomes lacks consensus due to the challenging presence of co-isolating contaminating proteins. Various techniques have been employed, each offering distinct advantages and limitations concerning factors such as yield, ease of use, purity, and cost. Currently, three published methods exist: Bellio et al. (2021) implemented a patented filtration system incorporating escalating differential centrifugation speeds and times culminating in ultracentrifugation, Sheller-Miller et al. (2020) adapted a protocol from exosome

isolation in other biofluids encompassing a dilution step, ultracentrifugation, and a size exclusion chromatography purification step, and Ebert et al. (2019) uses differential centrifugation and the addition of dithiothreitol (DTT) [71, 91, 98].

Despite the suitability of some isolation methods for research laboratory applications, their appropriateness for therapeutic use is limited. For instance, ultracentrifugation, a standard lab method, necessitates expensive equipment (capital cost of $50-100k and running cost of $3k per year), consumes considerable time (> 4 hours), and yields are relatively low (5%-25% recovery) [99, 100]. Alternatively, commercially available polymer precipitation kits (e.g., ExoQuick™) have demonstrated increased purity by reducing background contamination of proteins like albumin and IgG, without requiring expensive equipment. However, they come with drawbacks such as low specificity, extended overnight incubations (>12 hours), and, notably, their warranty statements currently specify that the product is "expressly not designed, intended, or warranted for use in humans or for therapeutic or diagnostic use".

For the prospective therapeutic use of isolated amniotic fluid exosomes, the chosen isolation method must be applicable to humans. Hence, despite its relatively low yield, ultracentrifugation currently stands as the optimal choice for our purposes. By incorporating a dilution step, we successfully demonstrated that amniotic fluid serves as a robust source of extracellular vesicles. To enhance purity, an additional ultracentrifuge step was introduced. Amniotic fluid is a viscous biofluid that contains many cellular and protein contaminants that are expected to co-precipitate when using ultracentrifuge. Therefore, it is even more important to

have proper characterization to show an enrichment of pure exosomes populations including exosome size, morphology and protein markers.

The distribution of exosome vesicle sizes was assessed using nanoparticle tracking analysis (NTA). NTA uses a laser to illuminate particles in solution under a constant flow rate. The laser light is scattered by the particles moving under Brownian motion and recorded by a camera. Since Brownian motion is particle size dependent, analysis of the recordings can approximate the size distribution of the particles in the sample. The samples exhibited a heterogeneous mix of vesicles, with the majority measuring <200 nm, aligning with the ISEV guideline for exosomes [94]. The data presented here showcases two representative distributions of amniotic fluid exosomes, with the expectation that all future exosome preparations will also display heterogeneity in size [91]. Existing literature supports our findings, indicating similar profiles for isolated amniotic fluid exosomes. Bellio et al. reported a mean size of 154.2 ± 7.3 nm and Gebara et al. reported two peaks around 150nm and 200nm [71, 101]. Notably, it's crucial to acknowledge that NTA does not differentiate between membrane vesicles and co-isolated non-membranous particles of similar size [102], emphasizing the need for evaluation in conjunction with an imaging modality.

Incorporating transmission electron microscopy (TEM), we observed cup-shaped circular morphologies in the exosomes, consistent with the ISEV guideline for exosomes. Noteworthy, the images revealed co-isolated protein contamination, but importantly, no contamination from vesicles exceeding 200 nm, such as ectosomes, microvesicles, microparticles, or apoptotic bodies.

Exosomes form by budding directly from the cell membrane or by inward budding of vesicles during endocytosis [92, 93]. In late endosomes, this process creates multivesicular bodies (MVBs). Here, specific sorting of lipids, proteins, RNA, and miRNA happens, managed by the endosomal sorting complex required for transport (ESCRT) or ESCRT-independent processes. This step enriches exosomes with specific proteins like CD9, CD63, and CD81, known as endosome-specific tetraspanins, which serve as markers for identifying the vesicles. For our amniotic fluid exosome samples, Western blots identified the exosome-specific proteins CD63 and CD81, following the guidelines of the ISEV. The Western blots indicated that an extra ultracentrifuge step, totaling two, increased the sample's purity by enhancing the detection of CD81. The absence of CD81 in the supernatant samples demonstrates the success of our isolation method in removing exosomes from the amniotic fluid. Silver stains revealed protein banding for second and third trimester amniotic fluid samples, highlighting differences in cargo sorted by ESCRT with gestational age. In the future, we could excise bands of interest and use Mass Spectrometry for specific protein identification.

Exosome uptake is a complex and selective process, and not all cells are equally capable of taking up exosomes. The ability of a cell to take up exosomes depends on various factors, including the cell type, the specific receptors on the cell membrane, and the specific receptors on the exosome. Exosomes can enter recipient cells through fusion, endocytosis, or interaction with cell membrane components [92]. They regulate recipient cells post-transcriptionally, influencing gene expression through miRNA [103, 104]. Proliferation experiments revealed that

amniotic fluid exosomes are either taken up or attach to intestinal epithelial cells inducing a functional change, leading to increased proliferation. This effect wasn't observed with exosome-free supernatant, amniotic fluid cell-cultured exosomes, or amniotic membrane cell-cultured exosomes. This suggests the need for further evaluation of amniotic fluid exosomes for therapeutic applications, especially in conditions like necrotizing enterocolitis (NEC), where increased proliferation is crucial. However, not all amniotic fluid exosome samples induced increased proliferation, highlighting sample variability and dynamic changes in exosomes. Assessing exosomes from cells derived from amniotic fluid and the amniotic membrane did not show a statistical difference compared to the control, emphasizing the differences in these exosome samples and cellular responses. Literature on breast milk exosomes as a pretreatment for NEC also did not show an increase in proliferation after 6 hours of pretreatment [105]. These findings reinforce the emphasis on amniotic fluid exosomes for therapeutic applications due to their consistent potential for measurable downstream effects on cellular proliferation. The proliferation experiment also raises the possibility that amniotic fluid exosomes swallowed by the fetus in utero could impact intestinal epithelial cells, influencing fetal development. Understanding how human amniotic fluid exosomes affect fetal gene expression is crucial for unraveling questions about fetal development and identifying potential therapeutic targets.

Limitations to this research include that there are no standard methods for the isolation, discrimination, and quantification of all the different sub-populations of extracellular vesicles [106]. Therefore, samples of varying vesicle and protein

contamination were used without knowledge of how the vesicle and protein contamination could affect functional testing like proliferation. As well as incomplete recovery of available material using ultracentrifuge method. Additionally, we are only getting samples from women who are electing to have Cesarean sections. We did not receive the information about why these births were Cesarean sections but we know abnormal changes of extracellular vesicle concentration and composition are involved in pregnancy-related diseases [106]. Because amniotic fluid is a dynamic fluid and changes during the whole pregnancy, the population of exosomes is also constantly changing and therefore we are limited by when we take the sample and the donor variability when it comes to reproducible results in functional tests like proliferation. Finally, the origin of these amniotic fluid exosomes is unknown and knowing would help elucidate their cargo and primary function for future therapeutics.

For future characterization, we can enhance our understanding of third-trimester exosomes by expanding the flow panel to identify the cells of origin within the mixed population. Additionally, we can delve into exosome cargo characterization through various methods such as proteomics, lipidomics, metabolomics, or next-generation sequencing. Further studies can dissect the heterogeneous exosome samples into distinct subtypes, including large exosomes, small exosomes, and exomeres. Comprehensive functional studies can be conducted to elucidate the biological relevance and specific functions of each subtype.

In preparation for functional testing and potential exosome therapeutics in humans, it's crucial to ensure that we are assessing the functional properties of exosomes and not the co-isolated contaminants. Therefore, reporting the purity of our isolations through methods like nanoparticle tracking analysis (NTA) and total protein quantification by BCA is important. Utilizing isolations with high vesicular purity, indicated by a ratio exceeding $3x10^{10}$ particles/µg protein [107], ensures accurate assessment. Lastly, dosing tests using both particle counts and total protein can be performed to determine a more reliable dosing metric.

3.5 Conclusion

In conclusion, we have successfully demonstrated the isolation of human amniotic fluid extracellular vesicles through ultracentrifugation, accompanied by a comprehensive characterization encompassing size, morphology, and surface proteins, aligning with the International Society for Extracellular Vesicles (ISEV) definition of "exosome." Furthermore, our findings highlight a favorable impact on the proliferation of intestinal epithelial cells. While these results provide promising insights, additional investigations into the biological relevance concerning development and potential therapeutic applications are warranted. Notably, third-trimester human amniotic fluid exosomes have exhibited promise in the treatment of pulmonary diseases, as evidenced in previous studies [71, 108]. However, their potential as a therapeutic intervention for necrotizing enterocolitis (NEC) remains unexplored, presenting an avenue for future research and clinical exploration.

4. Cell Viability, TEER, and RT-qPCR in an In-Vitro Model of NEC

4.1 Study Design

Despite medical and engineering innovations, the rate of prematurity mortality at 22-27 weeks gestational age remains disproportionally high [109]. One of the leading causes of mortality is due to immaturity of the gastrointestinal (GI) tract and immune system leading to Necrotizing enterocolitis (NEC). Complications of NEC results in the death of the intestinal tissue and increases the risk of sepsis infection [110]. Despite extensive research there are still no preventative therapies or cures for NEC.

Exosomes are vesicles ranging from 100-150 nm in size and are secreted by all cell types [91]. They contain many bioactive components such as mRNA, miRNA, lipids, and proteins. Exosomes mediate intercellular functioning. McCulloh et al. reported that amniotic fluid derived mesenchymal stem cells exosomes and amniotic fluid derived neural stem cell exosomes reduce the incidence and severity of experimental NEC [57]. Some experiments using human breast milk exosomes have been able to promote intestinal epithelial viability, proliferation, stimulate intestinal stem cell activity, and maintain intestinal epithelial barrier integrity in an in vitro model of NEC [111]. However, there is no research correlating amniotic fluid exosomes and their effect on integrity of the intestinal epithelium.

The purpose of this study is to investigate third trimester human amniotic fluid exosomes's prophylactic effect in an in vitro model of NEC. The in vitro model will consist of T84 intestinal epithelial cells exposed to a

Lipopolysaccharides (LPS) insult to induce inflammation and cell death similar to NEC symptoms. Cell viability and tight junction function will be evaluated using a cell viability assay and Trans-Epithelial Electrical Resistance (TEER) system. It is hypothesized that with exposure to amniotic fluid exosomes cell viability and tight junction function will be preserved compared to cells insulted with LPS. In addition, quantitative reverse transcription polymerase chain reaction (RT-qPCR) will be used to evaluate mRNA gene regulation of tight junction genes of interest (ZO-1, Claudin-1, Occludin) in experimental groups.

4.2 Materials and Methods

4.2.1 Cell Culture, Treatment, and Groups

T84 cells were maintained and cultured per protocol adapted from Motyka et al. [112]. The T84 intestinal epithelial cell line, derived from human colon carcinoma, were cultured in complete growth media made from Dulbecco's Modified Eagles Medium (DMEM) F12 (Hyclone, Logan, UT, cat # SH30272.01), 5% fetal bovine serum (FBS, Hyclone, Logan, UT, cat # SH30070.03IH25-40), and 1% antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA, cat # 15240062). The cell cultures were maintained in an incubator at 37°C with 5% CO_2. All experiments requiring T84 intestinal epithelial cells were conducted below passage 10 at approximately 80% confluence when grown in T25-T75 culture flasks and when transepithelial resistance (TER) was greater than 1,000 $\Omega \cdot cm^2$ when grown on Transwell inserts.

Experimental Groups: The T84 cells were categorized into four groups:

- **Untreated Control Group:** Cells with no added agents.

- **LPS-Stimulated Group:** Cells stimulated by lipopolysaccharide (LPS) for 24 hours.
- **Amniotic Fluid Exosome Pre-treatment + LPS-Stimulated Group:** Cells pre-treated with amniotic fluid exosomes for 48 hours, followed by LPS stimulation for 24 hours.
- **Exosome-Free Amniotic Fluid Pre-treatment + LPS-Stimulated Group:** Cells pre-treated with exosome-free amniotic fluid for 48 hours, followed by LPS stimulation for 24 hours.

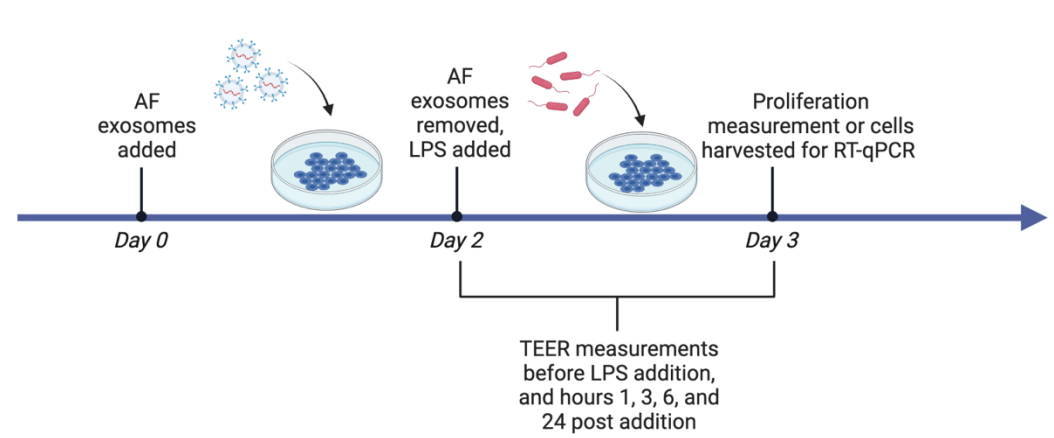

Figure 24: Treatment.

Treatment of intestinal epithelial cells. Created with BioRender.com.

4.2.2 Cell Viability

A cell viability assay adapted from He et al. was used to test amniotic fluid exosomes in an in vitro model of NEC [105]. T84 cells were seeded in 96-well plates in concentrations of 1×10^3 cells/cm^2 with complete growth media made from Dulbecco's Modified Eagles Medium (DMEM) F12 (Hyclone, Logan, UT, cat # SH30272.01), 5% fetal bovine serum (FBS, Hyclone, Logan, UT, cat # SH30070.03IH25-40), and 1% antibiotic/antimycotic (Thermo Fisher Scientific,

Waltham, MA, cat # 15240062). The experimental setups were performed in triplicate and maintained under controlled conditions at 37°C with 5% CO_2. Quantitative assessment of cell viability was conducted using a cell counting kit, CCK-8 (Sigma-Aldrich, Burlington, MA, cat # 96992). CCK-8 uses a tetrazolium salt, WST-8, that is reduced by cellular dehydrogenases to produce a water-soluble orange formazan dye. The amount of formazan dye produced is directly proportional to the number of living cells. Cells were exposed to 150-200µg of amniotic fluid exosomes, determined by Pierce™ BCA Protein Assay Kits following manufactures protocol (Thermo Scientific, Waltham, MA, cat # 23227), for 24-48 hours before insulting with 50-100µg/mL of Lipopolysaccharides (LPS, Invitrogen, Carlsbad, CA, cat # 00-4976-03). The cells were incubated for 1-2 hours with 10uL of CCK-8 at 37°C with 5% CO_2. After 1-2 hours, the fluorescence intensity was measured using excitation and emission at 450nm with a SpectraMax i3X plate reader (Molecular Devices, Silicon Valley, CA).

4.2.3 Trans-Epithelial Electrical Resistance (TEER)

TEER is a widely accepted method to quantify and model barrier function, we used a protocol adapted from Nighot et al. [113]. Before creating the epithelial cell barrier, the transwell inserts were coated overnight with 100 µl of collagen (Gibco, Grand Island, NY, cat # A1048301) diluted to 34 µg/ml in deionized water. One extra well was added to serve as a "blank" and did not receive cells or treatments. When T84 cells were 80% confluent in a T75 flask they were collected for passaging onto 24-well plate transwell inserts (Corning, Corning, NY, cat # 3470). First the collagen suspension was removed and 600 µl of complete growth

media made from Dulbecco's Modified Eagles Medium (DMEM) F12 (Hyclone, Logan, UT, cat # SH30272.01), 5% fetal bovine serum (FBS, Hyclone, Logan, UT, cat # SH30070.03IH25-40), and 1% antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA, cat # 15240062) was added to the bottom chamber. Next, we added $1/12^{th}$ of the total cell suspension in 100 µl of complete growth media to the top chamber. TEER of T84 cells was measured every other day from the day after cell seeding using an EVOM-2 Epithelial Voltohmmeter (World Precision Instruments, Inc., Sarasota, FL, cat # EVOM2) and manually placing two chopstick-style electrodes (World Precision Instruments, Inc., Sarasota, FL, cat # STX2) on each side of a confluent cell layer, seen in Figure 25.

Once the cell membrane layer reached above 1000 $\Omega \bullet cm^2$, cells were considered confluent. Confluent cells were then treated with 200µg amniotic fluid exosomes in 100µl of complete growth media and added to the apical side of the cell membrane for 48 hours. After 48 hours, the media was changed on the apical (200µl) and basolateral (600µl) sides and 100µg/mL of Lipopolysaccharides LPS (Invitrogen, Carlsbad, CA, cat # 00-4976-03) was added to the apical chamber for 24 hours. TEER measurements were taken when exosomes were added (0hr), before LPS was added (48hr), and 1h, 3h, 6h, and 24 hours after LPS addition. The total electrical resistance measured includes the ohmic resistance of the cell layer (R_{TEER}), the ohmic resistance of the cell culture media (R_M), the ohmic resistance of the semipermeable membrane insert (R_I), and the ohmic resistance of the electrode media interface (R_E). Since R_M, R_I, R_E do not change over time, changes in R_{TEER} directly relate to the changes in barrier integrity of the cell layer [114]. The

TEER experiments were conducted in triplicate, with three measurements of electrical resistance recorded for each well at every timepoint.

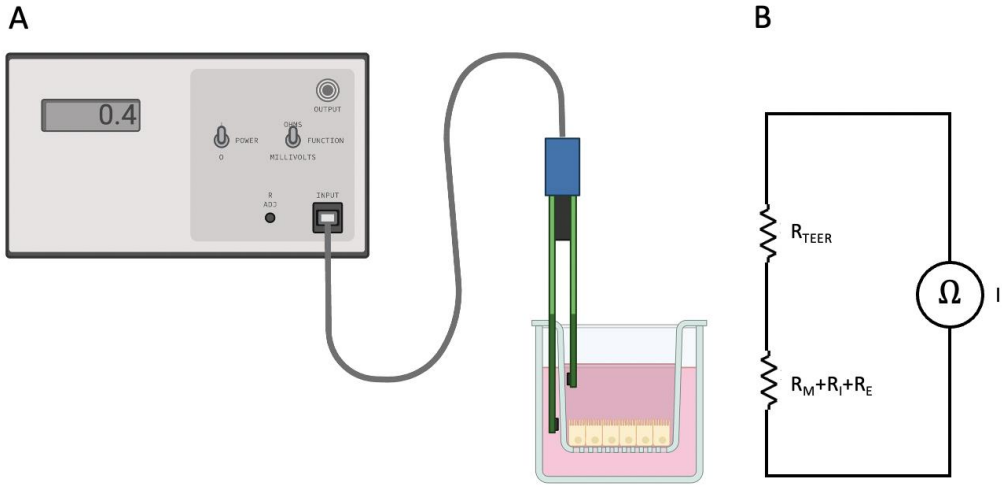

Figure 25: Schematic for the TEER measurement system.

(A) Experimental set up using cells seeded on a semipermeable membrane and a chopstick electrode. (B) Schematic of the total electrical resistance of the system. The total ohmic resistance includes the cell layer (R_{TEER}), the cell culture media (R_M), the semipermeable membrane insert (R_I), and the electrode media interface (R_E). Created with BioRender.com.

4.2.4 Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

In order to measure changes in gene expression, a protocol was adapted from He et al. and Chiba et al. to measure RT-qPCR [105, 115]. First, 1-2x10^6 T84 intestinal epithelial cells were seeded in four T25 flasks with complete growth media made from Dulbecco's Modified Eagles Medium (DMEM) F12 (Hyclone, Logan, UT, cat # SH30272.01), 5% fetal bovine serum (FBS, Hyclone, Logan, UT, cat # SH30070.03IH25-40), and 1% antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA, cat # 15240062). One of the flasks received 100µg/mL amniotic fluid exosomes. Then two flasks were insulted with 10µg/mL LPS. So that three experimental groups were created; untreated control, LPS-stimulated, and

amniotic fluid exosome pre-treatment + LPS-stimulated. RNA extraction was done using the RNeasy Micro kit (Qiagen, Germantown, MA, cat # 74004) according to the manufacturer's protocol. RNA was DNased using a TURBO DNA-free™ Kit (Invitrogen, Carlsbad, CA, cat # AM1907) according to the manufacturer's protocol. Reverse transcription to complementary DNA (cDNA) of 1 µg of Dnased RNA was completed using qScript cDNA SuperMix (QuantaBio, Beverly, MA, cat # 95048-025). qPCR was performed with PerfeCTa® SYBR® Green SuperMix Reaction Mixes (QuantaBio, Beverly, MA, cat # 95054-500) with custom primers ordered from Integrated DNA Technologies (IDT, Newark, NJ). Fold change in gene expression was calculated using the -ΔΔCt method with reference to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene and compared to the control of untreated cell cultures. Gene primer sequences are listed in Table 2.

Table 2: Gene primers and sequences for RT-PCR.

Name	**Forward (5'-3')**	**Reverse (5'-3')**
GAPDH	TTAAAAGCAGCCCTGGTGAC	CTCTGCTCCTCCTGTTCGAC
ZO-1	TGCCATTACACGGTCCTCTG	GGTTCTGCCTCATCATTTCCTC
Claudin-1	TGTCATACCTGTCCATCTTTCTTC	AACATCTCCTGGCATCCTCTTC
Occludin	AGTGTGATAATAGTGAGTGCTATCC	TGTCATACCTGTCCATCTTTCTTC

4.2.5 Statistical Analysis

To ensure robust reproducibility, accuracy, and the identification of genuine phenomena, all experiments within this study underwent rigorous repetition. Replicates were categorized as either "biological replicates" or "technical

replicates" to clarify their nature and facilitate statistical analysis. "Biological replicates" refer to distinct samples that share biological characteristics, such as the same cell type grown under identical conditions, capable of exhibiting biological differences [116]. On the other hand, "technical replicates" involve repeated measurements of the same biological sample, mitigating random variations introduced by protocols and instrumentation noise [117]. For instance, in our study, each well, flask, or plate of cells treated with amniotic fluid exosomes is considered one biological replicate. Specifically, each well in the TEER assay represents a biological replicate, while repeated measures on a qPCR plate are technical replicates, as illustrated in Figure 26.

It is important to note that data from individual women donors cannot be amalgamated with data from other women, as statistical analyses can only be conducted at the level of biological replicates during cell treatment. Our results are specific to individual women and cannot be generalized to the entire pregnant population. Furthermore, the absence of designated "healthy controls" stems from the lack of a universally defined "normal" amniotic fluid. Standard deviation, rather than standard error, is employed for all experiments, as we are measuring biological differences in sample treatments, not the error associated with the measuring equipment. Large standard deviations are expected for patient samples, a common occurrence in human research.

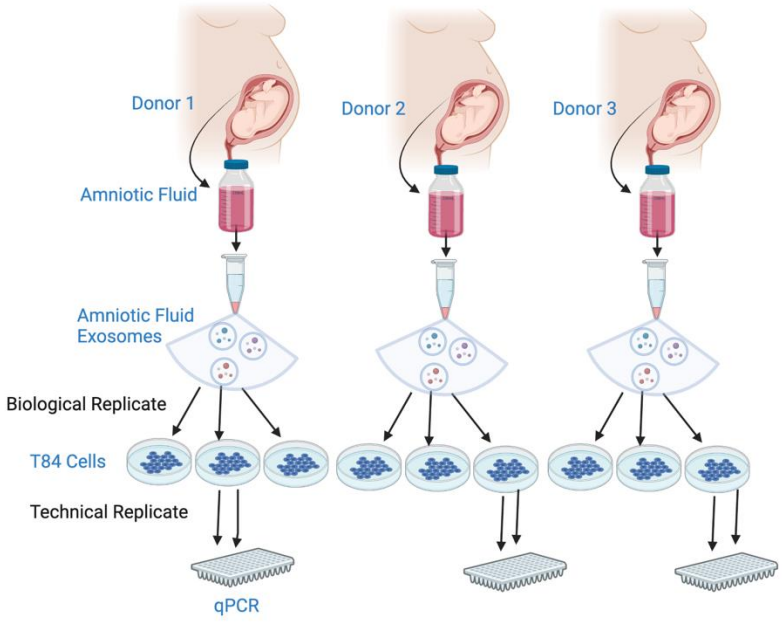

Figure 26: Experimental Design.

Diagrammatic illustration depicting the experimental design for this study utilizing amniotic fluid exosomes and T84 cell culture. Created with BioRender.com.

Data were analyzed using Microsoft Excel and PRISM 9 software (GraphPad, Inc., Boston, MA). Data are presented as mean ± standard deviation from the mean and all experiments were performed in triplicate. Statistical analysis was conducted using either a one-way analysis of variance (ANOVA) for a single independent variable or a two-way ANOVA for two independent variables to identify statistical differences. Asterisks indicates statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

4.3 Results

4.3.1 Cell Viability

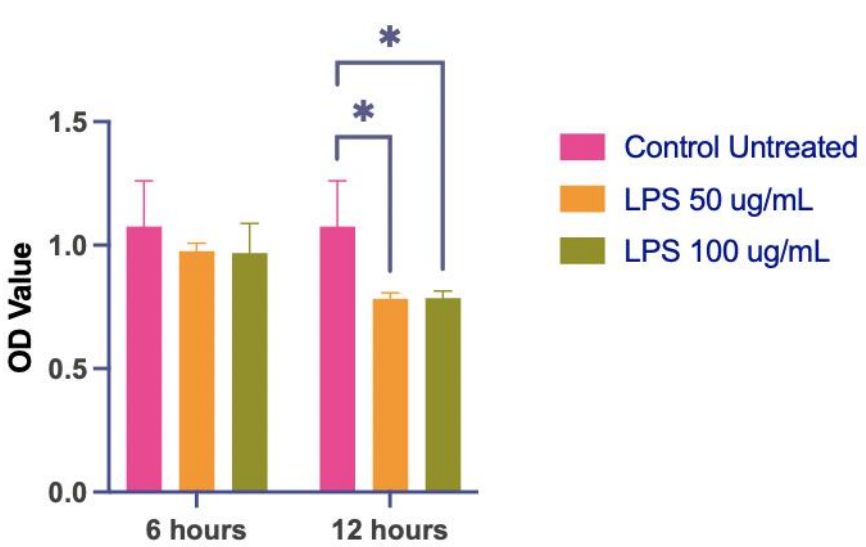

Figure 27: Cell viability.

Viability of intestinal epithelial cells after 6 and 12 hours when exposed to 50µg/mL and 100µg/mL of LPS.

Figure 27 presents the viability of intestinal epithelial cells after 6 and 12 hours when exposed to 50µg/mL and 100µg/mL of LPS. This shows that both concentrations of 50µg/mL and 100µg/mL demonstrated a statistically significant reduction in the viability of T84 intestinal epithelial cells after a 12-hour exposure.

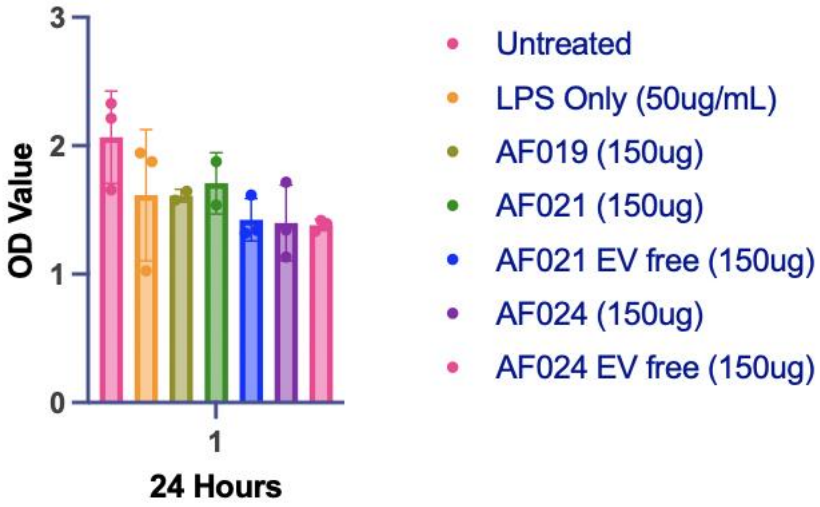

Figure 28: Cell Viability with AF exosomes.

Viability of intestinal epithelial cells exposed to 150µg of amniotic fluid exosomes for 48 hours and 50µg/mL LPS for 24 hours.

Figure 28 presents the cell viability of T84 intestinal epithelial cells pre-treated with 150µg of amniotic fluid exosomes or exosome-free amniotic fluid for 48 hours and exposed to 50µg/mL LPS for 24 hours. This shows that subjecting the intestinal epithelial cells to a 48-hour exposure of 150µg of various amniotic fluid exosome samples (AF019, AF021, and AF024) and their exosome-free supernatant did not change cell viability compared to the LPS only group or control at the 24-hour timepoint. Notably, the group that received LPS only was also not statistically significantly different compared to control.

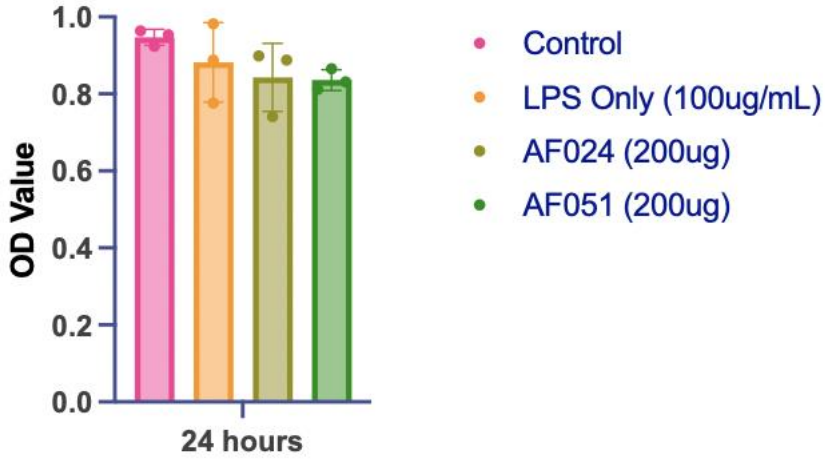

Figure 29: Cell viability with AF exosomes.

Cell viability of intestinal epithelial cells exposed to 200µg amniotic fluid exosomes for 48 hours and 100µg/mL LPS for 24 hours.

Figure 29 presents the cell viability of intestinal epithelial cells exposed to 200µg of amniotic fluid exosomes for 48 hours and 100µg/mL of LPS for 24 hours. This shows a 48-hour exposure to 200µg amniotic fluid exosomes does not change the cell viability of intestinal epithelial cells compared to LPS only or control groups at 24 hours. Additionally, the LPS-only group did not significantly reduce cell viability compared to the control at 24 hours.

4.3.2 Trans-Epithelial Electrical Resistance (TEER)

Figure 30 presents the resistance changes over time in the TEER assay using T84 intestinal epithelial cells pretreated with amniotic fluid exosomes and then insulted with LPS. This shows that the addition of LPS reduces the TEER of the T84 intestinal epithelial cells in a time-dependent manner. The mean TEER of T84 intestinal epithelial cells pretreated with sample AF045 and AF051 amniotic fluid exosomes increased TEER during the culture period and was significantly higher

when compared to untreated control cells at 48-49 hours (Fig. 30B, Fig. 30F). Additionally, the mean TEER of T84 intestinal epithelial cells pretreated with sample AF045, AF049, AF051 amniotic fluid exosomes maintained a relatively stable TEER during LPS insult and was significantly higher when compared to LPS only treated cells at 54-72 hours (Fig. 30B, D, F). Finally, not all treatments increased the mean TEER after 48 hours pretreatment or kept TEER stable after LPS insult; seen in amniotic fluid exosome sample AF024 (Fig. 30H) and exosome free amniotic fluid supernatants (Fig. 30F).

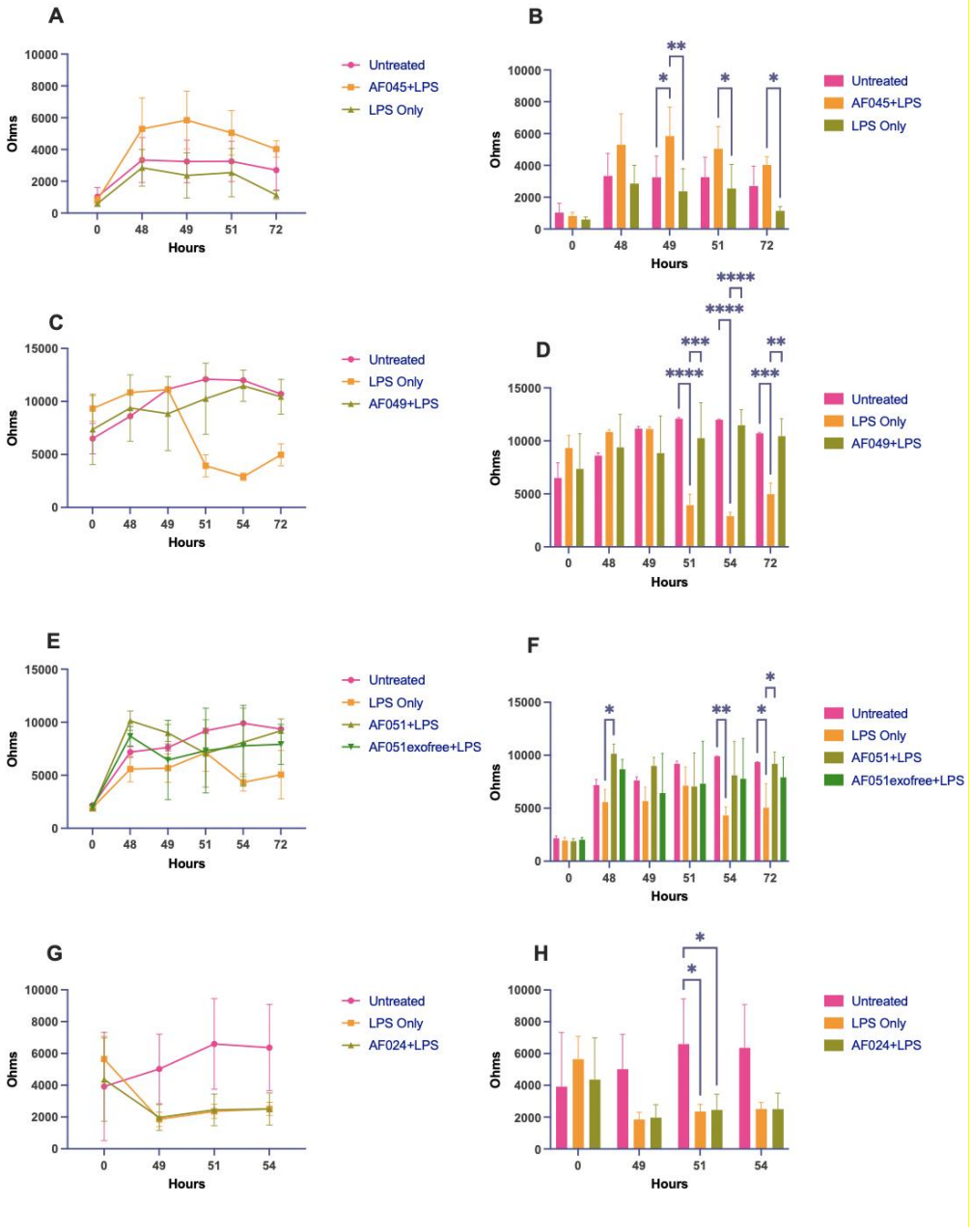

Figure 30: TEER.

Influence of amniotic fluid exosome treatment on TEER. (A) Time-dependent changes in TEER in T84 cells treated with AF045 amniotic fluid exosomes for 48 hours and LPS insult for 24 hours. Values are presented as mean ± SD (n=3). (B) Results of AF045 amniotic fluid exosome treatment two-way ANOVA. (C) Time-dependent changes in TEER in T84 cells treated with AF049 amniotic fluid exosomes for 48 hours and LPS insult for 24 hours. Values are presented as mean ± SD (n=3). (D) Results of AF049 amniotic fluid exosome treatment two-way ANOVA. (E) Time-dependent changes in TEER in T84 cells treated with AF051

amniotic fluid exosomes for 48 hours and LPS insult for 24 hours. Values are presented as mean ± SD (n=3). (F) Results of AF051 amniotic fluid exosome treatment two-way ANOVA. (G) Time-dependent changes in TEER in T84 cells treated with AF024 amniotic fluid exosomes for 48 hours and LPS insult for 24 hours. Values are presented as mean ± SD (n=3). (H) Results of AF024 amniotic fluid exosome treatment two-way ANOVA.

4.3.3 Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

Figure 31 presents the cellular mRNA expression levels of ZO-1, claudin-1, and occludin in an in vitro model of NEC tested with samples AF051 and AF045 by a two-step RT-qPCR. This shows that LPS did not reduce the mRNA expression of epithelial tight junction proteins ZO-1, claudin-1, or occludin. Groups treated with AF051 and AF045 amniotic fluid exosomes did not change tight junction gene expression as compared to control. The RT-qPCR showed relatively similar expression of these proteins for all groups within the variations of the technique.

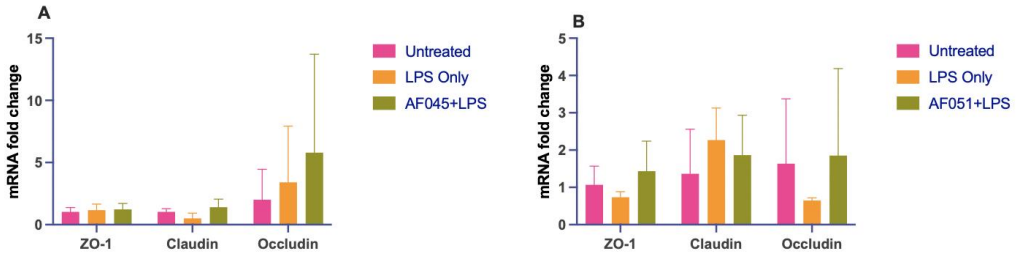

Figure 31:RT-qPCR.

The transcription of the intestinal epithelial tight junction proteins of different groups carried out from cell lines. (A) The difference in mRNA expression levels of the intestinal epithelial tight junction proteins of untreated control, LPS treated, and AF045 pretreated plus LPS treated carried out from the T84 cell line. (B) The difference in mRNA expression levels of the intestinal epithelial tight junction proteins of untreated control, LPS treated, and AF051 pretreated plus LPS treated carried out from the T84 cell line.

4.4 Discussion

Since the pathophysiology of NEC is not fully understood, there are no animal models or in-vitro models that perfectly mimic NEC either. However, a combination of environmental and microbial factors applied to intestinal epithelial cells can trigger or imitate NEC for research purposes [118]. Adding products secreted from colonizing bacteria like bacterial LPS, a cell wall component characteristic of gram-negative bacteria, can lead to the production of inflammatory mediators and reduce cell viability [118]. We used this in-vitro model of applied LPS with a colonic adenocarcinoma-derived epithelial cell line, T84, to test the functionality of an intestinal epithelial barrier [119]. NEC is known for its rapid progression, often advancing from initial symptoms to full-blown disease and death within 24–48 hours [21]. Consequently, we have opted to evaluate the prophylactic potential of amniotic fluid exosomes, focusing on prevention rather than therapeutic intervention.

In healthy individuals, typical plasma concentrations of LPS fall within the range of 0-0.2 ng/mL [113, 120]. In contrast, patients with intestinal permeability disorders, such as NEC, may exhibit higher plasma concentrations of LPS, ranging from 2-10 ng/mL [113, 120]. It's worth noting that extremely elevated pharmacological concentrations of LPS, ranging from 50-1000 µg/mL, can lead to sudden cell death in intestinal cells and result in the loss of intestinal barrier integrity, resembling symptoms observed in NEC [120]. To align with these considerations, we opted to test concentrations of 50-100 µg/mL in our cell viability and TEER experiments. In assessing the in vitro model of NEC, our aim was to

choose a sufficiently long exposure time and a high enough concentration of LPS to ensure detectable alterations in the viability of intestinal epithelial cells. Viability testing showed that cytotoxicity of both 50 µg/mL and 100 µg/mL LPS was sufficient to statistically reduce the number of viable intestinal epithelial cells after 12 hours.

However, in subsequent experiments measuring both LPS cytotoxicity and T84 cell proliferation (with a doubling time of 33.9 hours), the detrimental effects of 50 µg/mL and 100 µg/mL of LPS on T84 cell viability were not distinguishable from the control at 24 hours [121]. Moreover, cells treated with amniotic fluid exosomes did not exhibit statistically significant differences compared to untreated or LPS-treated cell groups. From literature it is seen that concentrations of up to 100 ng/mL did not induce cell death on Caco-2 intestinal epithelial cells, but a concentration of 1 mg/mL did [120]. Therefore, it is possible that our concentration of LPS was too low to cause a large enough negative effect on cell viability at 24 hours. However, other studies have exposed 10 µg/mL of LPS for 12 hours with Caco-2 and NCM460 intestinal epithelial cells and seen a difference between the LPS group and untreated controls. Therefore, it could also be the cell type we are using in our model. Finally, the initial reduction in cell viability induced by LPS at 12 hours might have been offset by subsequent cell proliferation at 24 hours, diminishing the overall impact of LPS and rendering the experimental groups indistinguishable from the untreated control.

Most of the current research involving NEC focuses on immunoregulatory changes but using an in vitro model of NEC with the TEER system allows us to

inspect the barrier function of intestinal epithelial cells in disease progression like NEC. The in-vitro model of NEC uses pro-inflammatory cytokine inducing LPS that causes epithelial injury (inflammation and cytokine release, disruption of tight junctions, and apoptosis) leading to increased permeability [3].

In the TEER studies prior to the LPS insult, the 48-hour pretreatment with amniotic fluid exosomes (AF045 and AF051) significantly increased transepithelial electrical resistance at the 48-hour mark as compared to control. This aligns with the proliferation data presented in Chapter 3, demonstrating that cells exposed to amniotic fluid exosomes exhibited increased proliferation compared to the control. Higher TEER values are associated with increased cell proliferation. Similar TEER improvements were reported in the literature for human milk exosome-treated Caco-2 cells compared to the control, although it took a longer duration to achieve significance—3 to 9 days after initiating treatment [115].

Pretreatment with amniotic fluid exosomes (AF045, AF049, AF051) significantly increased transepithelial electrical resistance compared to the LPS-only experimental group between 6 and 24 hours of LPS exposure. This indicates that exposure of intestinal epithelial cells to amniotic fluid exosomes facilitated cellular recovery from the LPS insult, maintaining resistance and intestinal barrier function similar to the untreated control cells. This is significant as the selected LPS insult concentration of 100 µg/mL is 10,000 times higher than concentrations typically observed in human plasma, even in cases of NEC [113, 120]. Despite this elevated challenge, exosomes demonstrated efficacy in preserving intestinal barrier function, aligning with the control group. However, it is important to note that not

all amniotic fluid exosome samples exhibited a prophylactic effect, underscoring the variability in sample collection and isolation.

The time it took for the LPS insult to induce a change in TEER varied across experiments. Nonetheless, existing literature indicates that when lower concentrations of LPS (0.3 ng/mL) are introduced to intestinal epithelial cells, it may take up to 4 or 5 days to observe alterations in TEER [120]. Therefore, the observed reduction in LPS resistance within our experiment over a few hours is reasonable.

Taking the cell viability data combined with TEER findings, suggest that the impact of LPS on T84 tight junction permeability may not solely be attributed to cell death causing disruptions in the integrity of the epithelial barrier. Per the literature, the changes in TEER measurements happen when LPS triggers the initiation of the TLR-4 signal transduction cascade, resulting in the phosphorylation and activation of membrane-associated adaptor protein FAK in intestinal epithelial cells. The activated FAK in enterocytes then governs the activation of MyD88, transmitting signals that ultimately lead to the opening of the intestinal tight junction barrier without causing cell death [122]. To investigate further, we looked at tight junction gene regulation between the experimental groups.

Crucial for maintaining the barrier function of intestinal cells are tight junction proteins, which include zonula occludins (ZOs), occludin, claudins, cinglin, tricellulin, and junction adhesion molecules [115, 123]. To represent these proteins, we focused our investigation on ZO-1, claudin-1, and occludin. However, the observed non-statistically significant increases in tight junction gene expression

following amniotic fluid exosome treatment suggest that the prophylactic effect observed in the TEER assay is not a result of enhanced tight junction gene expression. This implies the need to investigate an alternative molecular process mediating the restoration of the intestinal tight junction barrier during LPS-induced inflammation. Existing literature suggests that the localization and expression of tight junction proteins, including ZO-1, claudin-1, and occludin, remain unaffected by LPS [113]. Instead, the LPS-induced increase in permeability is mediated by the TLR-4/Myd88 signaling pathway and an upregulation of MLCK protein expression [113]. This cellular process warrants further investigation. It is also possible that an insufficient amount of amniotic fluid exosome samples were delivered to the cells, preventing a significant difference in mRNA gene regulation. Further exploration of exosome dosing for this experiment is also warranted.

Understanding the pathophysiology of necrotizing enterocolitis (NEC) remains a challenge, and replicating it in vitro is inherently complex. In the absence of a comprehensive understanding, creating in vitro models that precisely mimic NEC is particularly challenging. Our in vitro model, for example, lacks multiple cell types including immune cells, a critical element in NEC development. While we focus on studying intestinal epithelial barrier function, our model lacks the mucus layer, the primary defense against external molecules reaching the gut lumen [118].

Another notable limitation is the absence of knowledge regarding the normal distribution, content, and functional role of amniotic fluid exosomes. This knowledge gap restricts our ability to interpret our findings accurately as compared

to a norm. Additionally, our study's outcomes are specific to individual women and should not be generalized to the entire pregnant population. Consequently, the therapeutic potential of most amniotic fluid remains uncertain. These limitations underscore the need for further research and a cautious interpretation of our study's implications.

Future research could examine exosome contents and try to identify the specific nucleic acid or protein messengers responsible for the observed beneficial effects. Exploring a dose-response curve would provide valuable insights into the optimal concentration of exosomes for eliciting these benefits. We could also look into the therapeutic value of amniotic fluid exosomes and treat cells after the addition of LPS. Also, there is an exciting avenue to explore the functional and therapeutic roles of third-trimester amniotic fluid exosomes in other diseases models where improved intestinal barrier function is needed, such as digestive disease-induced colitis, leaky gut syndrome, inflammatory bowel diseases, irritable bowel syndrome, and Celiac disease. However, these amniotic fluid exosomes have also been implicated in various other potential therapeutic applications, including improved spermatogenesis, mitigating bronchopulmonary dysplasia, and alleviating Covid-19 symptoms therefore further investigation into various indications is also warranted [71, 108, 124]. Lastly, a crucial step forward involves determining whether amniotic fluid exosomes can prevent necrotizing enterocolitis (NEC) in vivo, investigating intestinal mucosal injury and inflammation in animal models. These future studies collectively aim to advance our knowledge and potentially pave the way for innovative therapeutic applications.

4.5 Conclusion

Our study demonstrates that selective amniotic fluid exosome samples exert a beneficial effect on intestinal epithelial cell barrier function and prevent barrier function injury caused by LPS. This effect was not solely due to exosome induced increase in proliferation or changes in mRNA expression of intestinal epithelial tight junction proteins ZO-1, claudin-1, or occludin. Therefore, other mechanisms of action need to be explored. NEC is characterized by intestinal tissue necrosis and bacterial translocation leading to systemic sepsis, if third trimester human amniotic fluid exosome exposure to intestinal epithelial cells maintained tight junction integrity it would warrant further therapeutic development.

5. Translational Value

5.1 Customer and Market Analysis

As of 2022, the preterm birth rate in the United States stood at 10.38% of total births, resulting in 380,034 preterm births [125]. Out of these, 9.5%, or 347,815 neonates require admission to neonatal intensive care units (NICUs) [126]. The NICU visit is structured under a bundle payment system with insurance companies, excluding coverage for enteral nutrition. Hospitals take on the responsibility of funding supplemental nutrition to mother's own breast milk (MOM). Their decisions in this regard includes minimizing the risk of Necrotizing enterocolitis (NEC), which is the most common and severe gastrointestinal emergency during the neonatal period.

A study examining US infant deaths from 1999 to 2020 revealed that out of 88,125,233 live births, 8,951 infants died of NEC, with higher rates among Black infants (16.1 per 100,000 live births) compared to White infants (6.4 per 100,000 live births) [127]. Surgery is necessary in 20-30% of NEC cases, with NEC surgery having fatality rates as high as 50%, and interventions and extended hospital stays for NEC accounting for 20% of all NICU costs [128]. Research indicates that an exclusive human milk-based diet (EHMD), excluding formula or bovine-derived fortifiers, significantly reduces the risk of NEC, mortality, and expected hospitalization costs, resulting in a net direct saving to the hospital of $8,167.17 per infant [129-131]. Fortifiers are mandated for all preterm infants under 30 weeks of gestation, and Prolacta Bioscience Inc. stands as the exclusive provider of a fortifier derived from human breast milk. However, the expense associated with

implementing EHMD with Prolacta's fortifier amounts to $7,731 per hospital stay, in stark contrast to the $226 cost for the standard care practice, involving the use of mother's expressed breast milk supplemented with a cow's milk-based fortifier [132]. Given that nutritional supplementation is funded by the hospital's budget, the inclusion of Prolacta's fortifier, even for only the most vulnerable extremely premature infants, can pose a significant financial challenge. This makes Prolacta a viable choice for only a small percentage of privately-owned hospitals with larger enteral nutrition budgets, leaving an opportunity for non-privately owned hospitals in need of a lower-cost preventative therapy that can be used with bovine fortifiers while still reducing the risk of NEC, mortality, and hospital expenses.

5.2 Product

Our amniotic fluid exosome therapy is a biologically advanced supplement to breastmilk, formula, and fortifiers for preterm babies in the NICU. The product formulation would be a vial of frozen exosomes from human amniotic fluid in sterile phosphate buffered saline (PBS). The exosomes will be isolated from donated third trimester amniotic fluid collected at Cesarean sections using ultracentrifuge. The concentration of exosomes will be optimized for the application of gastrointestinal (GI) epithelial cell health, proliferation, and barrier integrity. The value of this therapy is the ability to mimic part of the natural environment of the developmental timeline in a hospital setting and specifically improve intestinal barrier integrity for reduced bacterial translocation. The final "off the shelf" product will be sold directly to hospitals and reimbursed by insurance as a drug needed for at-risk neonates. The product will be stored in their

NICU floor freezers in aliquots that could be thawed and added directly to breastmilk or formula before enteral feedings by nurses. If enteral feedings are reduced or removed, the product could be administered by enema. This product could then transition to an adult market and treat gastrointestinal disorders such as Crohn's disease, inflammatory bowel disease, malabsorption syndromes, and ulcerative colitis.

5.3 Competitive Technologies with a NEC Indication

The primary strategy for reducing NEC in the Neonatal Intensive Care Unit (NICU) is the utilization of mother's own milk (MOM), recognized for its protective effects [133]. Initiatives promoting MOM production, with a focus on reducing care disparities, involve health programs and breastfeeding education [134]. In instances where MOM is unavailable, donor breast milk (DBM) serves as a valuable supplement, significantly lowering NEC occurrence compared to formula feeding [135]. Hospitals save costs by minimizing the length of stay when instances of NEC and sepsis are reduced. Estimates suggest an 18-day stay for medical NEC and a 50-day stay for surgical NEC [136]. The confirmed cost of medical NEC is an additional $138,000 per surviving infant, while surgical NEC costs an additional $238,000 per infant [134]. However, MOM and DBM may not consistently meet nutritional goals for very low birth weight (VLBW) infants in the NICU. The American Academy of Pediatrics (AAP) feeding guidelines allow for the addition of either a bovine milk-based milk fortifier or a human milk-based human milk fortifier to ensure adequate nutrition. However, a 10% increase in the

volume of cow milk-containing milk elevates the risk of NEC, surgical NEC, and sepsis [137].

Prolacta Bioscience Inc makes a breast milk caloric fortifier with pasteurized human milk cream derived from human milk. The breast milk is 25% fat and provides 2.5 Cal/mL. Prolacta CR human milk is the only completely human solution that adds calories to MOM or DBM without increasing non-human based nutritional products. Clinical evidence supports that an exclusive human diet (EHMD: the addition of human milk-based fortifiers to MOM or DHM), can develop healthy immune systems, and decrease rates of comorbidities such as NEC and decrease mortality [138][139]. The products costs about $180 an ounce, and a baby would typically consume $10,000 worth over its several week stay in the NICU. Generally, the cost is paid by the hospital or insurers, not the parents. The EHMD offers savings to the hospitals with cost avoidance strategies reducing medical and surgical NEC rates of $515,113-$3,369,515 annually per institution [139]. The EHMD's ability to reduce the length of stay is what causes the most sizable impact on the total cost. These cost concerns such as reimbursement, as well as a lack of standard feeding guidelines are just a few of the obstacles preventing EHMD adoption.

Alternative strategies include Noveome Biotherapeutics Inc.'s ST266, an amnion-derived cellular cytokine solution created from amnion-derived multipotent cell secretome, encompassing Vascular Endothelial Growth Factor (VEGF), angiogenin, Platelet-Derived Growth Factor (PDGF), Transforming Growth Factor-β (TGF-β), Tissue Inhibitor of Metalloproteinase-1 (TIMP-1), and

Tissue Inhibitor of Metalloproteinase-2 (TIMP-2) [140]. ST266 is produced through novel cell selection and culture in a bioreactor, enabling scalable production compared to breast milk or amniotic fluid products. Preclinical studies demonstrate ST266's efficacy in preventing and treating NEC in mouse and pig models, promoting intestinal barrier integrity, anti-inflammatory effects, and improved gut development, ultimately reducing mortality [53]. The FDA has granted Rare Pediatric Disease Designation (RPDD) and Orphan Drug Designation (ODD) to Noveome Biotherapeutics Inc.'s for ST266 in NEC treatment, providing benefits like a Priority Review Voucher (PRV), expedited review, discounted registration fees, and seven years of market exclusivity post-approval. Noveome Biotherapeutics Inc.'s has submitted an investigational new drug (IND) application for ST266, having recently secured a $40 million Series E funding round to support NEC clinical trials [141]. However, as of now, there is no human data, necessitating further exploration of potential adverse side effects. Despite being more likely to receive reimbursement as a drug rather than a nutritional supplement like breast milk and fortifier, it is essential to await human trial results for a comprehensive assessment.

While other applications like breast milk exosomes [142][105, 143] and stem cells exosomes [57] have shown promise in pre-clinical scientific publications, they have not applied for a clinical trial and no companies are championing them as products. Most NEC related clinical trials are focused on clinical biomarkers, surgical interventions, feeding and NICU protocol changes, imaging, blood transfusions, and probiotics.

5.4 Competitive Technologies without a NEC Indication

Organicell Regenerative Medicine, Inc. has introduced Zofin, a cell-free amniotic fluid enriched with a high concentration of exosome particles. This formulation comprises more than 300 growth factors, cytokines, chemokines, 102 unique microRNAs, and additional exosomes/nanoparticles sourced from donated third trimester amniotic fluid. The product is manufactured using centrifugation, ultracentrifugation, and sterile filtration. The final exosome pellet is resuspended with amniotic fluid supernatant saved from an earlier centrifugation step. The scaling up of this product would require more amniotic fluid donations.

Zofin has obtained Investigational New Drug (IND) approval from the FDA and recently completed Phase 1 studies, focusing on Bronchopulmonary Dysplasia and patients with moderate-to-severe COVID-19 [71, 108]. The trials involved over 20 patients, with no reported therapy-related safety events or significant adverse events [144]. The therapeutic potential of Zofin is due to the presence of anti-inflammatory nucleotides and proteins within extracellular vesicles (EVs). Notably, the product has not undergone testing for gastrointestinal indications.

Merakris Therapeutics Inc. is a biotechnology company focused on the use of cell-derived proteins and nano vesicles derived from amniotic fluid and placental membranes for a variety of applications including regenerative allograft coverings and buffers for chronic and acute wound care. Their flagship product, Dermacyte, is a wound repair matrix made from the preserved epithelial basement membrane and compact fibroblast layer of the amnion membrane. Dermacyte is used for wounds such as diabetic foot ulcers, surgical incisions, burns or trauma. Current

research has shown Dermacyte used with Dermacyte Liquid, an injectable cell free amniotic fluid containing exosomes, are effective tools in the treatment of non-healing wounds like venous leg ulcers [145]. Dermacyte Liquid's actual mechanism of action has not been elucidated. The Dermacyte Liquid is made by the isolation of amniotic micro-vesicles using ultracentrifugation, sucrose density gradient centrifuge, column chromatography, size exclusion, and filtration.

5.5 Patent Landscape

Prolacta Bioscience has 23 patents; however, all these patents are related to breast milk manipulation and therefore do not interfere with the development of our therapy. Organicell Regenerative Medicine, Inc filed a utility patent application on April 10, 2020 on their product, Zofin, that includes oral admission, miRNA composition, and target applications including to the GI tract but NEC is not listed as an indication, see Table 1. Merakris Therapeutics Inc have a patent around the isolation and use of amniotic fluid extracellular vesicles for wound healing, but it does not mention any indication for NEC, see Table 1. Patents for exosome isolation methods are becoming more abundant. There are currently no patents that would inhibit our use of exosomes from amniotic fluid in an oral or rectal application indicated for prevention of NEC by improved barrier integrity. A provisional patent was filed for our product in November of 2023 with the title "Amniotic Fluid/Membrane Therapy (AFMT) for the Prevention of Necrotizing Enterocolitis (NEC)" (Application Number 63011146).

Table 3: Patents Similar including Amniotic Fluid Exosome

Company	Patent Number	Title
Organicell Regenerative Medicine Inc.	US 2023/0092673 A1	Compositions Comprising Nanoparticles, Methods of Making and Uses Thereof
Merakris Therapeutics Inc.	US 2022/0133806 A1	Amniotic Fluid-derived Extracellular Vesicles and Uses Thereof for Wound Healing

5.6 FDA Regulatory Pathway

The development of human extracellular vesicle-based therapeutics, including exosomes, are subject to regulatory review due to their biological medical product status. In the US, these products will be evaluated by The Center for Biologics Evaluation and Research (CBER) within the Food and Drug Administration (FDA). Regulatory frameworks for Good Manufacturing Practices (GMP) already exist and include adequate technical equipment, established engineering systems, trained personnel, and quality management systems [68]. Donor safety, recipient safety, and release criteria including extracellular characterization, hypothesized mechanism of action, and microbiological controls will all be evaluated before clinical trials begin [68]. Currently, there are no standardized protocols for isolation or storage of extracellular vesicles, we will

need to develop tailor-made protocols. The stability of stored extracellular vesicles must be investigated for our product as well.

To sell this product we will require an Investigational New Drug (IND) application to begin Phase I safety and efficacy trials. Phase I-II will evaluate safety, toxicity, and immunogenicity while Phase III-IV will evaluate long-term adverse effects and efficacy [68]. After the IND application, a New Drug Application (NDA) and a Biologics/Product License Application (BLA) would be needed to formally propose a new pharmaceutical for sale and marketing in the U.S. We can concurrently apply for Rare Pediatric Disease Designation and Orphan Drug Designation which would allow for priority review, tax credit for qualified clinical trials, exemption from user fees, and potential seven years of market exclusivity after approval. The timeline for our therapy to get to market as a biologic is 10 years.

5.7 Proposed Exit Strategy

Therapeutic efficacy, pre-clinical studies, and patent applications can be completed within Tulane University (TU) supported by grant funding. Once our formulation is protected, we can exclusively license our technology from TU to other companies. We will look for regenerative medicine companies like Organicell Regenerative Medicine, Inc and convince them of the therapeutic efficacy and market value of third trimester human amniotic fluid exosomes as a preventative therapy for NEC in the hopes they will add it to their portfolio.

6. Conclusion and Future Studies

6.1 Conclusion

This research underlines the pivotal role of amniotic fluid in fetal gastrointestinal development and the heightened health risks, particularly the occurrence of necrotizing enterocolitis (NEC), in prematurely born infants removed from amniotic fluid. NEC, a leading cause of gastrointestinal disease-related mortality in preterm infants, is associated with exaggerated toll-like receptor 4 (TLR4) signaling, leads to intestinal inflammation, weaken intestinal barrier function, and bacterial translocation. Diverse studies on amniotic fluid, including whole amniotic fluid, stimulated amniotic fluid, cell-free amniotic fluid, and amniotic fluid stem cells and extracellular vesicles, reveal promising preventive and therapeutic potential for NEC.

The investigation into third-trimester amniotic fluid cells uncovered donor variability and heterogeneity within cell populations. While these cells do not meet the criteria for true MSCs due to their lack of differentiation potential, they may represent another class of progenitor cells not aligning with established criteria for adult MSCs. Given the absence of a clearly defined cell population and the limited translational potential of stem cell therapies approved by the FDA, these cells were not further investigated for their preventative or therapeutic potential against NEC.

After our successful isolation of human amniotic fluid extracellular vesicles through ultracentrifugation and the comprehensive characterization of extracellular vesicles as exosomes, we were able to show a favorable impact of amniotic fluid exosomes on the proliferation of intestinal epithelial cells. While promising, these

results call for further investigations into the biological relevance for development and potential therapeutic applications. NEC is known for its rapid progression, often advancing from initial symptoms to full-blown disease and death within 24–48 hours [21]. Due to this, we opted to evaluate the prophylactic potential of amniotic fluid exosomes, focusing on prevention rather than therapeutic intervention.

In our final studies, selective amniotic fluid exosome samples exhibited a beneficial effect on intestinal epithelial cell barrier function and prevented injury caused by lipopolysaccharide (LPS). This preventative effect is not solely attributed to increased proliferation or changes in mRNA expression of tight junction proteins. Therefore, other mechanisms of action need exploration.

Standardizing processes for isolating, purifying, quantifying, and characterizing amniotic products, including stem cells and extracellular vesicles, is crucial for advancing research and translational products. Regulatory changes by the FDA post-May 31, 2021, necessitate Biological License Applications (BLA), emphasizing the need for robust evidence of repeatable isolation and characterization, hypothesized mechanism of action, patient safety, and effectiveness.

6.2 Future studies

Our cellular work highlights the necessity for standardized collection protocols and improved isolation techniques for homogenous cell populations. Additional characterization, manufacturing, and medical investigations are imperative for a comprehensive understanding and utilization of cultured amniotic

fluid cells in clinical applications.

Future characterizations of third-trimester exosomes, an expanded flow panel can identify cells of origin within the mixed population. Cargo characterization through proteomics, lipidomics, metabolomics, or next-generation sequencing can provide insights into origin and function. Further studies can distinguish exosome subtypes, such as large exosomes, small exosomes, and exomeres, with comprehensive functional analyses to understand their specific roles.

Future research on amniotic fluid exosomes could focus on identifying specific nucleic acids or proteins responsible for observed beneficial effects. Exploring a dose-response curve would offer insights into optimal exosome concentrations for desired benefits. Investigating the therapeutic value of amniotic fluid exosomes in treating cells after LPS addition is another avenue. Additionally, exploring the functional and therapeutic roles of third-trimester amniotic fluid exosomes in various disease models with intestinal barrier dysfunction, such as colitis, leaky gut syndrome, inflammatory bowel diseases, irritable bowel syndrome, and Celiac disease, presents exciting opportunities. These exosomes have potential applications in improving spermatogenesis, mitigating bronchopulmonary dysplasia, and alleviating Covid-19 symptoms, warranting further investigation into various indications [71, 108, 124]. Lastly, determining whether amniotic fluid exosomes can prevent necrotizing enterocolitis (NEC) in vivo by investigating intestinal mucosal injury and inflammation in animal models

is a crucial step forward. Collectively, these future studies aim to advance knowledge and potentially open doors to innovative therapeutic applications.

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[Appendix]

Table 1: Donor Information

Sample ID	Age (years)	Height	Weight (Kg)
TM2001-22	29	4'6''	111
TM1912-10	24	5'6''	122
TM1912-24	22	5'8''	98
TM1912-32	29	5'4''	127
TM1912-15	34	5'4''	52
TM1912-12	27	5'2''	81
TM1912-08	22	5'4''	84
TM1912-03	36	5'3''	75
TM1911-49	32	5'5''	100
TM1911-48	26	5'1''	87.9
TM1911-32	25	5'2''	N/A
TM1911-20	25	5'0''	90.7
TM1911-14	37	5'4''	100
TM1911-17	21	5'9''	80
TM1911-07	34	5'6''	77
TM1910-28	19	5'4''	77
TM1910-20	27	N/A	104
TM1910-09	25	5'9''	128.8
TM1908-20	32	5'5''	112

TM1908-07	24	5'2''	95
TM1908-18	37	5'2''	76.2
TM1908-08	22	5'8''	113
TM1908-03	27	5'4''	85.3
TM1907-45	31	5'2''	97
TM1907-35	30	5'5''	70
TM1906-27	31	5'9''	127.27
TM1906-05	22	5'7''	79
TM1905-22	28	5'7''	68.95
TM1902-28	31	5'7''	171
TM1905-20	30	5'10''	157
TM2001-14	30	N/A	83
TM1912-18	23	5'0''	75

Table 2: Amniotic Fluid Donation Volumes

Sample	Volume (mL)
AF007	170
AF008	275
AF009	100
AF010	300
AF011	325
AF012	250
AF013	125
AF014	299
AF015	275
AF016	130
AF018	195
AF019	321

AF020	95
AF021	145
AF023	180
AF024	340
AF025	115
AF026	280
AF027	127
AF028	190
AF029 T1	81
AF029 T2	110
AF030	300
AF031	195
AF032	140
AF033	340
AF034	150
AF035	335
AF036	177
AF037	270
AF038	150
AF039	356
AF040	100
AF041	75
AF042	325
AF043	200
AF044	125
AF045	350
AF046	165
AF047	245
AF048	200
AF049	280
AF050	200
AF051	700