Bronchopulmonary Dysplasia

Bronchopulmonary Dysplasia: New Developments in Treatment and Prevention

Dissertation

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Prof. dr. Rianne M. Letschert

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by

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Justification of the Thesis

Bronchopulmonary dysplasia (BPD) is a form of chronic lung disease, peculiar to the extremely preterm infant, born at the early stages of lung development (1). Up to 40% of the infants below 29 weeks' gestation are diagnosed with BPD (2), while the composite outcome death and/or BPD occurs in 65% of the infants below 29 weeks' gestation (2). BPD lungs consist of enlarged and simplified breathing structures, as opposed to the alveolar ducts at term, characterized by smaller, more numerous and more complex alveoli, essential to ensure adequate gas exchange (1). BPD is also characterized by an abnormal distribution of the pulmonary vessels and a reduction in the number of the small arteries, which are functionally hyper-reactive and hypertonic, culminating in pulmonary arterial hypertension (3). Impaired pulmonary vascular growth may contribute to the irreversible arrest of lung development typical of BPD (4). Early and late pulmonary hypertension, assessed by indirect echocardiographic measurements, is detected in approximately 20% to 25% of the infants suffering from BPD, worsening their outcome (3, 5, 6).

Early birth itself primarily interrupts the intricate cascade of biochemical and hormonal factors that hesitate in full and proper lung development. Consistently, low gestational age (GA) and low birth weight (BW) are the major determinants of BPD (7). However, BPD is a multifactorial disease, being the consequence of prenatal and post-natal inflammatory, oxidative, anti-angiogenic and pro-fibrotic stimuli on the developing lung (8-11). Multiple modifiable factors have been associated with an increased risk of BPD. These include, among others, maternal smoking (12, 13), placental dysfunction (14), duration of mechanical ventilation (15), cumulative supplemental oxygen (16), postnatal growth failure (17), necrotizing enterocolitis (15), and late-onset sepsis (18, 19). Other elements, such as gestational hypertension (20-22), chorioamnionitis (23, 24) and patent ductus arteriosus (22, 25) have been inconsistently linked to BPD.

Despite the continuous advances of perinatal care, BPD remains a significant burden of extreme prematurity, because it lacks a safe and effective treatment and/ or prevention strategies. The structural arrest of lung development makes traditional therapies ineffective in treating this disease (26). In the past few years, increasing insight into stem cell biology has generated excitement about the potential of stem cells to regenerate damaged organs. Among stem cells, mesenchymal stromal (stem) cells (MSCs) have attracted much attention because of their ease of isolation, multilineage potential, and immunomodulatory properties (27). Perivascular cells (PCs) from diverse human tissues give rise to adherent multilineage progenitor cells that exhibit all the features of MSCs and may embody the precursors of MSCs (28, 29). MSCs and PCs may represent a novel therapeutical option for so far untreatable diseases, including BPD.

While the definitive prevention of BPD could only be obtained by avoiding preterm birth, prenatal and postnatal preventive efforts are also directed at the reduction of the other

stressors that may worsen the injury to the developing lung (30). Among other, human milk and probiotics have been proposed as promising options (31).

Aim and Outline of the Thesis

The present thesis is a collection of studies aimed at investigating the potential use of **mesenchymal stem cells and pericytes** in the treatment of BPD and evaluating the possible role of **probiotic supplementation** and **mother's own milk** in the prevention of BPD.

Chapter I is a general introduction in which we reviewed the pathophysiology and treatment of BPD with special emphasis on the role of MSCs.

In **Chapter II** we tested the potential of MSCs and their precursors (pericytes) in preventing and treating oxygen-induced lung injury in a murine model of BPD.

In **Chapter III** we performed a Cochrane meta-analysis to investigate the role of MSCs in the treatment of BPD.

In **Chapter IV** we performed a systematic review and meta-analysis to test the potential of probiotics in preventing BPD.

In **Chapter V** we performed a systematic review and meta-analysis to test the potential of mother's own milk in preventing BPD.

Finally, in **Chapter VI** a general discussion of the thesis is outlined.

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CHAPTER I:

INTRODUCTION*

*Based on:

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1. Definition and incidence of bronchopulmonary dysplasia

The revolutionary progress of perinatal care during the past few decades has led to a remarkable reduction of neonatal mortality (1, 2). However, this progress has created new challenges. Bronchopulmonary dysplasia (BPD), the chronic lung disease of prematurity, now occurs in extreme premature infants, disrupting normal lung growth at an early developmental stage (3). The first report of BPD dates back to the pre-surfactant era, when Northway described a form of chronic lung disease (4), defined as oxygen need for at least 28 days in conjunction with specific radiographic changes, in late preterm infants surviving from respiratory distress syndrome at birth (5). Subsequently, oxygen dependency at 36 weeks post-menstrual age (PMA) was shown to better predict long-term respiratory outcomes (6). Roughly 10 years later, in the NICHD Workshop Summary, infants below 32 weeks requiring supplemental oxygen for at least 28 days were stratified into three severity groups (mild, moderate and severe BPD), depending on the presence and the amount of supplemental oxygen and mode of respiratory support at 36 weeks PMA (7). The 'physiologic' definition of BPD was proposed in the attempt to compensate for the significant inter-center variability in oxygen administration (8). To be categorized as BPD patients according to the physiological definition, infants receiving less than 30% of supplemental oxygen and no respiratory support at 36 weeks PMA are challenged by reducing the fraction of administered oxygen during a standardized test. Infants who are unable to maintain saturations above 90% in room air during the test are diagnosed with BPD (9).

Although, by definition, BPD cannot be diagnosed before 28 days of life, the respiratory disease characterized by oxygen and/or ventilator-dependency from 7 to 28 days of life, represents the initial phase of the chronic process leading to BPD, thus classified as "evolving BPD" (10). Recently, the definition of BPD has been refined in order to include in the severity classification newer modes of noninvasive ventilation that were not included in the previous definitions. The severity classification has been changed from mild, moderate and severe into the new terms of grades I, II, and III. Grade III would refer to the most severe form of BPD. Early death (between 14 days of postnatal age and 36 weeks) owing to persistent parenchymal lung disease and respiratory failure that cannot be attributable to other neonatal morbidities has also been included in the definition as grade IIIa (11).

While some data suggest a trend towards a reduction of the BPD rates, most studies report a slight increase over the past two to three decades. This is likely linked to the higher survival of more immature infants at higher risk for BPD (12, 13). Moreover, incidence of BPD varies depending on the definition. The NICHD Neonatal Research Network has compared the BPD rates in extremely preterm infants (22-28 weeks gestation) according to the three definitions currently in use. The NICHD classification is associated with the highest rates (68%) due to the inclusion of infants requiring oxygen for 28 days, although breathing room air at 36 weeks PMA. Oxygen need at 36 weeks PMA and the physiological definition obtained similar results (42 and

40% respectively) (12, 13). It is still controversial which definition better predicts the long-term outcomes and if grading system actually improves its positive predictive value (14-16).

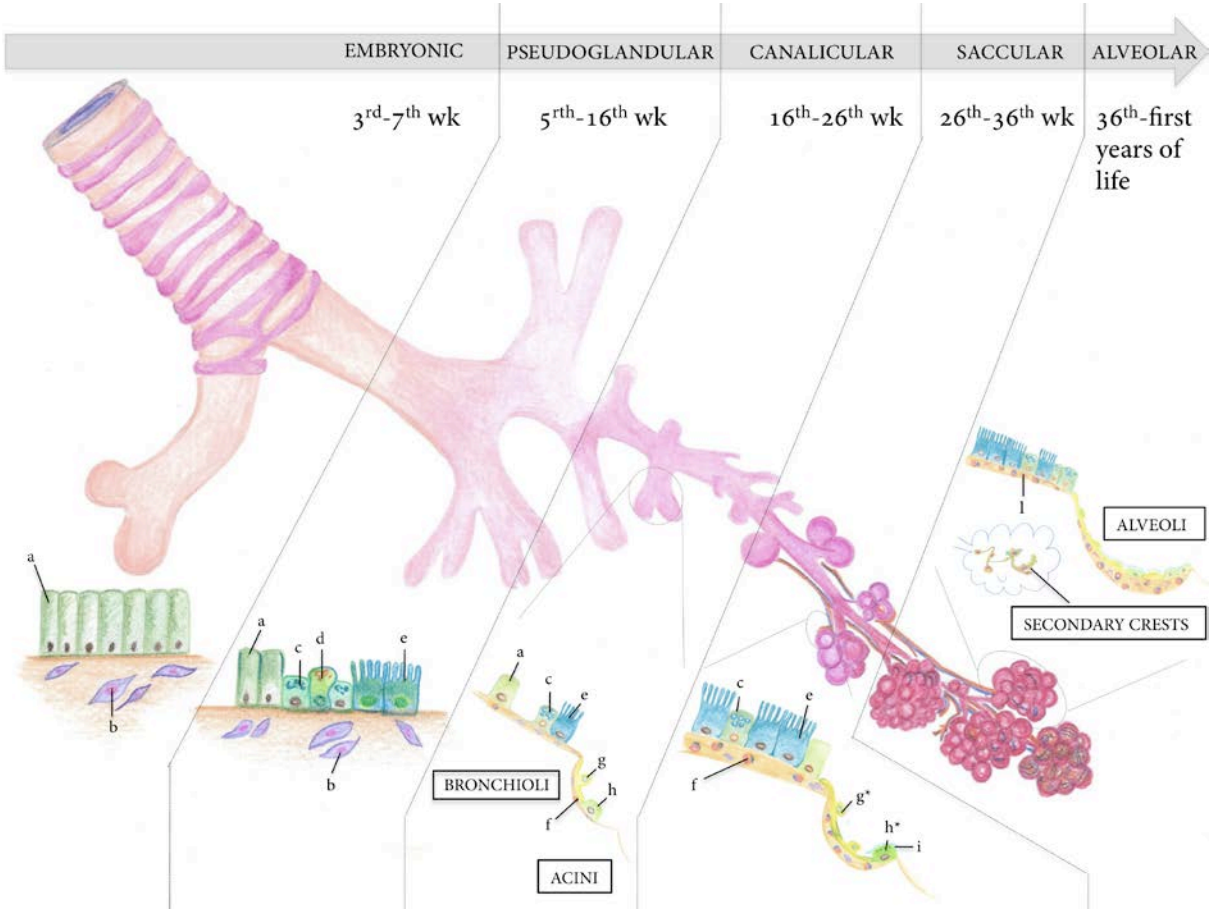

Figure 1. Stages of lung development

Embryonic phase (3rd-7th weeks of gestation): formation of the two lung buds (primary bronchi) further branching into the secondary bronchi and into the major airways. Histologically, these structures are lined with undifferentiated columnar epithelium (a). Mesenchymal cells (b) are crucial for epithelial-mesenchymal "cross-talk" cell interactions that regulate branching morphogenesis and cellular differentiation.

Pseudoglandular stage (5-16th weeks of gestation): formation of the conductive airways. The epithelium starts to differentiate into non-ciliated columnar Clara cells (c), pulmonary neuroendocrine cells (d), and ciliated cells (e).

Canalicular phase (16-26th weeks of gestation): formation of the respiratory bronchioles and the pulmonary acinus. Histologically, the epithelium of the distal lung begins to differentiate from cuboid type II cells (g) into squamous type I cells (h).

Saccular stage (26-36th weeks of gestation): Maturation of the distal epithelium. Mature type 2 cells (g*) start producing surfactant (i), while type 1 cells form the thin layer. In the proximal epithelium ciliated (e), non-ciliated Clara (c), and neuroendocrine cells (d) increase in number.

Alveolar stage (36 weeks of gestation-first few years of life): alveolarization and formation of the secondary crests. The microvascular maturation takes place and the capillary bilayer (f) merge into the single-layer vascular network (f*).

2. Pathogenesis of BPD

Since the major pathogenetical clue of BPD is the disruption of normal lung development, a thorough insight into the normal lung development, is a prerequisite to deeply understand the pathological process leading to BPD.

Lung development is typically divided into five stages: embryonic, pseudoglandular, canalicular, saccular, and alveolar (*figure 1*). During the embryonic phase (third-seventh week of gestation), the human lung originates from the primitive foregut as a ventral endodermal bud, which will divide into the two lung buds (primary bronchi) and then into the secondary bronchi, further branching into the major airways. Histologically, these structures are lined with undifferentiated columnar epithelium. The pseudoglandular stage begins at the end of the fifth week of fetal life, with the branching of all conductive airways down to the terminal bronchi. The epithelium of the conducting airways starts to differentiate into non-ciliated columnar Clara cells, pulmonary neuroendocrine cells, and ciliated cells. The distal cuboidal epithelium differentiate into type II epithelial cells. Between 16 and 26 weeks, the canalicular phase takes place, leading to the formation of the respiratory bronchioles and the pulmonary acinus. During this time, the capillary bed of the distal lung remarkably increases. Histologically, the epithelium of the distal lung begins to differentiate from cuboid type II cells into squamous type I cells. During the saccular stage (26-36 weeks), the distal epithelium further mature: type 2 cells start producing surfactant, while type 1 cells form the thin layer, needed for future gas exchange. The interstitial mesenchyme becomes thinner. Saccules and ducts, the characteristic elements of this stage, consist of thick primary septae, containing a double pulmonary capillary layer. In the proximal epithelium ciliated, non-ciliated Clara, basal and neuroendocrine cells increase in number. The larger vessels of the pulmonary vasculature start to muscularize.

Starting from 36 weeks up to the first few years of life, the process of alveolarization and secondary septation ensure the formation of mature and well-organized alveoli. Equally important, the microvascular maturation takes place and the capillary bilayer merge into the single-layer vascular network, creating the efficient air-blood gas-exchange unit (17).

The form of chronic lung disease described by Northway, now referred to as "old BPD", was mainly a consequence of the aggressive ventilatory approach on a relatively mature lung, although deficient in surfactant. Histologically, "old BPD" was characterized by a diffuse injury, with significant inflammation and parenchymal fibrosis, despite numerically and structurally normal, mature and complex alveoli (4). Thanks to the improvement of neonatal care, in particular to the introduction of antenatal steroids in order to induce lung maturation, the discovery of exogenous surfactant and the use of “gentle” ventilatory techniques, nowadays infants born after the canalicular stage of lung development exceptionally suffer from chronic lung disease (7). At the same time, the increased survival of extremely premature infants has led to the appearance of a new form of chronic lung disease ("new BPD"), typical of the infants born at the early stages of lung development (22-28 weeks of gestation) (7, 18). The "new BPD" is the expression of the lung immaturity, rather than the iatrogenic damage. Histologically, the BPD lungs are characterized by less numerous, enlarged, and simplified alveoli, typical of the

early canalicular stage (*figure 2*), while fibrosis and inflammation are less represented (19). BPD is also characterized by an abnormal distribution of pulmonary vessels and a significant reduction in the number of small arteries, which are functionally hyper-reactive and hypertonic, culminating in pulmonary arterial hypertension and right ventricular hypertrophy (20-22).

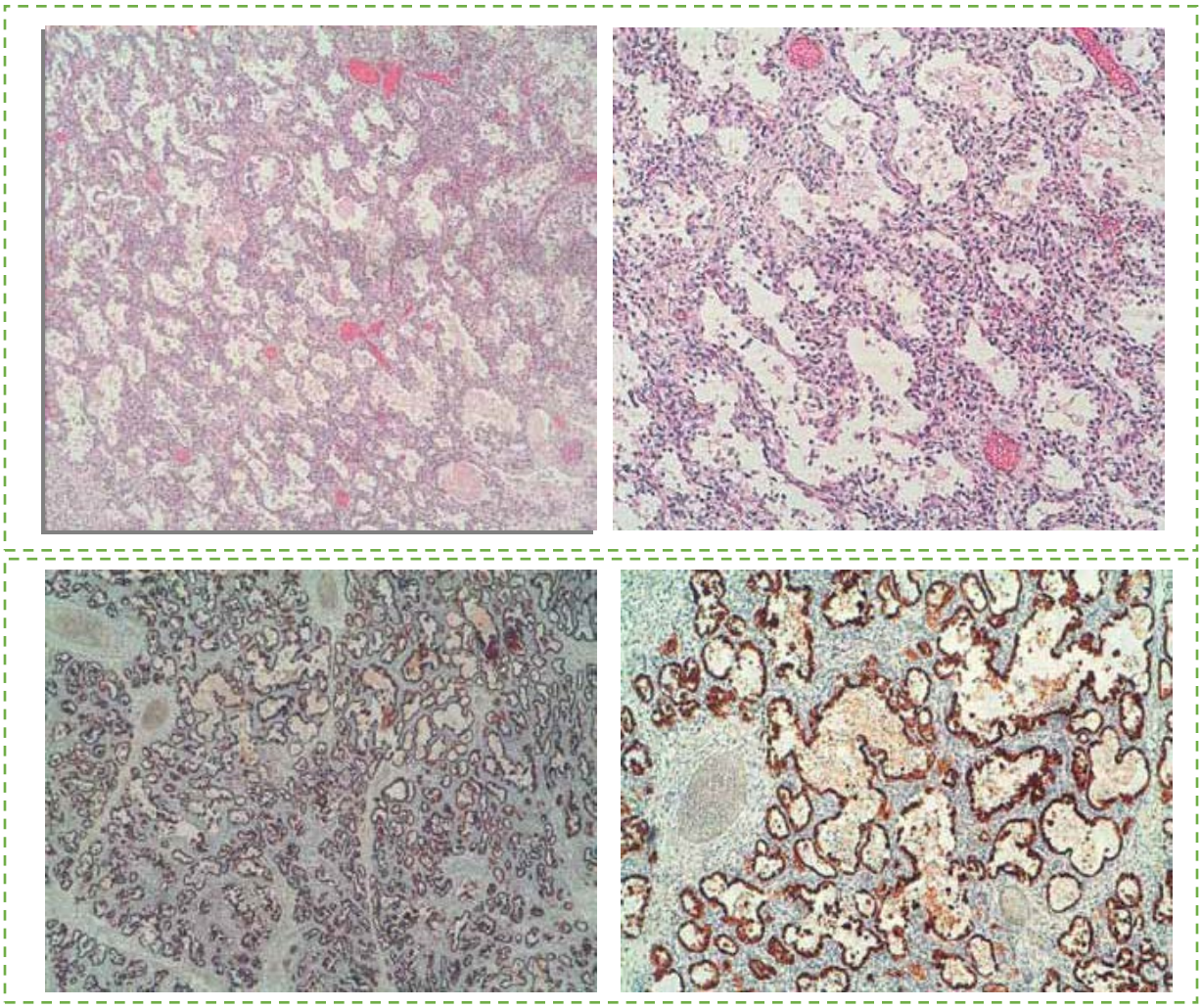

Figure 2. Lung sections of a very preterm infants died from BPD

A. Lung sections in hematoxylin-eosin of a lung of a very preterm infant (23 weeker) died from BPD; 10x (A) e 20x (B). Lungs are in the in the canalicular-saccular stage with no secondary crests typical of the alveolar phase.

B. Lung sections from the same patient stained with con cytokeratin-7; 10x (A) e 20x (B), better showing the epithelium

Gentle concession of Prof. Ezio Fulcheri, IRCCS, Istituto Giannina Gaslini, Genova

Moreover, the imbalance between anti-oxidant defense mechanisms and increased exposure to oxygen reactive species (23, 24) exacerbates cell apoptosis and disruption of the extracellular matrix, leading to tissue remodeling. These events contribute to the development of BPD, by further perturbing the developing lung and intensifying the alveolar and vascular dysregulation, primarily caused by the premature birth (3) (*figure 3*).

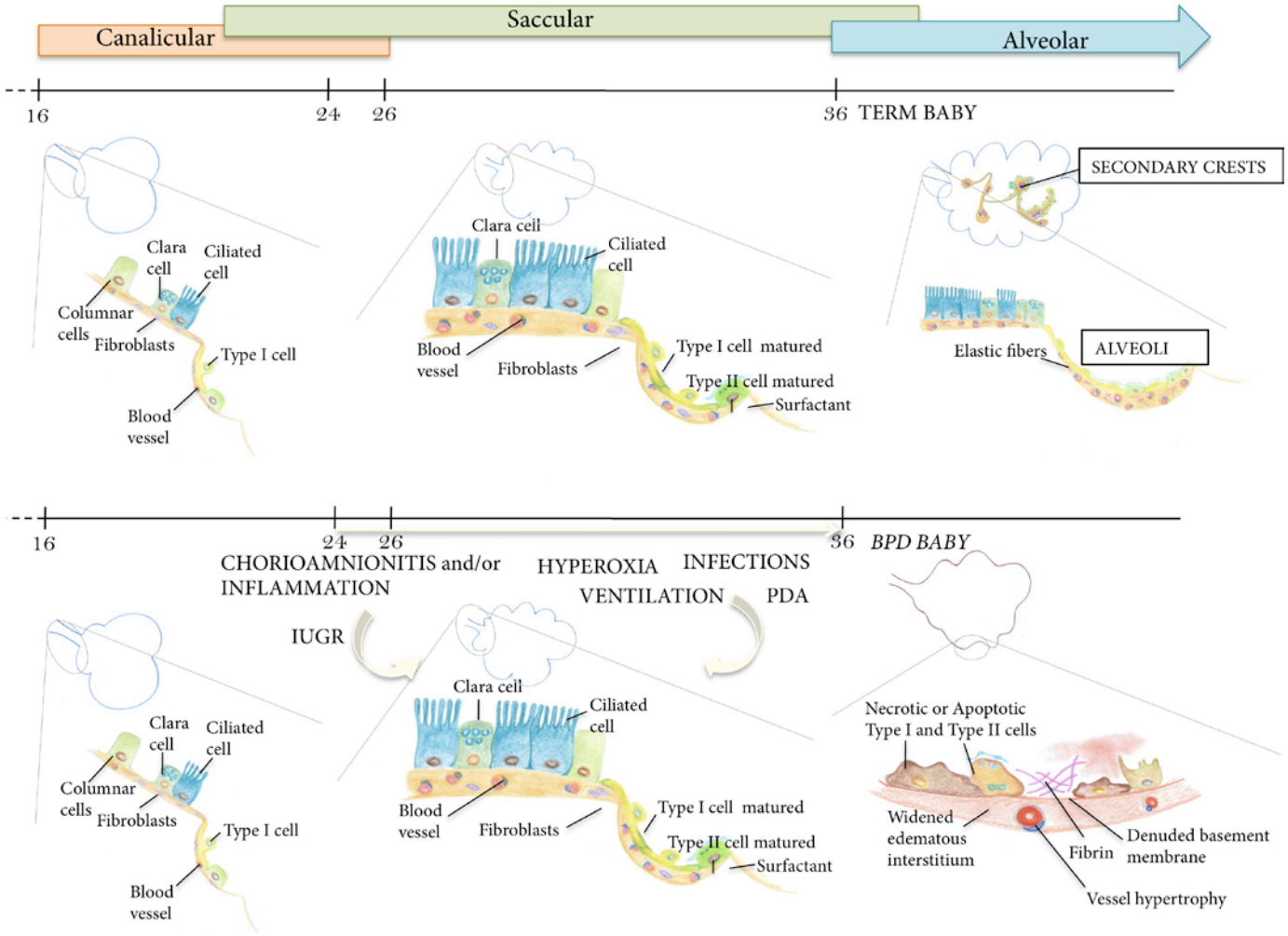

Figure 3. Events leading to the development of BPD

The "new BPD", typical of the infants born at the early stages of lung development (22-28 weeks of gestation), is the expression of the lung immaturity. Prenatal and postnatal inflammatory stimuli, such as chorioamnionitis, ventilator induced lung injury, oxygen toxicity and neonatal sepsis contribute to the development of BPD. Histologically, the BPD lungs are characterized by less numerous, enlarged, and simplified alveoli, with no secondary crests, typical of the early canalicular stage. BPD is also characterized by an abnormal distribution of pulmonary vessels and a significant reduction in the number of small arteries

3. Outcomes of BPD

Premature infants, with or without BPD, continue to show worse respiratory performances compared to their peers up to late childhood (25, 26). Among premature infants, BPD increases the risk for re-hospitalization during the first 2 years of life, due to acute respiratory distress and respiratory tract illness (27). Later in childhood, ex-preterm infants affected by BPD need more often respiratory medications and present more frequently with respiratory symptoms (28). Respiratory symptoms have been objectified by signs of airway obstruction at lung function tests (15). Airway obstruction in these patients seems to be caused by structural changes rather than airway hyperreactivity (29). Response to asthma medication is less pronounced and levels of exhaled nitric oxide are lower in symptomatic BPD patients as compared to asthmatic children born at term with similar grade of airway obstruction (29). In agreement with these results, structural BPD findings have been documented at the autopsy of a child diagnosed with BPD, died from asthma attack at 12 years of life (30). BPD is also associated with worse long-term developmental outcomes, including higher rates of cerebral palsy at 18–22 months' corrected age and psychomotor delay, attentional impairments, speech and language disorders, executive deficits and behavioral problems at school age (31).

4. Current Strategies for prevention and treatment of BPD

While the definitive prevention of BPD could only be obtained by avoiding preterm birth, prenatal and postnatal preventive efforts should be directed at the reduction of the other stressors, such as postnatal infections and inflammation that may lead to the injury of the developing lung (32). Inflammatory events, such as necrotizing enterocolitis (NEC) and late-onset sepsis (LOS), are not only life-threatening for preterm infants but can likely mediate major short- and long-term adverse outcomes (33), including BPD. It is well accepted that sepsis and infections pose the premature population at increased risk of developing BPD (13, 14). Pro-inflammatory cytokines, released during NEC and LOS, may exert a direct effect on lung development or sensitize the lung to the effects of oxygen, mechanical ventilation and other stressors (34-37). On the other hand, infants suffering from NEC and LOS often require more aggressive and prolonged mechanical ventilation, which, although life-saving, is burdened by various degrees of lung injury (34-37). It has been suggested that the reduction of postnatal systemic infections has a higher impact in decreasing the inflammatory response in developing lungs, rather than avoiding invasive mechanical ventilation (37). Consistently, studies directed at evaluating the impact of quality improvement efforts to reduce LOS in preterm infants showed that a reduction in LOS is accompanied by decreased BPD rates (38, 39). Moreover, the immature immune system of the premature infants is unable to balance pro-inflammatory responses, leading to a sustained status of inflammation, that significantly contributes to several neonatal diseases, including BPD (36). Decreased number of T regulatory cells (Tregs), which constitute the anti-inflammatory T subset designed to limit and suppress excessive innate and adaptive immune responses, in the cord blood (40) and higher proportions of activated pro-inflammatory T cells in the peripheral blood during the first week of life (41) can predict the development of BPD. In experimental BPD, macrophages are polarized towards the pro-inflammatory M1 phenotype, while the anti-inflammatory M2 phenotype is inhibited (42).

Approaches aimed at reducing the pro-inflammatory stimuli, including “gentle“ rather than aggressive ventilatory techniques starting from the delivery room, adequate oxygen saturation targets and optimal timing for surfactant administration, seem to be partially effective in preventing BPD, by reducing inflammation to the lung. On the other hand, optimized nutrition and other strategies able to restore to pro-inflammatory/ant-inflammatory balance and the immunological status may open new therapeutical options.

In terms of medications, most of the therapies are either ineffective, inconsistently evaluated or unsafe. A meta-analysis (43) analyzed all the therapies available to prevent or treat BPD. Among the 21 drugs, 16 showed no efficacy. Out of the 5 effective drugs, only three (vitamin A, caffeine and dexamethasone) have been assessed in large or multi-centric randomized controlled trials (RCTs). However, none of them is currently recommended for prevention or treatment of BPD. The need for a long course of intramuscular injections of vitamin A may not be balanced by the modest reduction of BPD rates (44). Treatment with caffeine was associated with a significant reduction in the incidence of BPD in one large RCT that enrolled more than 2,000 patients (45). However, BPD was only a secondary measure and the results need to be evaluated in further trials. Early dexamethasone is the only drug that has been repeatedly shown to significantly reduce the incidence of BPD. However, the higher risk of cerebral palsy at 18 months of life after this treatment (especially if administered during the first week of life) have greatly limited its use. On the other hand, patients at higher risk for BPD (ie ventilator dependent after 2 weeks of life) seem to benefit from a short course of low-dose dexamethasone (46). In case they survive, these patients will be almost certainly diagnosed with BPD. Dexamethasone, by reducing the incidence of BPD, reduces its associated neurological complications as well, improving the overall outcome (47). However, dexamethasone use should be cautious and restricted to severe cases, with ventilator dependency after the first two-three weeks of life. In order to avoid the adverse consequences of dexamethasone, hydrocortisone had been increasingly used as an alternative. However a recent large RTC showed no beneficial effects of hydrocortisone as compared to placebo in reducing the composite outcome of death or BPD in preterm infants at high risk for BPD (48). In summary, no effective and safe treatment has yet been developed for BPD and novel therapies are urgently needed.

5. EXPERIMENTAL MODELS OF BPD

In order to test the efficacy and safety of a novel therapy, it is paramount to dispose of reliable animal models. This section briefly reviews the experimental settings adopted to investigate innovative approaches for neonatal lung diseases. Few models in different animal species have been able to mimic BPD or some of its aspects. The hyperoxia-induced lung injury (*figure 4*). is the most adopted model and displays several features of BPD, such as alveolar and vascular disruption, inflammation, fibrosis and oxidative stress (49, 50). Rats and mice are born at the canalicular stage of lung development, similarly to premature infants at risk for BPD. Hyperoxia-induced lung remodeling persists histologically and functionally at least up to 10 months (equivalent to 25-30 years in humans) (51). Animals exposed to hyperoxia in the neonatal period show functional impairment later in life, as documented by significantly reduced distance running on the treadmill (51). Neonatal exposure of mouse pups to hyperoxia worsens the severity of subsequent viral infections and increases mortality rates following adult influenza A inoculation (52). Few other models, although much less employed, may approximate BPD. Postnatal injection of intraperitoneal bleomycin in newborn rats from day 4 to 14 induces significant pulmonary hypertension and alveolar simplifications, with no fibrosis (53), as opposed to adult rats that suffer mostly from fibrotic lung lesions after bleomycin administration (54).Larger animals (lamb, sheep and baboon) can be delivered prematurely and undergo mechanical ventilation, reproducing the chain of events that leads to BPD. These animals show acute lung inflammation, abnormal pulmonary function, altered lung architecture, alveolar simplification, and mild fibrotic changes (55-57). However only few facilities are equipped to work with large animals and most of the data on stem cell therapy are obtained with the murine models, particularly the hyperoxia-induced lung injury.

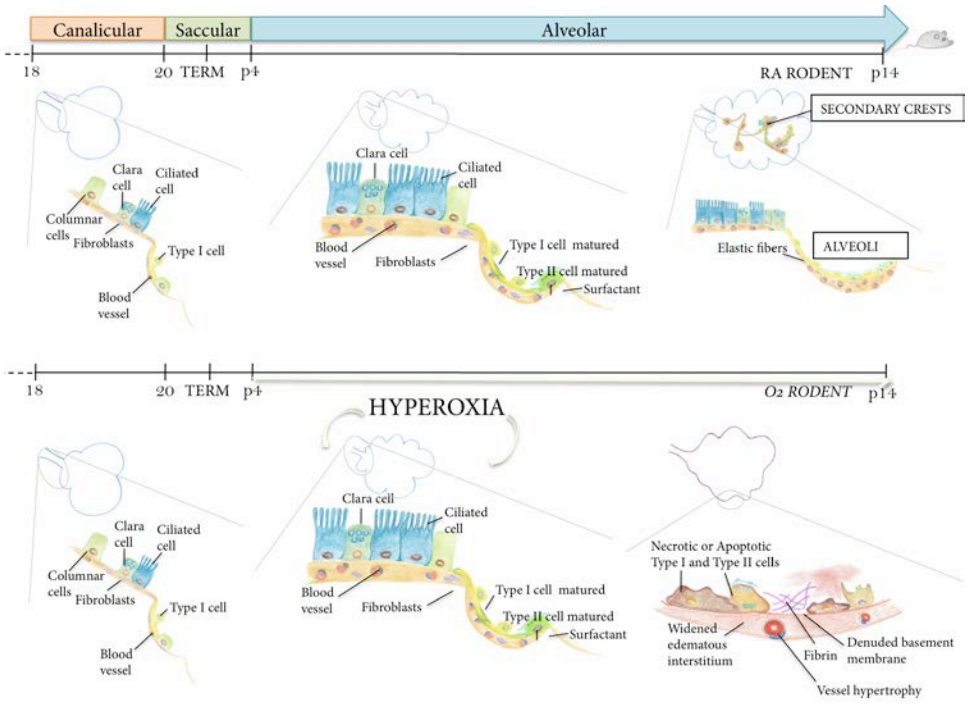

Figure 4. Experimental BPD: the hyperoxia-induced lung injury
Rat pups are naturally born during the saccular stage of lung development. Exposure of the developing rat lung to various concentration of hyperoxic gas (O2 rodent), during the alveolar stage (day 4 to day 14 of life) impairs alveolarization, resulting in fewer and enlarged alveolar air spaces, resembling BPD lungs, as opposed to controls in room air (RA rodent).

6. REGENERATIVE MEDICINE FOR BPD: MESENCHYMAL STEM/STROMAL CELLS

Regenerative medicine appears as promising option for so far untreatable diseases. Several studies have already proven stem cell efficacy in a variety of experimental settings, including neonatal lung diseases. Stem cells, thanks to their unique possibility of restoring and regenerating damaged tissue, may structurally revert the alveolar and vascular architectural changes that make traditional therapies unsuccessful in treating BPD. Among stem cells, mesenchymal stromal cells (MSCs) have been extensively investigated, thanks to their peculiar properties that make them particularly interesting for clinical practice. MSCs are easy to isolate and display multilineage potential and compelling immunomodulatory properties (58).

6.1 Characterization and origin of MSCs

MSCs represent a broad and heterogeneous cell population, defined by three minimum criteria: (i) adhesion to plastic when cultured in a tissue culture flask under standard culturing conditions (ii) expression of specific surface markers (CD73, CD90, CD105) and lack of expression of hematopoietic markers [CD45 (leukocytes), CD34 (hematopoietic progenitors), CD14 or CD11b (monocytes/macrophages), CD19 or CD79a (B-cells), or HLA-DR (human leukocyte antigen DR major histocompatibility complex type II], (iii) ability to differentiate into mesodermic (osteogenic, chondrogenic, and adipogenic) lineages upon in vitro stimulation (58). The prevailing hypothesis, is that MSCs originate from perivascular precursors, named pericytes (*figure 5*) (59). Long-term cultured perivascular cells from a variety of tissues start expressing typical markers of MSCs and become able to differentiate into osteocytes, chondrocytes, and adipocytes (59). Pericytes surround the blood vessels throughout the body and protrude into the endothelial lumen to sense chemotactic signals and allow prompt and efficient MSC recruitment and response to injury (*figure 5*) (60).

6.2 Source of MSCs

Bone marrow is the first described and best known source of MSCs (*figure 6*). However, bone marrow as a source of MSCs demonstrate some limitation due to the aging of the cells with the donor aging (61), the paucity of the MSCs among the other cells of the bone marrow (62) and the painful and invasive procedure needed to obtain the cells (iliac crest bone marrow aspirate).

Adipose tissue is an emerging source of MSCs (*figure 6*). It offers a greater number of cells and it is more accessible than the bone marrow (63).
Recently, perinatal tissues have emerged as a promising source of MSCs (*figure 6*). The use of perinatal stem cells would be particularly suitable and practical in treating neonatal diseases. A significant advantage of perinatal tissues is their availability without invasive and painful harvesting procedures (64). Being discarded at birth, most of these tissues and fluids are entirely free from ethical concerns. Furthermore, MSCs from perinatal tissues, as compared to adult MSCs, display superior cell biological properties, such as stronger immunomodulatory and

immunosuppressive potential (65), improved proliferative capacity, life span (66) and stemness (67), higher trophic (68) and anti-inflammatory (66) activity as well as improved cardiac function after myocardial infarct (69).

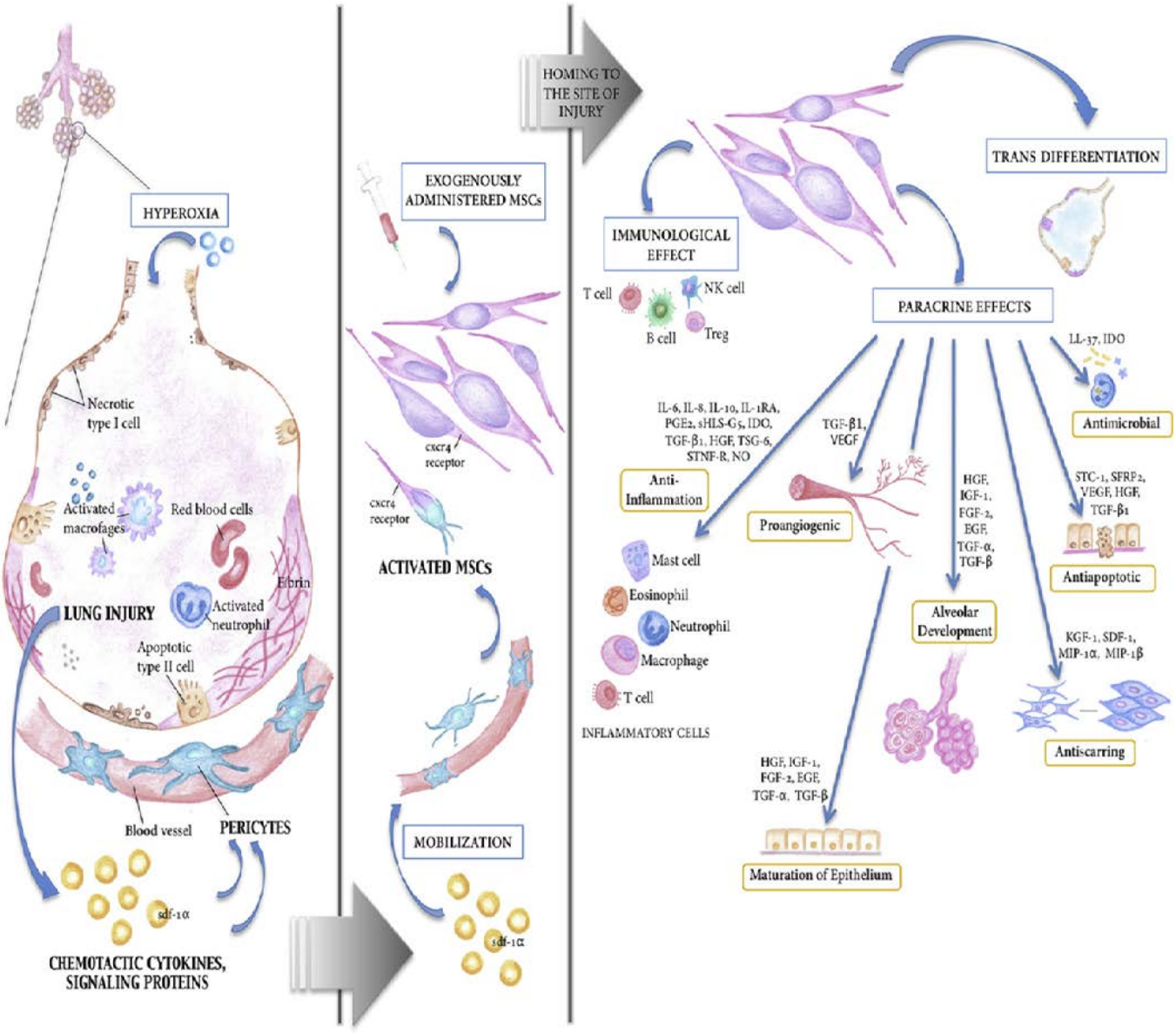

Figure 5. Mechanism of action of MSCs.

Chemokines, cytokines, and growth factors released on injury mobilize perivascular cells (pericytes), which surround the blood vessels throughout the body. Pericytes are believed to be precursors of MSCs. Activated MSCs, derived from pericytes, or exogenously delivered MSCs are recruited to the site of injury through interactions between chemokines, such as stromal cell-derived factor-1a (SDF-1a) and MSC receptors (ie, C chemokine receptor type 4, CXCR4, for SDF-1a) in a process called homing. Once homed to the damaged organ, MSCs can exert their therapeutic benefit through multiple mechanisms of action: (1) paracrine secretion of molecule to induce cell proliferation and angiogenesis and (2) interaction with immune cells.

Perinatal tissues are divided into extra-embryonic tissues (chorion, amniotic membranes, and umbilical cord) and placental fluids (amniotic fluid and umbilical cord blood); MSCs have been isolated from any of them, with some differences between one another. Amniotic membrane-derived MSCs cells show a pronounced inter-donor variability (70). Chorion-derived MSCs may be contaminated with maternal cells, which present lower proliferative potential as compared to cord MSCs from the same donor (71). Amniotic fluid is an interesting source of MSCs, although isolation and characterization protocols need further investigation (72). MSCs from the umbilical cord blood and umbilical tissue display high regenerative and immunosuppresive potential. However, cell presence in the cord blood is extremely rare (64). To date, among the different perinatal sources, the most practical and effective one is the cord tissue (in particular the Wharton's Jelly), disposing of robust and reproducible techniques for harvesting and expansion (64, 73).

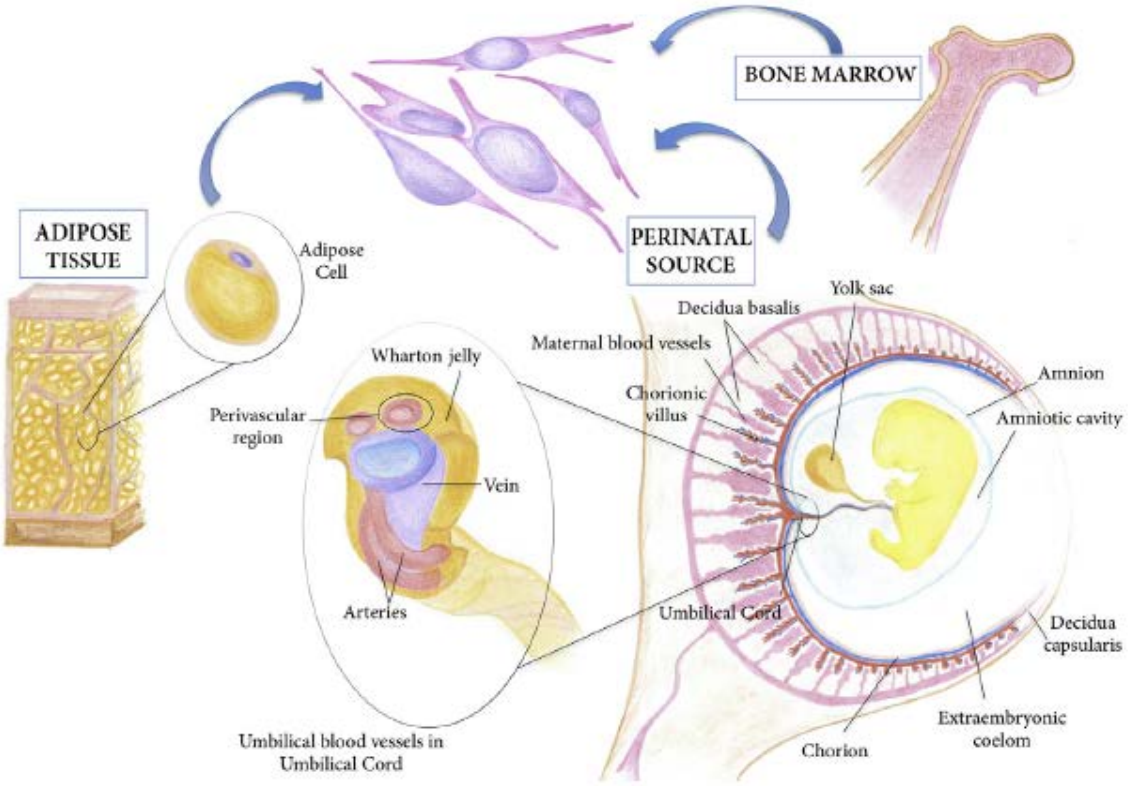

Figure 6. Clinically relevant sources of MSCs
Clinically relevant sources of MSCs include adult sources (adipose tissue and bone marrow) and perinatal sources (chorion, amniotic membranes, amniotic fluid, and umbilical cord or cord blood).

6.3 Lung Resident MSCs (L-MSCs) are involved in lung development and lung homeostasis

MSCs can be isolated virtually from any tissue (74). Although most of the harvesting sites are not clinically relevant due the difficult access, the understanding of the tissue-specific MSC role in organ homeostasis and disease pathogenesis, may optimize the impact of novel therapies. MSCs from any tissue share the three minimum criteria needed to be defined as MSCs. In addition, tissue-specific MSCs, including lung resident MSCs, also display distinct functional characteristics to support their specific microenvironment (75). In particular, L-MSCs display higher gene expression of lung-specific extracellular matrix proteins, growth factors, and chemokines (76), promote proliferation of epithelial progenitor cells (77) and differentiate into epithelial cells in vitro (78). Moreover, lung mesenchyme actively guide branching of the airways and alveolar maturation during lung development, thanks to the ''cross-talk'' interactions with the epithelial cells (79). Interstitial myofibroblasts during the pseudoglandular stage and secondary crests myofibroblasts during the alveolar stage, both derived from mesenchymal precursors, have a key, yet elusive, role in lung development (80). Interestingly, MSCs from the tracheal aspirate of preterm infants undergo myofibroblastic differentiation upon TGF-beta1 stimulation, as opposed to bone marrow derived MSCs (81). L-MSCs can be isolated from fetal and adult animals (77, 82), and human lung (83, 84). The presence of donor MSCs years after a sex-mismatched lung transplant in the recipients further confirms the lung origin of L-MSCs that reside and self-renew in the adult lung (83). The existence of lung specific MSCs and the evidence of their contribution to normal lung development have provided rational support to the documented MSC therapeutic efficacy in experimental diseases, corroborating the great potential of exogenous MSC administration and/or endogenous MSC restoration in the treatment of neonatal lung disorders.

6.4. Mechanism of action of MSCs

6.4.1.Homing to site of injury and regeneration

Chemotactic cytokines and signaling proteins, released by different types of injury, recruit resident and remote host MSCs in a process called "homing" (85). MSCs migrate into the local or systemic circulation through the endothelium upon interaction of their membrane receptor with chemokines (86). Once recruited into the damaged area, MSCs exert their therapeutic function (*figure 7*). Although MSCs have been proven to differentiate in vitro along different lineages, including lung epithelial cells and alveolar type 2 cells (87, 88), this property has not been convincingly confirmed in vivo. Moreover, independently from the ability to generate various differentiated cell lineages, it has been repeatedly shown that only few exogenously-administered cells engraft and differentiate into the damaged organs in neonatal (89) or adult lung injury models (90, 91), despite a significant beneficial effect, suggesting that MSC therapeutic benefits cannot be ascribed to cell replacement.

6.4.2. Paracrine effect

The discrepancy between the impressive preclinical results and the low rate of engraftment has introduced the hypothesis that the few engrafted cells may secrete healing factors in a paracrine fashion to boost the local response to injury (60, 92) (*figure 7*). Growth factors and cytokines that can induce cell proliferation and angiogenesis, as well as anti-apoptotic, anti-oxidant, anti-fibrotic, anti-inflammatory and anti-microbial factors have been detected in the MSC media (93). These polymorphic factors are likely to explain the pleiotropic effect of the cells, making MSCs particularly appealing for the treatment of a multifactorial disease such as BPD. Recently, a new intriguing theory has been suggested. MSC may "reprogram" the injured tissue by delivering nanoparticles in the form of extracellular vesicles, subsequently incorporated by other cells (*figure 7*). These nanoparticles, also named exosomes, are membrane vesicles, formed through the fusion of the endosomes with the plasma membrane, and can be distinguished by their size (range in size from 30 to 100 nM) (94). Exosomes are secreted by several cell types, including stem cells and are involved in the cell-to-cell communication. They can be considered as nano-packages of bioactive molecules and non-coding microRNA (non-coding RNA involved in transcriptional regulation of gene expression) that can been transferred from one cell to another and could mediate tissue repair and remodelling (95). Exosomes can be isolated from cultured cells and delivered in vivo (96).
Mitochondrial transfer from MSCs to resident lung cells via nanotubes seems to be another mechanism of action of MSCs (97) (*figure 7*).

6.4.3.Immunomodulatory properties

The interaction with the immune system is a striking component of the MSC function and it contributes to their clinical outstanding appeal (*figure 7*). Undifferentiated MSCs express low levels of human leukocyte antigen (HLA) Class I and low levels of HLA Class II, enabling MSCs to avoid recognition by the immune system (98). MSCs also exert immunomodulatory effects by direct cell-to-cell contact, preventing proliferation and function of many inflammatory immune cells, including T cells, natural killer cells, B cells, monocytes, macrophages and dendritic cells (99). Although, immune rejection cannot be entirely ruled out (100), MSC are the good candidates for allogeneic therapies, thanks to their immunomodulatory potential.

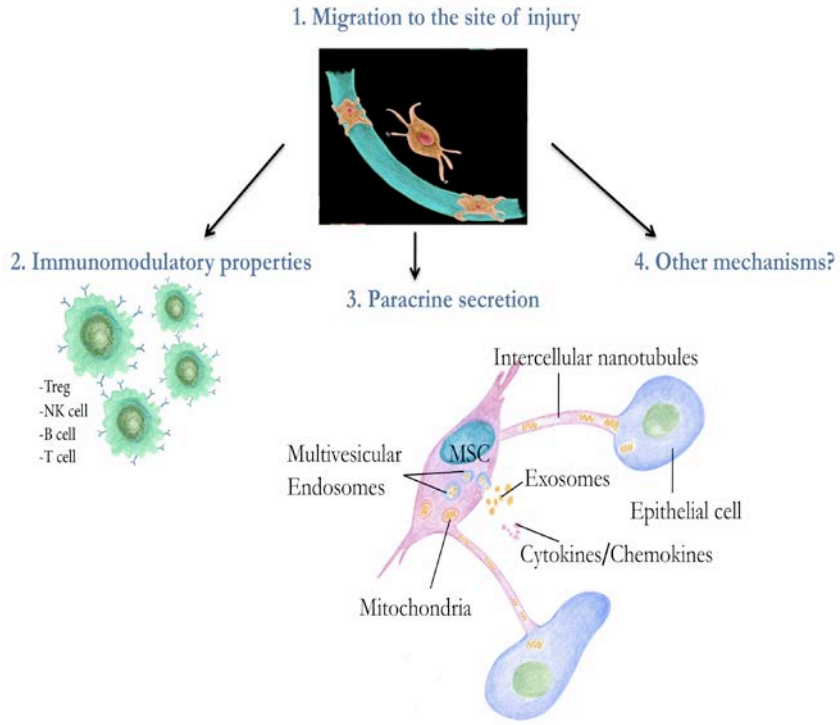

Figure 7.
MSC mechanism of action
Activated MSCs, derived from pericytes, which surround the blood vessels, are recruited to the site of injury. Once homed to the damaged organ, MSCs can exert their therapeutic benefit through the interaction with immune cells, including T cells, natural killer cells, B cells, monocytes, macrophages and dendritic cells by cell-to-cell contact (2) and the paracrine secretion of anti-inflammatory, antimicrobial, anti-apoptotic and anti-scarring molecules and growth factors and other chemokines to induce cell proliferation and angiogenesis. MSC may "reprogram" the injured tissue by delivering nanoparticles in the form of extracellular vesicles (exosomes), subsequently incorporated by other cells and also through mitochondrial transfer from MSCs to resident lung cells via nanotubules (3). Other uninvestigated mechanisms may be involved in MSC mechanism of action (4).

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CHAPTER II

SHORT-TERM, LONG-TERM AND PARACRINE EFFECT OF HUMAN UMBILICAL CORD-DERIVED STEM CELLS IN LUNG INJURY PREVENTION AND REPAIR IN EXPERIMENTAL BRONCHOPULMONARY DYSPLASIA

Pierro M, Ionescu L, Montemurro T, Vadivel A, Weissmann G, Oudit G, Emery D, Bodiga S, Eaton F, Péault B, Mosca F, Lazzari L, Thébaud B.

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ORIGINAL ARTICLE

Short-term, long-term and paracrine effect of human umbilical cord-derived stem cells in lung injury prevention and repair in experimental bronchopulmonary dysplasia

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ABSTRACT

Background Bronchopulmonary dysplasia (BPD) remains a main complication of extreme prematurity and currently lacks efficient treatment. Rat bone marrow-derived mesenchymal stem cells (MSC) prevent lung injury in an oxygen-induced model of BPD. Human cord is an advantageous source of stem cells that is especially appealing for the treatment of neonatal diseases. The therapeutic benefit after established lung injury and long-term safety of cord-derived stem cells is unknown.

Methods Human cord-derived perivascular cells (PCs) or cord blood-derived MSCs were delivered prophylactically or after established alveolar injury into the airways of newborn rats exposed to hyperoxia, a well-established BPD model.

Results Rat pups exposed to hyperoxia showed the characteristic arrest in alveolar growth with air space enlargement and loss of lung capillaries. PCs and MSCs partially prevented and rescued lung function and structure. Despite therapeutic benefit, cell engraftment was low, suggesting that PCs and MSCs act via a paracrine effect. Accordingly, cell free-derived conditioned media from PCs and MSCs also exerted therapeutic benefit when used either prophylactically or therapeutically. Finally, long-term (6 months) assessment of stem cell or conditioned media therapy showed no adverse lung effects of either strategy, with persistent improvement in exercise capacity and lung structure.

Conclusions Human umbilical cord-derived PCs and MSCs exert short- and long-term therapeutic benefit without adverse lung effects in this experimental model and offer new therapeutic options for lung diseases characterised by alveolar damage.

Key messages

What is the key question?

- Is cord-derived cell-based therapy efficient and safe for the prevention and/or treatment of chronic lung disease of prematurity?

What is the bottom line?

- Currently there is no effective treatment for the most common complication of extreme prematurity.

Why read on?

- Human cord-derived perivascular cells and cord blood-derived mesenchymal stem cells partially prevent and restore lung structure and function in newborn rats with experimental oxygen-induced arrested alveolar growth through a paracrine effect. Neither whole cell therapy nor cell-free conditioned media therapy adversely affect lung structure and function at 6 months post-treatment.

INTRODUCTION

Lung diseases characterised by alveolar damage such as chronic lung disease of prematurity (or bronchopulmonary dysplasia, BPD) and emphysema in adults currently lack efficient treatments. A common denominator of these diseases is the absence of injury resolution leading to distorted tissue repair resulting in arrested alveolar growth in BPD or alveolar destruction in emphysema. Despite intense investigations, current clinical management remains devoid of treatments specifically promoting lung repair.[1]

Recent insight into stem cell biology has generated excitement over the potential of stem cells to regenerate damaged organs.[2] Mesenchymal stem cells (MSCs) have attracted much attention because of their ease of isolation, multilineage developmental potential and immunomodulatory properties.[3] Adult rat bone marrow-derived MSCs prevent lung injury in various experimental lung disease models[2] including experimental BPD.[4 5] MSCs can be isolated from different sources including umbilical cord and cord blood, two neonatal cell sources which show unique advantages over the adult MSC counterpart.[6]

Perivascular cells (PCs) from diverse human tissues give rise to adherent multilineage progenitor cells that exhibit all the features of MSCs and may represent precursors of MSCs, the native identity of which has long been elusive.[7] We previously showed that PCs derived in culture from human umbilical

cord vessels are candidates for lung repair due to their ability to migrate towards an alveolar type II cell line damaged with bleomycin,[8] but their therapeutic potential remains unknown. In this context, we tested two human stem cell populations derived from the perivascular compartment of the umbilical cord (PCs) and from cord blood (MSCs) in newborn rats exposed to hyperoxia, a well-established model mimicking BPD.[9] In addition, to select the best possible approach for future clinical applications, we compared two different administration strategies—one prophylactic and one therapeutic—after established lung injury. In order to investigate the mechanisms underlying the beneficial effects and with the perspective of a 'pharmaceutical' cell-based therapy, we also tested the therapeutic potential of conditioned media (CdM) from cord-derived PCs and cord blood-derived MSCs. Finally, we evaluated the so far unknown long-term effects of cord-derived cell-based therapies on exercise capacity and lung structure at 6 months of age.

MATERIALS AND METHODS

More details of the methods are available in the online supplement. Procedures were approved by the Institutional Animal Care and Use Committee at the University of Alberta.

PC and MSC isolation, culture and CdM generation

PCs were isolated from the umbilical cords after parental consent as previously described (see online supplementary figure S1).[8] CdM was obtained as previously described.[5]

Animal model of oxygen-arrested lung growth

Rat pups were exposed to normoxia (21% oxygen, control group) or hyperoxia (95% oxygen, BPD group) from birth to P14 in sealed Plexiglas chambers (BioSpherix, Redfield, New York, USA) as described elsewhere.[5 10]

In vivo cell administration

For prevention experiments, newborn rat pups were randomised into seven groups: (1) room air (RA); (2) RA+MSCs; (3) RA+PCs; (4) hyperoxia (oxygen injury model); (5) hyperoxia+human neonatal dermal fibroblast (HNDF); (6) hyperoxia+MSCs; and (7) hyperoxia+PCs. For subsequent rescue experiments only the RA, hyperoxia, hyperoxia+MSC and hyperoxia+PC groups were analysed. Cells were administered at P4 (prevention studies) or P14 (regeneration studies) via an intratracheal injection (300 000/20 µl and 600 000/40 µl, respectively). Lungs were harvested on P22 (prevention studies) or P35 (regeneration studies). Long-term study animals were treated at P4 and lungs were harvested at 6 months.

In vivo CdM administration

CdM was administered daily intraperitoneally at a dose of 7 µl/g from P4 to P21 (prevention studies) or from P14 to P28 (regeneration studies). Lungs were harvested on P22 (prevention studies) or P35 (regeneration studies). Long-term study animals were treated from P4 to P21 and lungs were harvested at 6 months.

Lung function tests

Tests were performed on anaesthetised and paralysed animals using Flexivent (Scireq, Montreal, Quebec, Canada).

Lung morphometry

Alveolar structures were quantified on systematically sampled formaldehyde-fixed lung sections using the mean linear intercept and septal counts.[10 11]

Barium-gelatin angiograms and vessel density counts

Barium was infused in the main pulmonary artery as previously described.[5 10]

Right ventricular hypertrophy and pulmonary artery remodelling

The right ventricle to left ventricle+septum ratio was used as an index of right ventricular hypertrophy.[5] Pulmonary artery remodelling was quantified by medial wall thickness.[5 10]

Exercise capacity

Rats were run on a treadmill according to a pre-established protocol.

Total body CT scan

Anaesthetised rats were imaged with a rodent SPECT-CT using Amira software package (Gamma Medica, Northridge, California, USA).

Real-time PCR

Real-time PCR was performed on frozen lungs from three animals per group harvested at various time points after injection as described elsewhere.[10]

Immunofluorescence

Staining was performed on non-adjacent 5 µm paraffin-embedded lung sections using rabbit anti-human β_2-microglobulin (Abcam, Cambridge, Massachusetts, USA) and appropriate secondary antibodies (Invitrogen, Carlsbad, California, USA).

Statistical analysis

Values are expressed as means±SEM. Intergroup differences were assessed using analysis of variance with post hoc test (Fisher probable least significant difference test) (SPSS V.18). A p value of < 0.05 was considered statistically significant. All investigators performing evaluations were blinded to the experimental groups.

RESULTS

Airway delivery of cord-derived PCs or cord blood-derived MSCs prevents and rescues arrested alveolar growth

A total of 42 animals were used in the prevention experiments. Exposure of newborn rats to oxygen from P4 to P14, a well-established model mimicking BPD, led to distal air space enlargement, alveolar simplification (figure 1A–C) and decreased lung compliance (figure 1D) compared with RA-housed animals. Prophylactic intratracheal delivery of PCs and MSCs partially preserved alveolar growth (figure 1A–C) and prevented the decrease in lung compliance (figure 1D). Conversely, HNDF used as control cells had no effect on lung function and structure (figure 1). PCs and MSCs had no adverse effect on lung function and structure in RA control animals (figure 1).

A total of 24 animals were used in the rescue experiments. Administration of both PCs and MSCs at P14 as rescue therapy after established arrested alveolar growth restored normal alveolar architecture (figure 2A–C).

Lung engraftment of PCs and MSCs is low

Immunofluorescent staining for human β_2-microglobulin in P22 lungs 18 days after administration of PCs and MSCs localised very few cells within the lung (figure 3A). Quantification of human cells using qRT-PCR confirmed the low rate of

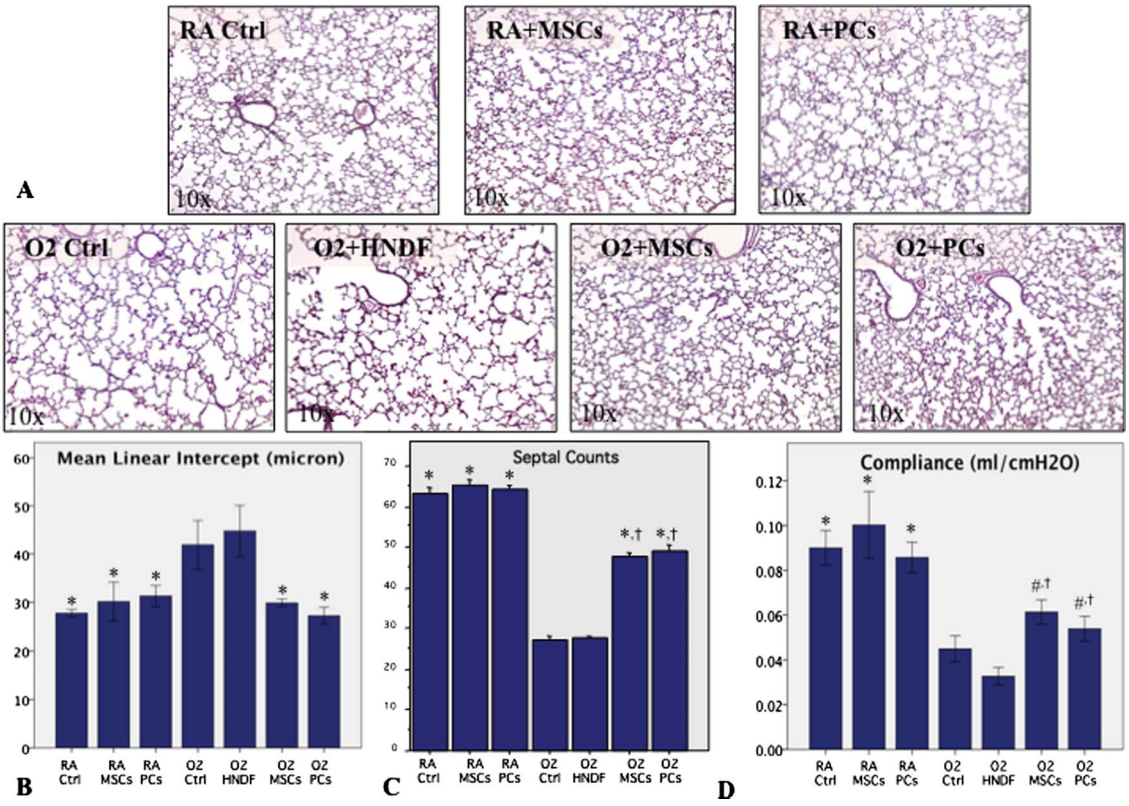

Figure 1 Perivascular cells (PCs) and mesenchymal stem cells (MSCs) prevent hyperoxia-induced lung injury. (A) Representative H&E-stained lung sections showing larger and fewer alveoli in hyperoxia-exposed lungs compared with lungs from rats housed in room air (RA) and RA animals treated with MSCs (RA MSCs) and PCs (RA PCs). Intratracheal administration of MSCs (O_2+MSCs) and PCs (O_2+PCs) in O_2-exposed animals partially preserved alveolar growth. Human neonatal dermal fibroblast (HNDF) administration (O_2 HNDF) did not show any improvement in oxygen-exposed animals. (B,C) Quantitative confirmation is provided by the mean linear intercept (n=6/group; *p<0.001 vs O_2 Ctrl and O_2 HNDF. No differences between O_2+MSCs and O_2+PCs and RA+MSC and RA+PCs) and the septal counts (n=6/group, *p<0.001 vs O_2 Ctrl and O_2 HNDF, †p<0.01 vs O_2+MSCs and O_2+PCs vs all RA groups). (D) Lung function testing shows decreased lung compliance in untreated oxygen-exposed animals compared with RA Ctrl and RA MSC and RA PC groups. Compliance was significantly improved in oxygen-exposed animals treated with MSCs and PCs. HNDF administration (O_2 HNDF) had no effect on lung compliance (n=6/group; #p<0.05 vs O_2 Ctrl; *p<0.001 vs O_2 Ctrl and O_2 HNDF; †p<0.01 O_2+MSCs and O_2+PCs vs all RA groups).

engraftment in recipient lungs with a dramatic decrease in detected human Alu sequences from the first day after injection to almost undetectable levels within 4 days (figure 3B). A total of 42 animals were used (3/time point/cell type).

Therapeutic benefit of PCs and MSCs is mediated via a paracrine effect

Low cell engraftment suggests the therapeutic benefit is unlikely to be due to cell replacement. Evidence suggests that stem cells act in a paracrine fashion. To verify this hypothesis, we assessed in vivo the therapeutic potential of CdM harvested from PC and MSC serum-free cultures. A total of 36 animals were used in the prevention experiments to assess lung morphometry, lung function and features of pulmonary hypertension. Prophylactic daily intraperitoneal CdM injections (7 µl/g) from P4 to P21 improved alveolar architecture (figure 4A–C) and lung function (figure 4D). CdM from PCs or MSCs had no adverse effects on lung function and structure in RA control animals.

Another hallmark of BPD is rarefaction of pulmonary vessels.[12] Lung CT scans of barium-injected pulmonary arteries showed severe rarefaction of pulmonary vessels in oxygen-exposed animals (figure 5A). PC and MSC CdM partially prevented the arrest in lung angiogenesis (figure 5A). Quantification of barium gelatin-injected pulmonary vessels (a total of 24 animals were assessed for lung vessel density) confirmed the severe decrease in pulmonary vascular density in the hyperoxic group (figure 5B). Both PC and MSC CdM significantly attenuated the decrease in pulmonary vascular density (figure 5B), but to a lesser extent than the improvements seen in lung morphometry and function.

Pulmonary hypertension is a common complication of BPD and significantly worsens the prognosis.[13] PC and MSC CdM were effective in preventing pulmonary arterial wall remodelling (figure 6A,B) and right ventricular hypertrophy (figure 6C), two structural features of pulmonary hypertension.

Similar to whole cell therapy, therapeutic administration of PC and MSC CdM (from P14 to P28 assessed in 24 animals) after established lung injury improved alveolar architecture (figure 7A–C) and lung function (figure 7D).

PCs and MSCs display long-term safety

Seventy-two animals were kept alive for 6 months for long-term evaluation of whole cell therapy (36 animals) and CdM therapy (36 animals).

Intrapulmonary delivery of PCs and MSCs at P4 was safe up to 6 months of life. Total body CT scans did not reveal any suspicious images suggesting tumour formation (figure 8A). A single suspicious CT scan picture was ruled out as a congested vessel at histology (figure 8B). Exercise capacity, using a graduated treadmill exercise protocol by a blinded observer, was significantly

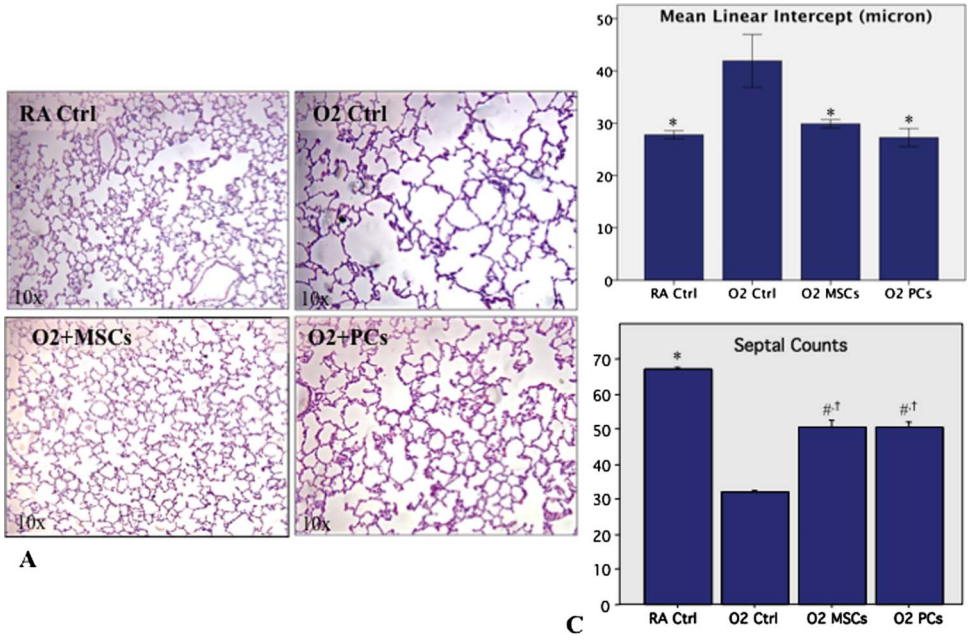

Figure 2 Perivascular cells (PCs) and mesenchymal stem cells (MSCs) rescue hyperoxia-induced lung injury. (A) Representative H&E-stained lung sections of animals treated intratracheally with MSCs and PCs at P14 after established lung injury and harvested at P28. MSCs (O_2+MSCs) and PCs (O_2+PCs) in oxygen-exposed animals partially restored alveolar growth. (B,C) This is confirmed by the mean linear intercept (n=6/group; *p<0.001 vs O_2 Ctrl; no differences between O_2+MSCs and O_2+PCs and room air (RA)+MSCs and RA+PCs) and the septal counts (n=6/group; #p<0.01 vs O_2 Ctrl; *p<0.001 vs O_2 Ctrl; †p<0.01 vs RA control).

decreased in untreated oxygen-exposed rats (figure 8C). This was associated with persistent enlarged and simplified distal airspaces (figure 8D–F). Rats treated with whole cell therapy exhibited significantly improved exercise capacity (figure 8C) and showed almost normal alveolar architecture (figure 9D–F) at 6 months of age. Control RA housed animals treated with whole cell therapy showed no adverse effect on exercise capacity (figure 8C) or lung structure (figure 8D–F).

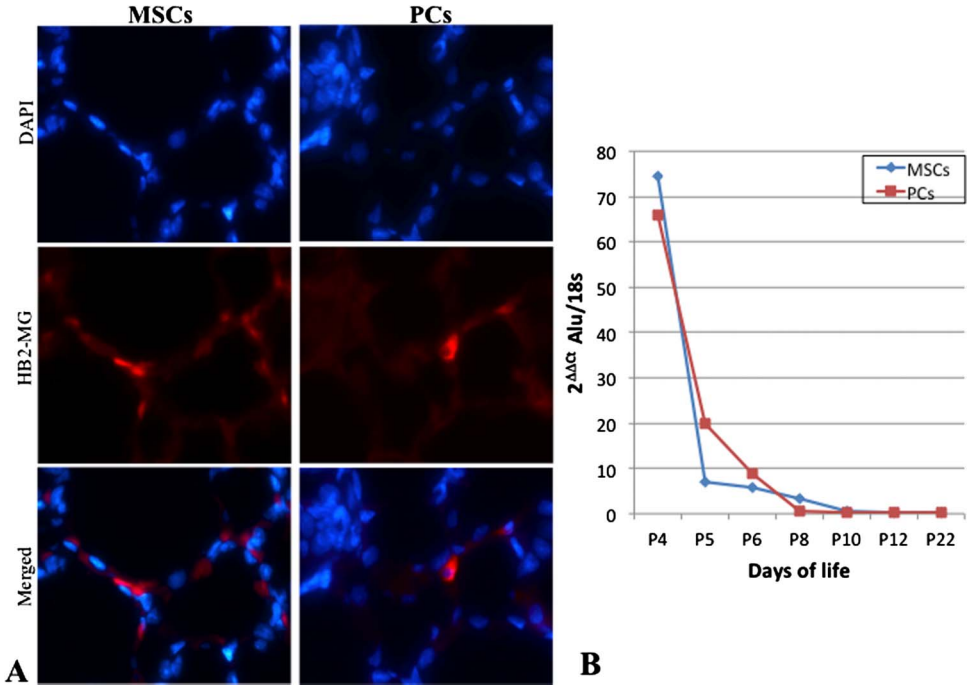

Figure 3 Low engraftment rate after intratracheal injection of perivascular cells (PCs) and mesenchymal stem cells. (A) Immunofluorescent staining for human β_2-microglobulin (HB2-MG) at P22, performed in order to detect cells of human origin in the recipient lungs, showed a low rate of engraftment of both cell types. (B) Quantitative RT-PCR for Alu sequences revealed a dramatic decrease during the first day after injection. Human DNA became almost undetectable 4 days after injection. Values indicate $2^{\Delta\Delta CT}$ for human/rat 18s and Alu sequences. The control samples were non-injected lungs (n=3 animals/time point).

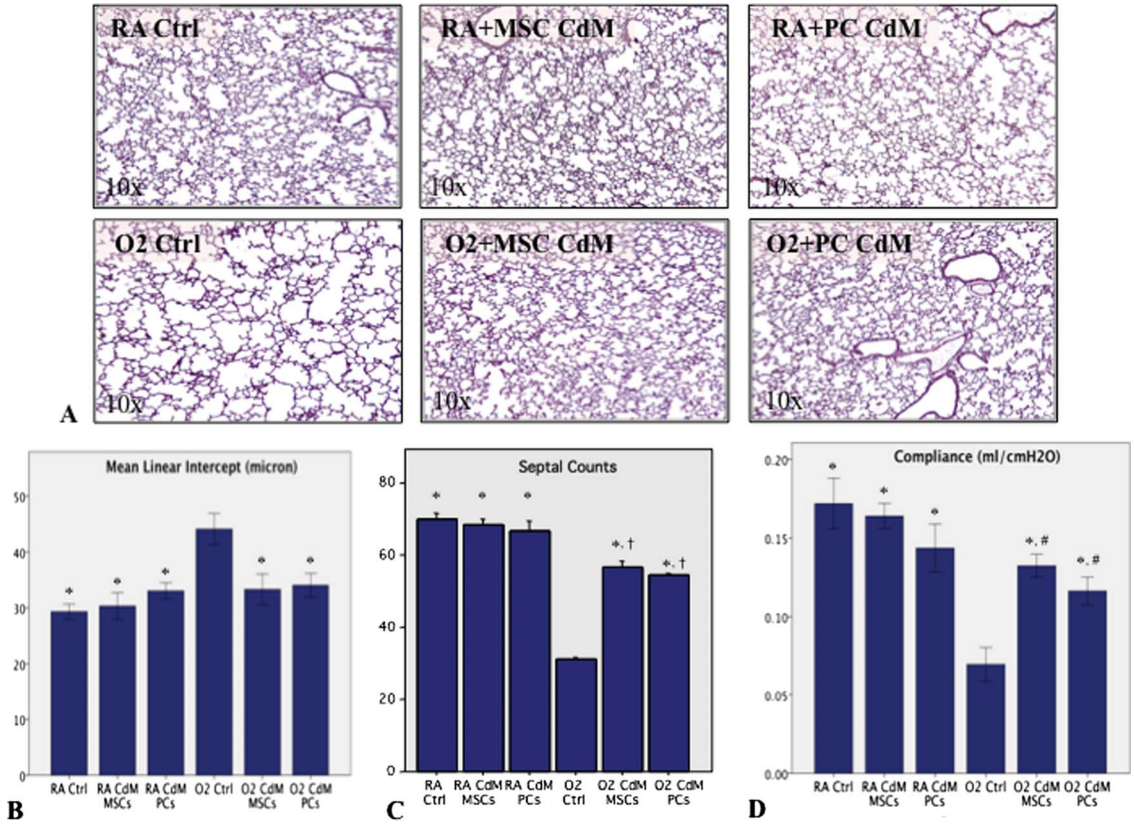

Figure 4 Conditioned media (CdM) from perivascular cells (PCs) and mesenchymal stem cells (MSCs) prevent hyperoxia-induced lung injury. (A) Representative H&E-stained lung sections showing larger and fewer alveoli in hyperoxia-exposed lungs compared with lungs from rats housed in room air (RA) and RA animals treated with MSC CdM and PC CdM. Daily intraperitoneal administration of MSC CdM and PC CdM in oxygen-exposed animals improved alveolar growth. (B,C) Quantitative confirmation is provided by the mean linear intercept (n=6/group; *p<0.001 vs O_2 Ctrl; no differences between O_2+CdM MSCs and O_2+PC CdM and RA+MSC CdM and RA+PC CdM) and the septal counts (n=6/group; *p<0.001 vs O_2 Ctrl; †p<0.01 vs all RA groups). (D) Invasive lung function testing shows decreased lung compliance in untreated oxyten-exposed animals compared with RA Ctrl and RA MSC CdM and RA PC CdM groups. Lung compliance was significantly improved in oxygen-exposed animals treated with MSC CdM and PC CdM (n=6/group; *p<0.001 vs O_2 Ctrl; #p<0.05 O_2+MSC CdM vs RA+CdM MSC and O_2+PC CdM vs RA+PC CdM).

Likewise, animals evaluated 6 months after PC- and MSC-derived CdM treatment showed no suspicious CT scans (figure 9A). These animals also had significantly improved exercise capacity (figure 9B) and alveolar architecture (figure 9C,D).

DISCUSSION

The major findings in this study include: (1) the efficacy of human cord-derived PCs and cord blood-derived MSCs in preventing and rescuing oxygen-induced arrested alveolar growth;

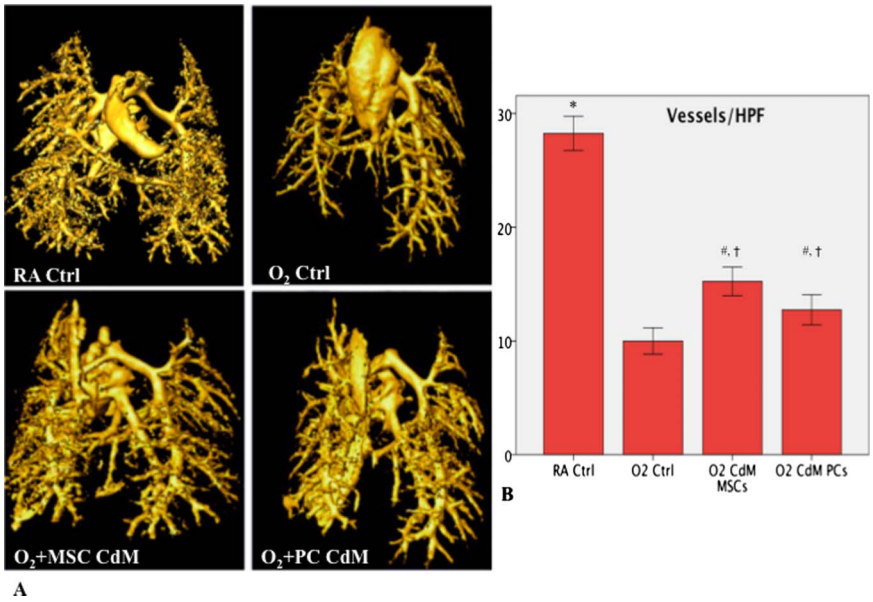

Figure 5 Conditioned media (CdM) from perivascular cells (PCs) and mesenchymal stem cells (MSCs) improve lung angiogenesis. (A) Representative micro-CT scans of the pulmonary vasculature after barium injection into the pulmonary artery. (B) Mean vessel density assessed on barium-injected lungs was significantly decreased in the lungs of oxygen-exposed animals. Daily intraperitoneal administration of MSC CdM and PC CdM improved lung vessel density (n=6 animals/group; *p<0.001 vs O_2 Ctrl; #p<0.05 vs O_2 Ctrl; †p<0.001 vs all room air Ctrl).

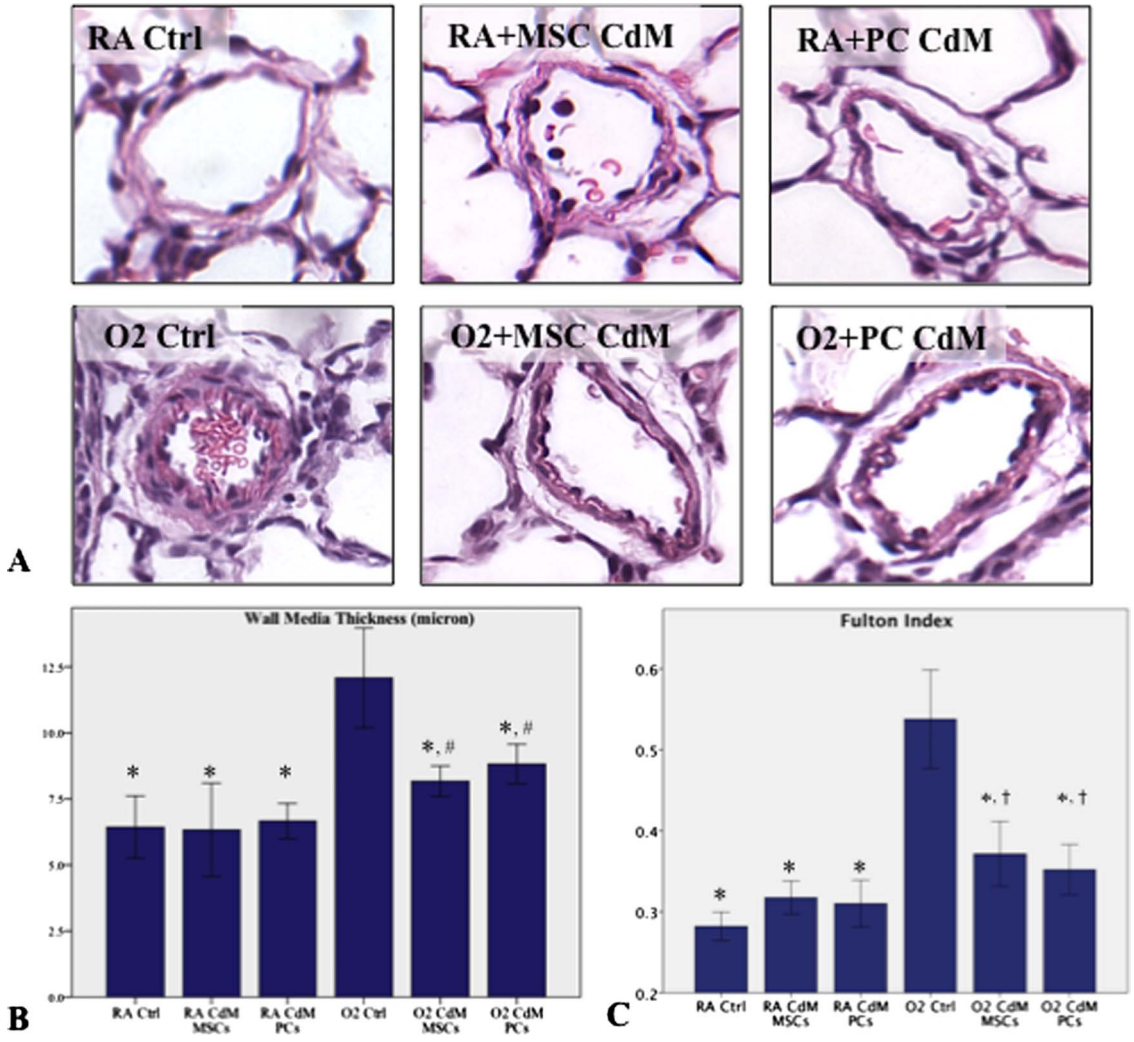

Figure 6 Conditioned media (CdM) from perivascular cells (PCs) and mesenchymal stem cells (MSCs) prevent features of pulmonary hypertension. (A) Representative H&E sections of pulmonary arteries from the six experimental groups. (B) Hyperoxic-exposed rats had a significant increase in media wall thickness (MWT) compared with room air (RA) housed rat pups. MSC CdM and PC CdM significantly reduced MWT (n=5 animals/group; *p<0.001 vs O_2 Ctrl; #p<0.01 vs all RA groups). (C) Fulton index, reflecting right ventricular hypertrophy, was significantly increased in untreated oxygen-exposed rats compared with RA Ctrl, RA MSC CdM and RA PC CdM groups. MSC CdM and PC CdM significantly reduced MWT (n=5 animals/group; *p<0.001 vs O_2 Ctrl; †p<0.05 vs all RA groups).

(2) PCs, similar to MSCs, exert their therapeutic benefit primarily through a paracrine effect; and (3) the long-term efficacy and absence of adverse lung effects of whole cell or CdM therapy at 6 months.

Therapeutic potential of cord-derived cells

We harnessed the potential of umbilical cord and cord blood as stem cell sources because of their numerous advantages, in particular for neonates. Indeed, among the various sources of stem cells, umbilical cord and cord blood represent an ethically non-controversial, clinically relevant and easily accessible source of potent stem cells.[6 14]

Among stem cells, MSCs have attracted most attention and numerous clinical trials are underway (http://clinicaltrials.gov/ct2/results?term=mesenchymal+stem+cells) to test their therapeutic potential for regenerative purposes.[3] Recently, PCs in numerous human organs have been characterised based on expression of CD146, NG2 and PDGFRβ and the absence of haematopoietic, endothelial and myogenic cell markers.[7] In addition to their vascular functions, human PCs are multilineage progenitor cells that natively exhibit features of MSC and give rise in culture to adherent cells indistinguishable from conventional MSCs, confirming previously documented similarities between pericytes and MSCs.[7] The capacity of human pericytes to generate skeletal muscle, bone, cartilage[7 15 16] and to form vascular grafts[15] has already been documented, but the therapeutic potential of these cells in lung diseases has not yet been investigated.

Here we show that, similar to MSCs, cord-derived PCs demonstrate repair potential by preserving lung function and preventing oxygen-induced arrested alveolar growth in newborn rats. Our observation, combined with previous findings showing that bone marrow- and cord-derived MSCs attenuate lung inflammation in this model,[4 17] make cord-derived cell-based therapies appealing for the prevention of lung injury. The prevention approach is legitimate in BPD as one can predict which premature infants are at high risk for developing the disease. Furthermore, we have shown that PCs and MSCs restore lung function and structure after established lung injury. This is relevant for lung diseases characterised by currently irreversible alveolar destruction, including emphysema.

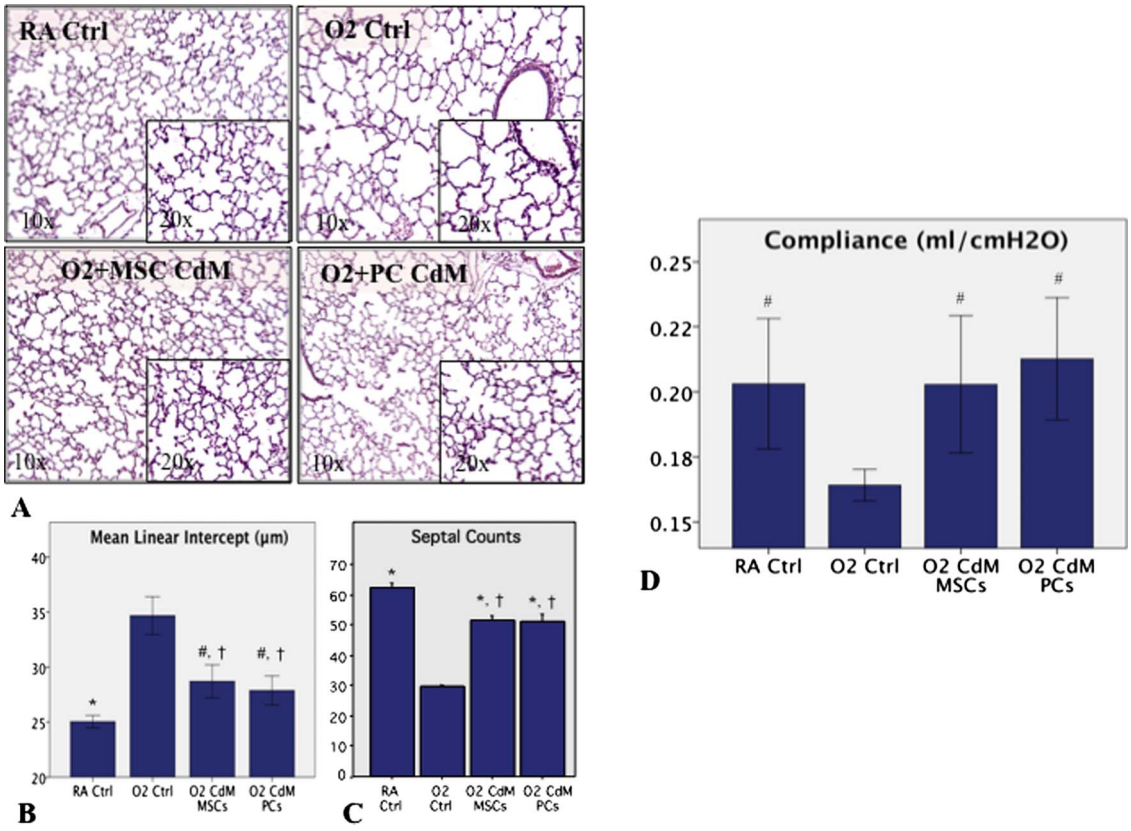

Figure 7 Conditioned media (CdM) from perivascular cells (PCs) and mesenchymal stem cells (MSCs) rescue hyperoxia-induced lung injury. (A) Representative H&E-stained lung sections showing larger and fewer alveoli in hyperoxia-exposed lungs compared with lungs from rats housed in room air (RA) and RA animals treated with MSC CdM and PC CdM. Daily intraperitoneal administration of MSC CdM and PC CdM after established arrested alveolar growth restored almost normal lung architecture in oxygen-exposed animals. (B,C) Quantitative assessment by the mean linear intercept (n=6 animals/group; #p<0.05 vs O_2 Ctrl; *p<0.001 vs O_2 Ctrl; †p<0.05 vs RA Ctrl) and the septal counts (n=6 animals/group; *p<0.001 vs O_2 Ctrl; †p<0.05 vs RA Ctrl) confirms larger and fewer alveoli in hyperoxia-exposed lungs compared with lungs from rats housed in RA and RA animals treated with CdM MSC and CdM PC. CdM MSC and CdM PC restored alveolar growth. (D) Invasive lung function testing shows decreased lung compliance in untreated oxygen-exposed animals compared with RA Ctrl and RA MSC and RA PC groups. Compliance was significantly improved in oxygen-exposed animals treated with CdM MSC and CdM PC (n=6 animals/group; # p<0.05 vs O_2 Ctrl).

Paracrine effect of cord-derived stem cells

Very few engrafted cells were detected by immunofluorescence and analysis of human-specific Alu sequences, consistent with previous reports suggesting that engraftment does not account for the therapeutic benefit.[18 19] The current concept supports the view that MSCs act via a paracrine effect.[20] Indeed, MSCs produce and secrete a variety of cytokines, chemokines and growth factors that may contribute to tissue repair.[21] There is evidence that pericytes exert their benefit through a similar mechanism. In the vicinity of peripheral nerves, pericytes secrete neurotrophic soluble factors facilitating axonal regeneration in peripheral neuropathy.[22] We have previously documented the secretion by cultured human pericytes of diverse cytokines[23] and observed that cord-derived PCs produce higher levels of keratinocyte growth factor (KGF)—a factor recently demonstrated to mediate the therapeutic benefit of human bone marrow-derived MSC CdM in endotoxin-induced acute lung injury in the ex vivo perfused human lung[24] and in ventilation-induced lung injury[25]—when co-cultured with damaged lung cells.[8]

In the present study we provide in vivo evidence for the therapeutic benefit of cord-derived PC CdM. Prophylactic CdM administration improved lung function and structure. Moreover, CdM preserved lung angiogenesis—known to contribute to normal lung growth and to be impaired in BPD[10]—and prevented pulmonary hypertension, a life-threatening complication of BPD.[13] We opted for daily intraperitoneal administration of CdM, reasoning that repetitive dispensation would be required to insure a constant release of protective factors to achieve a therapeutic benefit. Accumulating evidence, however, suggests that a single dose of CdM is enough to prevent oxygen-induced lung injury in neonatal mice.[4 26] This is consistent with recent data suggesting that MSCs act through the release of microparticles[27] or via mitochondrial transfer.[28] These observations may explain why a single injection of CdM may be sufficient to obtain a therapeutic benefit. This also opens new therapeutic avenues for cell-based therapies. Indeed, the recognition of MSC release of microparticles acting as micropackages containing a combination of healing factors may circumvent the complex task of identifying each of the various healing compounds and determining the most potent healing combination of these factors. This may also be relevant for the design of clinical trials to determine the most efficacious and safest stem cell-based approach: whole cell therapy versus cell-derived CdM versus single or multiple identified CdM-derived compounds. Ex vivo preconditioning may further enhance the efficacy and also facilitate the discovery of MSC-derived repair molecules.[29]

The discrepancy between the striking improvement in lung morphometry and lung function with CdM and a more

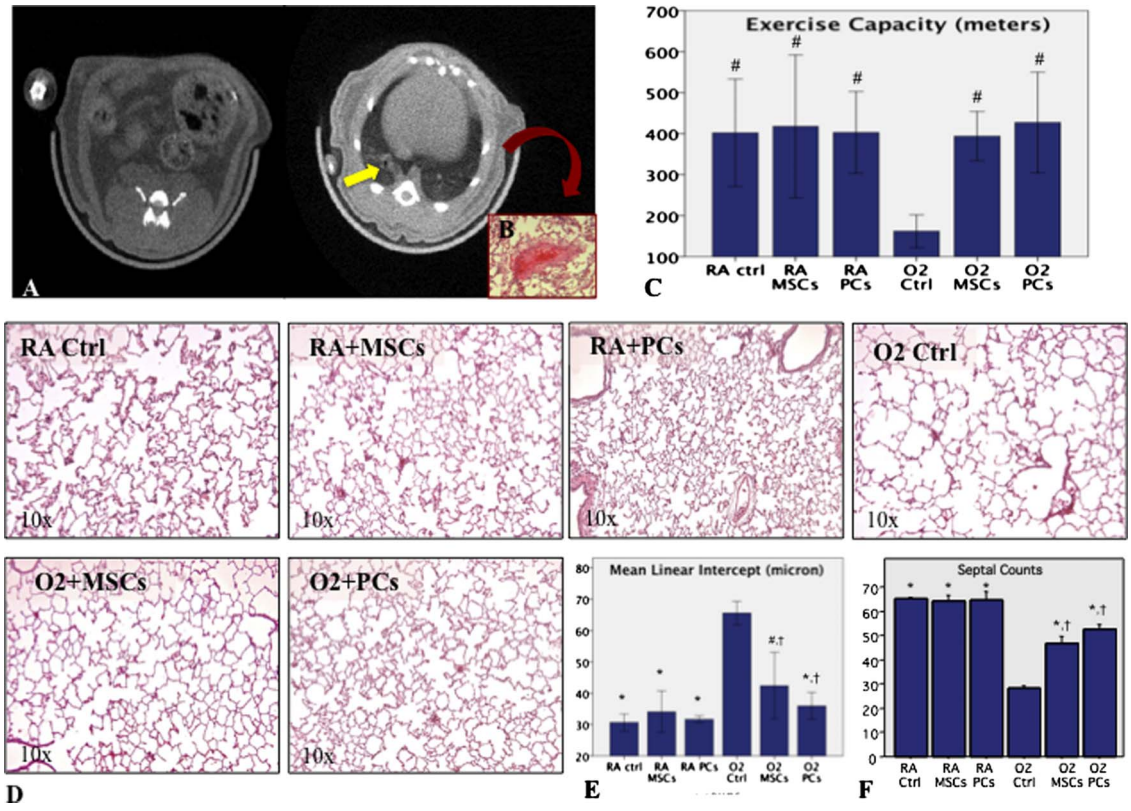

Figure 8 Long-term (6 months) safety and efficacy of stem cell therapy. (A) Representative CT scan performed at 6 months of age showed no suspicious images in the perivascular cell (PC) group and one doubtful image in the mesenchymal stem cell (MSC) group (n=4 animals/group). The corresponding histology samples (B, insert) ruled out the presence of a possible tumour and indicated a congested blood vessel. (C) Oxygen-exposed animal experienced reduced exercise capacity at 6 months of age compared with animals housed in room air (RA). Oxygen-exposed animals treated with MSCs and PCs had improved exercise capacity (n=6 animals/group; # $p<0.05$ vs O_2 Ctrl; no differences between O_2+MSC and RA+MSC and O_2+PC and RA+PC). (D) Representative H&E-stained lung sections show persistent alveolar simplification at 6 months of age in hyperoxia-exposed animals compared with lungs from rats housed in RA. Oxygen-exposed animals treated with MSCs and PCs presented with improved lung histology. (E,F) The mean linear intercept (n=6 animals/group; #$p<0.05$ vs O_2 Ctrl; *$p<0.001$ vs O_2 Ctrl; †$p<0.05$ O_2+MSC vs RA+MSC and O_2+PC vs RA+PC) and the septal counts (n=6 animals/group; *$p<0.001$ vs O_2 Ctrl, †$p<0.01$ O_2+MSC vs RA+MSC and O_2+PC vs RA+PC) confirm arrested alveolar growth in untreated oxygen-exposed animals and preserved alveolar structure with MSC and PC treatment.

moderate effect on pulmonary vessel density is unexpected. One may speculate that secretion of epithelial growth factors by MSCs—KGF in particular—promotes preferential alveolar epithelial cell protection leading to improved lung histology.[24 25] However, MSCs also produce many pro-angiogenic factors to stimulate vascular growth and Hansmann *et al* recently showed efficient restoration of the pulmonary vascular bed with intravenous MSC CdM in neonatal mice exposed to hyperoxia.[26 30] Further studies in various animal models of chronic neonatal lung injury are required to clarify these observations.

Long-term effects of whole cell therapy and CdM

BPD can have life-long consequences including impaired lung function, asthma, early onset emphysema and pulmonary hypertension.[31] Similarly, our model showed long-lasting alterations in lung function and structure following neonatal hyperoxia. Animals exposed to oxygen from P4 to P14 still displayed altered exercise capacity and arrested alveolar growth at 6 months of age (life span 2–3 years). More importantly, the therapeutic benefit of both whole cell therapy and CdM administration was sustained, showing improved exercise capacity and lung histology 6 months after treatment. Furthermore, 6 months after injection of PCs and MSCs or their CdM, no tumours were detectable on total body CT scans.

A limitation of the well-established neonatal rodent model to mimic BPD via hyperoxic exposure is that oxygen represents only one among many deleterious factors contributing to BPD such as mechanical ventilation and pre- and postnatal inflammation. Recent observations suggest that MSCs prevent ventilation-induced lung injury in adult rats.[25] In the developing lung, human amnion epithelial cells prevent ventilation- and inflammation-induced lung injury in fetal sheep.[32 33] These studies add another interesting reparative cell source for cell-based therapies.

In conclusion, human umbilical cord-derived PCs, as whole cell therapy or growth factor producers, show promise as a new cell-based therapy for lung diseases characterised by arrested alveolar growth/loss of alveoli.

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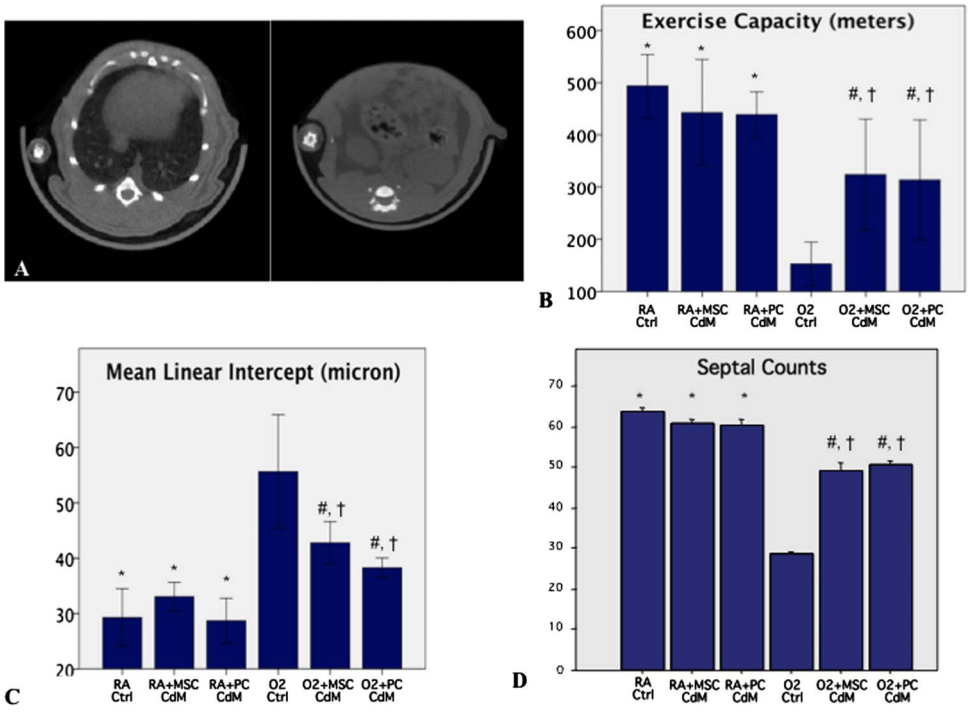

Figure 9 Long-term (6 months) safety and efficacy of conditioned media (CdM) therapy. (A) Representative CT scan performed at 6 months of age in a CdM perivascular cell (PC) treated animal. There were no suspicious images in the treated groups (n=4 animals/group). (B) Oxygen-exposed animals experienced reduced exercise capacity at 6 months of age compared with animals housed in room air (RA). Oxygen-exposed animals treated with CdM mesenchymal stem cells (MSCs) and CdM PCs had improved exercise capacity (n=6 animals/group; #$p<0.05$ vs O_2 Ctrl; *$p<0.001$ vs O_2 Ctrl; †$p<0.05$ O_2+MSC vs RA+MSC and O_2+PC vs RA+PC). (C, D) Quantitative assessment of lung structure confirms the persistent alveolar simplification at 6 months of age in hyperoxia-exposed animals compared with lungs from rats housed in RA. Oxygen-exposed animals treated with CdM MSC and CdM PC presented with improved lung histology (n=6 animals/group; #$p<0.05$ vs O_2 Ctrl; *$p<0.001$ vs O_2 Ctrl; †$p<0.01$ O_2+MSC vs RA+MSC and O_2+PC vs RA+PC).

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Contributors MP, LI, GO, FM and BT designed the study. MP, LI, AV, GW and FE performed the experiments. SB carried out the western blots. DM read the CT scans. TM and LL harvested and characterised the cord-derived cells. MP, BP, LL, TM and BT drafted the manuscript and are guarantors of the paper.

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Competing interests None.

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Online Supplement:

Methods

Human umbilical cord isolation and culture of pericytes. Pericytes (PCs) were isolated from the umbilical cords after parental consent as previously described (Supplemental Figure).[1] Briefly, human umbilical cords were dissected longitudinally to expose the vein and the two arteries and digested with 1mg/mL collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) at 37°C, for a maximum of 18 hours. The cell suspension was washed and the cell pellet was resuspended and cultured in EGM2 medium (Lonza, Walkersville, MD, USA) on a pre-coated gelatin layer (Sigma-Aldrich; St. Louis, MO, USA). After 1 week, the medium was replaced with DMEM high-glucose (Invitrogen, Carlsdad, CA, USA), supplemented with 20% fetal bovine serum (FBS; Biochrom, AG, Berlin, Germany) and 1% penicillin/streptomycin (P/S, Sigma-Aldrich) and the cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2. Adherent PCs, 80% confluent, were passaged by treatment with trypsin-EDTA (Gibco, Grand Island, NY, USA), and split 1:3 in uncoated plates in the same culture conditions. Medium was changed every 3 days.

Human umbilical cord blood isolation and culture of mesenchymal stem cells (MSCs). Human cord blood (CB) was collected from newborns after parental consent and MSC isolation was performed within 12 hours as previously described (Supplemental Figure).[2] First, CB was centrifuged and plasma discarded. An enrichment protocol was performed by a negative immunodepletion of CD3+, CD14+, CD38+, CD19+, glycophorin A and CD66b+ using a commercial kit (RosetteSep Mesenchymal Stem Cell, StemCell Technologies, Vancouver, BC, Canada), and followed by a density gradient centrifugation (Ficoll-Paque Premium, GE Healthcare, Amersham Place, UK). After washing, cells were cultured in Modified Eagle alpha-medium (Invitrogen) supplemented with 20% FBS (Biochrom) and 2mM L-glutammine (Gibco). Cultures were maintained at 37°C in humidified atmosphere containing 5% CO2. After overnight incubation, non-adherent cells were removed

and fresh medium was added; culture medium was changed every 3 days.

Generation of PCs and MSC-derived CdM. Cells were grown in 75t flask up to 90% confluence (MSC 1.500.000 cells/ fask, PCs 1.000.000 cells/flask). Then cells were rinsed 3 times with PBS and serum free media was added. After 24 hours the supernatant was harvested and centrifuged at 4000 RPM for 40 minutes in ultrafiltration tubes (Millipore, US) to obtain a 25 times concentrated CdM.[3] CdM was also obtained from human neonatal dermal fibroblasts (HNDF, ATCC, Manassas, VA, USA) and cultured in Fibroblast Basal Medium supplemented with FGM bulletkit (Lonza, Basel, Switzerland).

Animal model of O_2-arrested lung growth. Rat pups were exposed to normoxia (21% O_2, control group) or hyperoxia (95% O_2, BPD-group) from birth to P14 in sealed Plexiglas chambers (BioSpherix, Redfield, NY) with continuous O_2 monitoring.[4,5] Dams were switched every 48 hours between the hyperoxic and normoxic chambers to prevent damage to their lungs and provide equal nutrition to each litter. Litter size was adjusted to 12 pups to control for effects of litter size on nutrition and growth. Rat pups were euthanized at various time points with intraperitoneal pentobarbital and lungs and heart were processed, according to the performed experiments.

***In Vivo* Cells Administration.** We performed short-term experiments using a prevention and a rescue approach. For the prevention studies, newborn rat pups were randomized into seven groups: (1) room air control (RA Ctrl), (2) room air+MSCs (RA MSCs), (3) room air+PCs (RA PCs), (4) hyperoxia (O_2 Ctrl, injury model), (5) hyperoxia+HNDF, (6) hyperoxia+MSCs (O_2 MSCs), and (7) hyperoxia+PCs (O_2 PCs). For these prevention studies, rat pups received 300.000 cells in 20µl at P4 via an i.t. injection and harvested at P22.

For subsequent rescue experiments, the control cell group (HNDF) was deleted because HNDFs had no effect. For the same reason, we also deleted the room air+MSC and room air+PC groups. Thus, for rescue studies, newborn rat pups were randomized into 4 groups: (1) room air control (RA Ctrl), (2) hyperoxia (O_2 Ctrl, injury model), (3) hyperoxia+MSCs (O_2

MSCs), and (4) hyperoxia+PCs (O_2 PCs). For these rescue studies, rat pups received 600.000 in 40µl at P14 and harvested at P35.

The cell dose was adjusted to animal weight and based on the literature.[6]

We also performed long-term studies to assess the effect of stem cell administration at 6 months. In these experiments animals were treated at P4 and harvested at 6 months. Animals were randomized into 6 groups: (1) room air control (RA Ctrl), (2) room air+MSCs (RA MSCs), (3) room air+PCs (RA PCs), (4) hyperoxia (O_2 Ctrl, injury model), (5) hyperoxia+MSCs (O_2 MSCs), and (6) hyperoxia+PCs (O_2 PCs).

***In Vivo* CdM Administration.** We performed short-term experiments using a prevention and a rescue approach. In the prevention studies, newborn rat pups were randomized into six groups: (1) room air control (RA Ctrl), (2) RA+MSC CdM, (3) RA+PC CdM, (4) hyperoxia control (O_2 Ctrl), (5) O_2+MSC CdM, and (6) O_2+PC CdM. In these prevention studies, CdM was administered daily IP at the dose of 7 µl/g from P4 to P21 and animals were harvested at P22 (prevention studies).

In the rescue studies, newborn rat pups were randomized into 4 groups: (1) room air control (RA Ctrl), (2) hyperoxia control (O_2 Ctrl), (3) O_2+MSC CdM, and (4) O_2+PC CdM. In these rescue studies, CdM was administered daily IP at the dose of 7 µl/g from P14 to P28 and animals were harvested at P35. The dose of the CdM was based on Aslam et al. [7]

Long-term study animals were treated from P4 to P21 and harvested at 6 months.

Lung function tests. Animals were anesthetized using ketamine (10 mg/kg i.p) and xylazin (5 mg/kg i.p) mixture and paralyzed using a pancuronium bromide injection (1 mg/kg i.p). Tracheostomy was performed and lung function was assessed using Flexivent (Scireq, Montreal, QC, Canada).

Lung Morphometry. Lungs were inflated and fixed via the trachea with zinc formalin

solution at a constant pressure of 20 cm H_2O.[4,5] Lungs were paraffin embedded and cut into 4-µm-thick serial sections, and lungsections were stained with hematoxylin and eosin. Alveolar structures were quantified using the mean linear intercept as described.[4,5] Six lungs/group, three sections/lung and 100 high-power fields/section were counted.

Barium-gelatin angiograms and vessel density counts. A barium-gelatin mixture (60ºC) was infused in the main pulmonary artery until surface filling of vessels with barium was seen uniformly over the surface of the lung as previously described.[4,5] Four to five lungs/group, five sections/lung and ten high-power fields/section were counted. Barium-injected lung vasculature was imaged with a rodent SPECT-CT (FLEX Pre-clinical platform) using Amira software package (Gamma Medica, Northridge, CA).

Right ventricular hypertrophy (RVH) and pulmonary artery remodeling. The right ventricle free wall was separated from the left ventricle and the septal wall. The tissue was dried overnight and weighed the next day to determine the right ventricle to left ventricle+septum ratio (RV/LV+S) as an index of RVH.[5] Pulmonary artery remodeling was quantified by the medial wall thickness (MWT).[4,5] Five pups/group, three sections/lung and ten high-power fields/section were counted.

Exercise capacity. Rats were run on a treadmill adjusting the speed according to the following protocol: 1 min at 10 meters/min, 1 min at 11 meters/min, 1 min at 12 meters/min, 2 min at 13 meters/min, 5 min at 15 meters/min, 17 meters/min until exhaustion. Exhaustion was defined by sitting on the shock panel longer than 5 seconds.

Total Body CT-Scan. Rats were anesthetized using inhaled isoflurane and 3 to 4 sections with 1028 slides/section were taken with a rodent SPECT-CT (FLEX Pre-clinical platform) using Amira software package (Gamma Medica, Northridge, CA).

Real-time PCR. Total RNA was extracted from pulverized frozen lungs using Qiagen RNeasy kit (Qiagen, Mississauga, ON). RNA was quantified using a Nanodrop system (ND-1000 ThermoFisher Scientific, Wilmington, DE) and cDNA was prepared from 1ung RNA

using random hexamers. PCR was performed on an ABI 7900 and using Taqman Universal PCR master mix (Applied Biosystems), Human Alu sequence primers and values determined from a standard curve prepared from pure pericytes and MSCs.[8] All results are expressed as a ratio of Alu sequences normalized to human 18S. Three animals/group were harvested 10 min after injection (P4), 1 day after injection (P5), 2 days after injection (P6) and at 22 days of life.

Immunofluorescence for β2-microglobulin. Immunofluorescent staining was performed on nonadjacent 5μm paraffin-embedded lung sections using rabbit anti-human β2-microglobulin (Abcam, Cambridge, MA, USA) and appropriate secondary antibodies (Invitrogen, Carlsbad, CA, USA). Nuclei were identified by DAPI staining. Five random fields of four sections per animal were analyzed by confocal microscopy.

Statistics. Values are expressed as means ± SEM. Intergroup differences were assessed using analysis of variance with post hoc test (Fisher's probable least significant difference test) (SPSS v 18). A value of $P<0.05$ was considered statistically significant. All investigators performing evaluations were blinded to the experimental groups.

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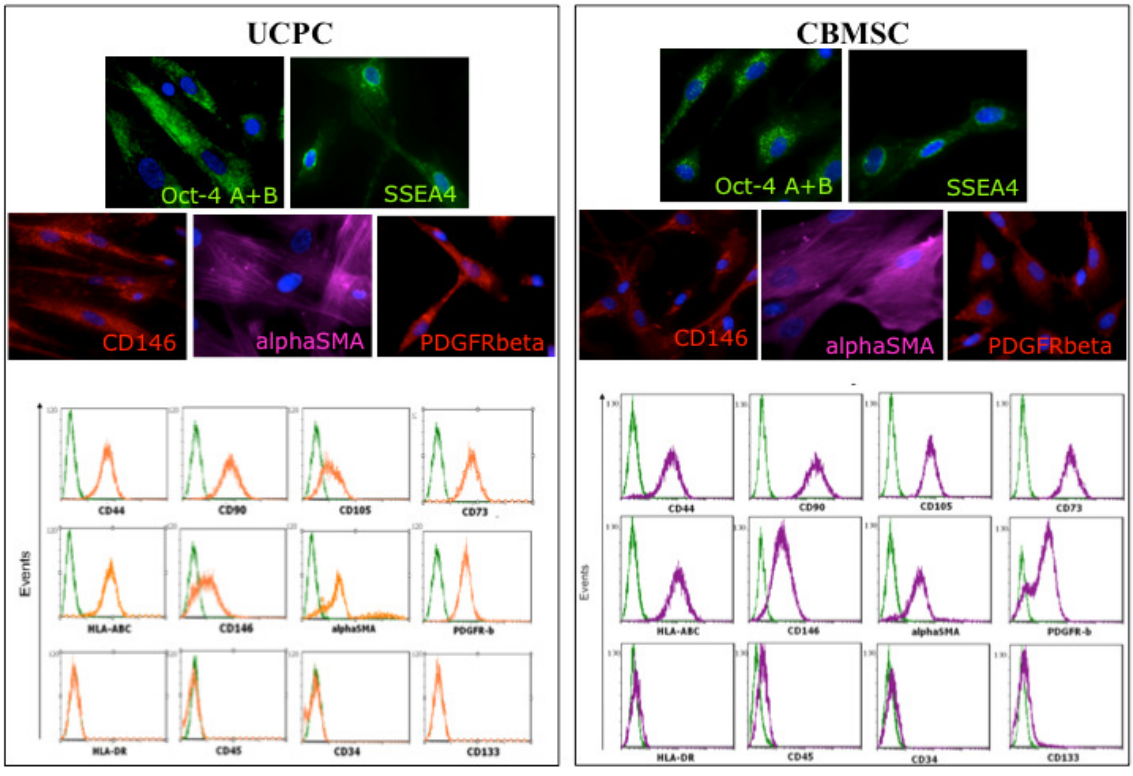
Supplemental Figure Legends

Supplemental Figure. A. Characterization of cord derived perivascular cells (UCPCs). Immunofluorescence and fluorescence intensity histograms with specific antibodies for membrane antigens (orange line) and irrelevant isotypic-matched Ab as negative control (green line). **B. Characterization of cord blood derived mesenchymal stem cells (CBMSCs).** Immunofluorescence and fluorescence intensity histograms with specific antibodies for membrane antigens (purple line) and irrelevant isotypic-matched Ab as negative control (green line).

Supplemental Figure



CHAPTER III

MESENCHYMAL STEM CELLS FOR THE PREVENTION AND TREATMENT OF BRONCHOPULMONARY DYSPLASIA IN PRETERM INFANTS

Pierro M, Thébaud B, Soll R.

Cochrane Database Syst Rev. 2017;11:CD011932.

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Mesenchymal stem cells for the prevention and treatment of bronchopulmonary dysplasia in preterm infants (Review)

Pierro M, Thébaud B, Soll R

Pierro M, Thébaud B, Soll R.
Mesenchymal stem cells for the prevention and treatment of bronchopulmonary dysplasia in preterm infants.
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[Intervention Review]

Mesenchymal stem cells for the prevention and treatment of bronchopulmonary dysplasia in preterm infants

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ABSTRACT

Background

Bronchopulmonary dysplasia (BPD) remains a major complication of prematurity and currently lacks efficient treatments. Mesenchymal stem/stromal cells (MSCs) have been extensively explored as a potential therapy in several preclinical and clinical settings. Human and animal MSCs have been shown to prevent and treat lung injury in various preclinical models of lung diseases, including experimental BPD.

Objectives

To determine if MSCs, administered intravenously or endotracheally, are safe and effective in preventing or treating BPD, or both, in preterm infants.

Search methods

We used the standard search strategy of the Cochrane Neonatal Review Group to search the Cochrane Central Register of Controlled Trials (CENTRAL 2016, Issue 10), MEDLINE via PubMed (1966 to 6 November 2016), Embase (1980 to 6 November 2016), and CINAHL (1982 to 6 November 2016). We also searched clinical trials databases, conference proceedings, and the reference lists of retrieved articles for randomized controlled trials (RCTs) and quasi-RCTs**.**

Selection criteria

We considered RCTs and quasi-RCTs investigating prevention or treatment of BPD, or both, in preterm infants.

Data collection and analysis

Two review authors independently assessed trial quality according to prespecified criteria.

Main results

We found no RCTs or quasi-RCTs addressing the use of MSCs for prevention or treatment of BPD in premature infants. Two RCTs are currently registered and ongoing.

Authors' conclusions

There is insufficient evidence to determine the safety and efficacy of MSCs in the treatment or prevention of BPD in premature infants. The results of the ongoing trials addressing this issue are expected in the near future.

PLAIN LANGUAGE SUMMARY

Stem cells for the prevention and treatment of bronchopulmonary dysplasia in preterm infants

Background

Bronchopulmonary dysplasia (BPD) is a chronic lung disease that often complicates the course of babies who are born too early. Bronchopulmonary dysplasia can lead to serious health issues during childhood and later in life. There is currently no effective and safe treatment for BPD. Mesenchymal stem cells (MSCs), cells that can multiply and turn into a different type of cell, can protect the damage to newborn lungs in experimental models of BPD. Mesenchymal stem cells may bring new hope for untreatable health issues in babies born too early, including BPD, and thus improve their survival and quality of life.

Review question

Are MSCs, administered intravenously or endotracheally, safe and effective in preventing or treating BPD, or both, in preterm infants?

Study characteristics

We found no clinical trials that addressed the use of MSCs for prevention or treatment of BPD in premature infants. However, some studies are currently underway.

Key results

There is insufficient evidence to determine the safety and efficacy of MSCs for the treatment or prevention of BPD in premature infants. The results of the ongoing trials are expected in the near future.

BACKGROUND

Description of the condition

Bronchopulmonary dysplasia (BPD) is considered one of the major complications of preterm birth (Farstad 2011; Jobe 2001). Bronchopulmonary dysplasia develops as a consequence of impaired lung development, exacerbated by the imbalance between pro-inflammatory stimuli and anti-inflammatory defense mechanisms typical of the preterm infant (Jobe 2001; Speer 2006).

The definition of BPD has been evolving since its first description as 28 days of oxygen exposure with characteristic radiographic changes (NIH 1979). Subsequently, oxygen dependency at 36 weeks' postmenstrual age was shown to better predict long-term respiratory outcomes (Shennan 1988). The current definition of BPD stratifies infants below 32 weeks requiring supplemental oxygen for at least 28 days into three severity groups (mild, moderate, and severe), depending on the presence and the amount of supplemental oxygen and the mode of respiratory support at 36 weeks' postmenstrual age (Ehrenkranz 2005; Jobe 2001). The 'physiologic' definition of BPD was proposed in an attempt to address the significant intercenter variability in oxygen administration (Lapcharoensap 2015). At 36 weeks' postmenstrual age, infants receiving less than 30% supplemental oxygen are challenged by reducing the fraction of administered oxygen during a standardized test. Infants who are unable to maintain saturations above 90% during the test are diagnosed with BPD (Walsh 2003). The incidence of BPD varies depending on the definition, complicating the course of up to 40% of the infants born between 22 and 28 weeks' gestation (Stoll 2010). It must be noted that, although by definition BPD cannot be diagnosed before 28 days of life, a respiratory disease defined as oxygen and/or ventilator-dependency from 7 to 28 days of life represents the initial phase of the chronic process leading to BPD, thus classified as "evolving BPD" (Walsh 2006). Infants suffering from BPD are at increased risk of death and long-term pulmonary and neurodevelopmental

morbidities (Anderson 2006; Bhandari 2006). Several treatments have been used in an attempt to prevent or treat BPD. Unfortunately, even the most promising strategies have not been able to confirm the initial enthusiasm in robust randomised controlled trials (RCTs). A recent meta-analysis combining all the available pharmacological options to prevent BPD found that only five out of the 21 drugs tested (vitamin A, caffeine citrate, dexamethasone, inositol, and clarithromycin) in RCTs may reduce the incidence of BPD. Among these, meta-analysis could confirm the data only for vitamin A and dexamethasone, due to the lack of multiple trials for the other drugs (Beam 2014). Moreover, vitamin A showed only a very modest effect (Darlow 2011), while the use of dexamethasone is limited in preterm infants by its well-known long- and short-term side effects (Watterberg 2010). Despite the continuous advance of neonatal care, BPD remains a significant burden for the preterm population, lacking a safe and effective treatment.

Description of the intervention

Stem cells are primitive cells capable of extensive self renewal with the potential to give rise to multiple differentiated cellular phenotypes (Blau 2001). Among stem cells, mesenchymal stem cells (MSCs) have been largely explored as a potential regenerative therapy in several preclinical and clinical settings. Mesenchymal stem cells are defined mainly by three criteria:

1. adherence to plastic in standard culture conditions;
2. expression and lack of specific surface markers;
3. multipotent differentiation potential along the osteogenic, chondrogenic, and adipogenic lineages (Dominici 2006; Krampera 2013).

Although bone marrow is the major source of isolation, MSCs can be obtained from any tissue. Although MSCs have been isolated from any tissue (da Silva Meirelles 2006), most of the sites are not clinically relevant due to the difficulty in harvesting. Bone marrow and adipose tissue are the most utilized adult sources of MSCs (Dmitrieva 2012). Lately, MSCs obtained from perinatal tissues and umbilical cord blood have attracted particular interest thanks to their unique advantages in terms of availability, reduced immunogenicity, and pronounced differentiating potential (Batsali 2013; Sullivan 2008).

Beyond the cell source, several factors involving the manufacturing of the final stem cell product can affect the efficacy of MSC therapy. Among these, the aging of the cells with increasing passages may have significant consequences on their function (Bellayr 2014; Wagner 2008). A passage is the process of removing cells from a culture flask and plating them into more culture flasks. Passaging and expansion is necessary to obtain a sufficient number of cells for transplantation. However, early-passage cells may have superior therapeutic potential and thus improve the chances of success of the stem cell therapy in clinical trials.

The type of transplant (autologous or allogeneic) may yield different results (Alagesan 2014). Autologous transplantation of MSCs (from the same patient) is particularly appealing in terms of lower risks for infections and immune rejection. However, MSCs are characterized by low immunogenicity, although immune rejection is still theoretically possible. On the other hand, allogeneic transplantation (from a donor) offers significant practical advantages. Human and animal bone marrow-derived and cord-derived MSCs have been shown to prevent and treat lung injury in various preclinical models of lung diseases, including experimental BPD (Baker 2014). Their broad-spectrum clinical potential in the respiratory field is currently under investigation in numerous clinical trials (Antunes 2014). Possible side effects of MSC administration are immune rejection and tumor formation, although these are rather hypothetical possibilities. Mesenchymal stem cell safety has been documented in several clinical trials and confirmed in a recent meta-analysis in adult patients (Lalu 2012). In preclinical models of BPD, MSC administration in the first days of life was not associated with long-term adverse lung effects or tumor formation at the total-body computed tomography scan (Pierro 2013). A recent phase I study showed that the intratracheal transplantation of human cord-derived MSCs in preterm infants at high risk for BPD seems to be safe and feasible (Chang 2014).

How the intervention might work

Mesenchymal stem cells represent a perfect candidate for allogeneic transplantation, thanks to their paramount immunomodulatory properties, which allow them to reduce the risk for immune rejection (Gebler 2012). The mechanism of action of MSCs is still under investigation. However, MSCs seem to exert their therapeutic effects thanks to the paracrine secretion of anti-inflammatory, antioxidant, anti-apoptotic, trophic, and pro-angiogenic factors (Murphy 2013).

Mesenchymal stem cells and their products seem to ameliorate many critical aspects of BPD pathogenesis in preclinical models, by mitigating lung inflammation, inducing vascular and alveolar growth, and inhibiting lung fibrosis (Aslam 2009; Chang 2011; van Haaften 2009). These effects are confirmed by the significant improvement of lung function tests and long-term exercise tolerance in MSC-treated animals (Pierro 2013).

Why it is important to do this review

Bronchopulmonary dysplasia represents a significant burden for the preterm population and lacks an effective treatment. Stem cells, particularly MSCs, may have the potential to regenerate lung tissue and substantially improve the outcome of this disease. To our knowledge this is the first review addressing MSC treatment in patients affected or at risk for BPD.

OBJECTIVES

To determine if MSCs, administered intravenously or endotracheally, are safe and effective in preventing or treating BPD, or both, in preterm infants.

We performed the following specific comparisons.

1. Prevention: to determine if MSCs administered intravenously or endotracheally within the first week of life can prevent BPD in extremely preterm infants less than 26 weeks' gestation.

2. Treatment of evolving BPD: to determine if MSCs administered intravenously or endotracheally at more than one week of age but less than 36 weeks' postmenstrual age can prevent BPD in preterm infants on supplemental oxygen or respiratory support.

3. Treatment of established BPD: to determine if MSCs administered intravenously or endotracheally at ≥ 36 weeks' postmenstrual age can reduce mortality in preterm infants on respiratory support and/or supplemental oxygen > 30%.

We would also conduct subgroup analyses including gestational age, source of stem cells, type of graft, route of administration, MSC dose, number of doses, passages, disease severity at time of study entry, and exposure to postnatal steroids (Subgroup analysis and investigation of heterogeneity).

METHODS

Criteria for considering studies for this review

Types of studies

We considered RCTs and quasi-RCTs.

Types of participants

Prevention studies

- High-risk preterm infants: extremely preterm infants (< 26 weeks' gestation) ≤ one week of age.

Treatment studies

- Preterm infants with evolving BPD: preterm infants > one week of age but < 36 weeks' postmenstrual age on supplemental oxygen and invasive (conventional mechanical ventilation or high frequency oscillatory ventilation) or non-invasive (nasal continuous positive airway pressure, bilevel positive airway pressure, non-invasive positive pressure ventilation, high flow nasal cannula) respiratory support.

- Preterm infants with established BPD: preterm infants ≥ 36 weeks' postmenstrual age on invasive or non-invasive respiratory support and/or supplemental oxygen > 30%.

Types of interventions

Mesenchymal stem cells compared to control (placebo, steroids, or no treatment), including all types of transplantation regardless of cell source (bone marrow, cord blood versus Wharton's jelly, placenta, adipose tissue, peripheral blood), type of graft (autologous or allogeneic), route of cell administration (endotracheal or intravenous), and dose.

Types of outcome measures

Primary outcomes

1. All comparisons (prevention and treatment trials)
    i) Mortality prior to hospital discharge (from any cause)
2. Prevention trials
    i) Neonatal mortality (mortality < 28 days of age) from any cause
    ii) Chronic lung disease/BPD
        a) Oxygen requirement at 28 to 30 days of age
        b) Oxygen requirement at 36 weeks' postmenstrual age
    iii) Death or chronic lung disease
        a) Death or oxygen requirement at 28 to 30 days of age
        b) Death or oxygen requirement at 36 weeks' postmenstrual age
3. Treatment of evolving BPD
    i) Oxygen requirement at 36 weeks' postmenstrual age
    ii) Death or oxygen requirement at 36 weeks' postmenstrual age

Secondary outcomes

1. Pneumothorax
2. Air leak syndromes (including pulmonary interstitial emphysema, pneumothorax, pneumomediastinum)
3. Pulmonary hemorrhage
4. Patent ductus arteriosus (that has been treated with cyclooxygenase inhibitor or surgery)
5. Culture-confirmed bacterial sepsis
6. Culture-confirmed fungal sepsis
7. Necrotizing enterocolitis (defined as Bell stage II or greater) (Bell 1978)
8. Periventricular leukomalacia

9. Retinopathy of prematurity in infants examined (all stages and severe (stage 3 or greater)) (ICCROP 2005)
10. Intraventricular hemorrhage (any grade and severe (grade 3 to 4)) (Papile 1978)
11. Pulmonary hypertension (defined by Doppler ultrasound)
12. Days on assisted ventilation
13. Days on supplemental oxygen
14. Length of hospital stay (days)
15. Cerebral palsy at 18 to 24 months' corrected age
16. Neurodevelopmental outcome at approximately two years' corrected age (acceptable range 18 months to 28 months) including: cerebral palsy, delayed neurodevelopment (Bayley Scales of Infant Development Mental Developmental Index < 70), legal blindness (< 20/200 visual acuity), and hearing deficit (aided or < 60 dB on audiometric testing)
    i) We defined the composite outcome 'neurodevelopmental impairment' as having any one of the aforementioned deficits.
17. Rehospitalization in the first two years of life
18. Safety outcomes defined as tumor formation, immune-rejection, or any serious adverse event (certain, probable, or possible according to the World Health Organization probability scale)

We considered post hoc analyses for any unexpected adverse effects reported by the studies.

Search methods for identification of studies

We used the criteria and standard methods of Cochrane and Cochrane Neonatal (see the Cochrane Neonatal search strategy for specialized register).

Electronic searches

We conducted a comprehensive search including the Cochrane Central Register of Controlled Trials (CENTRAL) (2016, Issue 10) in the Cochrane Library, MEDLINE via PubMed (1966 to 6 November 2016), Embase (1980 to 6 November 2016), and CINAHL (Cumulative Index to Nursing and Allied Health Literature) (1982 to 6 November 2016) using the following search terms: (stem cell OR Mesenchymal) AND (BPD OR bronchopulmonary dysplasia OR lung disease OR CLD OR chronic lung disease OR respiratory distress syndrome), plus database-specific limiters for RCTs and neonates (see Appendix 1 for the full search strategies for each database). We did not apply language restrictions.

We searched the bibliography cited in each publication obtained in order to identify additional relevant articles.

Searching other resources

We handsearched the abstracts of the Society for Pediatric Research for the years 1985 to 1999 using the keyword 'stem cells'. We electronically searched the abstracts of the Pediatric Academic Societies from 2000 to 2014 through the Pediatric Academic Societies' 2000 to 2014 Archive Abstracts2View site (www.abstracts2view.com/pasall/).

We checked references and cross-references from identified studies. We handsearched abstracts from the proceedings of the Pediatric Academic Societies Meetings (from January 1990 to present). We did not impose any language restrictions.

We searched clinical trials registries (6 November 2016) for ongoing or recently completed trials: US National Institutes of Health Ongoing Trials Register ClinicalTrials.gov (clinicaltrials.gov), the World Health Organization International Clinical Trials Registry Platform (WHO ICTRP) (www.who.int/ictrp/en/), and the ISRCTN registry (www.isrctn.com/).

Data collection and analysis

Selection of studies

Two review authors (MP, BT) independently searched the literature as described above. We considered only RCTs and quasi-RCTs fulfilling the above criteria for inclusion in the review. We excluded studies published only in abstract form unless the final results of the trial were reported and all necessary information could be ascertained from the abstract or authors, or both. Two review authors (MP, BT) selected studies separately. Any disagreements were resolved by discussion involving all review authors.

Data extraction and management

Two review authors (MP, BT) independently extracted, assessed, and coded data using a form designed specifically for this review. We collected information regarding the method of randomization, blinding, drug intervention, stratification, and whether the trial was single-center or multicenter for each included study. We noted information regarding trial participants including gestational age criteria, birth weight criteria, and other inclusion or exclusion criteria. We extracted information on primary and secondary outcomes (Primary outcomes; Secondary outcomes). When any questions arose or additional data were required, we contacted the authors.

For each study, one review author (MP) entered data into Cochrane's statistical software Review Manager 5 (RevMan 2014), and a second review author (BT) checked the entered data. We planned to replace any standard error of the mean by the corresponding standard deviation. We resolved any disagreements by discussion.

Assessment of risk of bias in included studies

We planned to assess risk of bias according to selection bias (quality of randomization, allocation concealment/blinding of randomization), performance bias (blinding of intervention), attrition bias (completeness of follow-up), and detection bias (blinding of outcome measurement), selective reporting bias, or other bias using Cochrane's tool for assessing risk of bias (Higgins 2011). We planned to assess each domain as 'low risk,' 'high risk,' or 'unclear risk.' Any disagreements would be resolved by discussion involving all review authors.

We planned to evaluate the following issues and enter the information into a 'Risk of bias' table (Higgins 2011).

- Sequence generation (checking for possible selection bias).
  - Was the allocation sequence adequately generated?
  - For each included study, we planned to categorize the method used to generate the allocation sequence as:
    - adequate (any truly random process, e.g. random number table, computer random number generator);
    - inadequate (any non-random process, e.g. odd or even date of birth, hospital or clinic record number);
    - unclear.
- Allocation concealment (checking for possible selection bias).
  - Was allocation adequately concealed?
  - For each included study, we planned to categorize the method used to conceal the allocation sequence as:
    - adequate (e.g. telephone or central randomization, consecutively numbered, sealed, opaque envelopes);
    - inadequate (open random allocation, unsealed or non-opaque envelopes, alternation, date of birth);
    - unclear.
- Blinding (checking for possible performance bias).
  - Was knowledge of the allocated intervention adequately prevented during the study? At study entry? At the time of outcome assessment?
  - For each included study, we planned to categorize the methods used to blind study participants and personnel from knowledge of which intervention a participant received.
  - We would assess blinding separately for different outcomes or classes of outcomes.
  - In some situations there might have been partial blinding, for example where outcomes were self reported by unblinded participants, but they were recorded by blinded personnel without knowledge of group assignment. Where needed, we would add 'partial' to the list of options for assessing quality of blinding.
  - We planned to categorize the methods as:
    - adequate, inadequate, or unclear for participants;
    - adequate, inadequate, or unclear for personnel;
    - adequate, inadequate, or unclear for outcome assessors.
- Incomplete outcome data (checking for possible attrition bias through withdrawals, dropouts, protocol deviations).
  - Were incomplete outcome data adequately addressed?
  - For each included study and for each outcome, we would describe the completeness of data including attrition and exclusions from the analysis.
  - We planned to note whether attrition and exclusions were reported, the numbers included in the analysis at each stage (compared with the total randomized participants), reasons for attrition or exclusion where reported, and whether missing data were balanced across groups or were related to outcomes.
  - Where sufficient information was reported or supplied by the trial authors, we would re-include missing data in the analyses.
  - We planned to categorize the methods as:
    - adequate (< 20% missing data);
    - inadequate ($\geq$ 20% missing data);
    - unclear.
- Selective reporting bias.
  - Were reports of the study free of the suggestion of selective outcome reporting?
  - For each included study, we planned to describe how we investigated the possibility of selective outcome reporting bias and what we found. We planned to assess the methods as:
    - adequate (where it was clear that all of the study's prespecified outcomes and all expected outcomes of interest to the review had been reported);
    - inadequate (where not all of the study's prespecified outcomes had been reported; one or more reported primary outcomes were not prespecified; outcomes of interest were reported incompletely and so could not be used; the study failed to include the results of a key outcome that would have been expected to be reported);
    - unclear.
- Other sources of bias.
  - Was the study apparently free of other problems that could put it at a high risk of bias?
  - For each included study, we planned to describe any important concerns we had about other possible sources of bias (e.g. whether there was a potential source of bias related to the specific study design or whether the trial was stopped early owing to some data-dependent process).
  - We planned to assess whether each study was free of other problems that could put it at risk of bias as:
    - yes;
    - no;
    - unclear.

We planned to explore the impact of the level of bias through undertaking sensitivity analyses if needed.

Measures of treatment effect

We planned to use risk ratios (RRs), risk differences (RDs), numbers needed to treat for an additional beneficial outcome (NNTB) or numbers needed to treat for an additional harmful outcome (NNTH) for categorical variables, and weighted mean differences (WMDs) for continuous variables. We planned to replace any within-group standard error of the mean (SEM) reported in a trial by its corresponding standard deviation (SD) using the formula SD = SEM x $\sqrt{N}$. We planned to report 95% confidence intervals (CIs) for each statistic.

We planned to perform the statistical analyses using Review Manager 5 (RevMan 2014). We planned to analyze categorical data using RRs and RDs. For statistically significant outcomes, we would calculate NNTB or NNTH. We planned to analyze continuous data using WMDs and standardized mean differences (SMDs). We planned to report the 95% CIs on all estimates.

Unit of analysis issues

We planned to include all RCTs and quasi-RCTs in which the unit of allocation was the individual infant.

Dealing with missing data

When any questions arose or additional data were required, we contacted the authors.

Assessment of heterogeneity

We planned to assess the magnitude of heterogeneity of treatment effects using the I^2 statistic. We planned to consider an I^2 value of greater than 60% as indicative of high heterogeneity. We also planned to inspect each forest plot carefully for heterogeneity, as indicated by lack of overlapping CIs of individual trials.

We planned to estimate the treatment effects of individual trials and examined heterogeneity among trials by inspecting the forest plots and quantifying the impact of heterogeneity using the I^2 statistic. We would grade the degree of heterogeneity as follows:

- less than 25%: no heterogeneity;
- 25% to 49%: low heterogeneity;
- 50% to 75%: moderate heterogeneity;
- greater than 75%: substantial heterogeneity.

In case of statistical heterogeneity (I^2 > 50%), we would explore the possible causes (e.g. differences in study quality, participants, intervention regimens, or outcome assessments).

Assessment of reporting biases

We planned to examine the possibility of within-study selective outcome reporting for each study included in the review. We searched for trial protocols of included trials on electronic sources such as PubMed, ClinicalTrials.gov, and the WHO ICTRP in order to assess whether outcome reporting seemed to be sufficiently complete and transparent. We planned to investigate publication bias using funnel plots if we included at least 10 clinical trials in the review (Egger 1997; Higgins 2011).

Data synthesis

We planned to use a fixed-effect model to pool data for meta-analyses.

If we identified multiple studies that we thought to be sufficiently similar, we planned to perform meta-analysis using Review Manager 5 (RevMan 2014). For categorical outcomes, we would calculate the typical estimates of RRs and RDs with their corresponding 95% CIs; for continuous outcomes we planned to calculate MDs or a summary estimate for SMDs with their respective 95% CIs. When we judged meta-analysis to be inappropriate, we planned to analyze and interpret individual trials separately.

Quality of evidence

We planned to use the GRADE approach, as outlined in the GRADE Handbook (Schünemann 2013), to assess the quality of evidence for the following (clinically relevant) outcomes:

- neonatal mortality (mortality < 28 days of age) from any cause;
- chronic lung disease/BPD defined as oxygen requirement at 28 to 30 days of age;
- chronic lung disease/BPD defined as oxygen requirement at 36 weeks' postmenstrual age;
- death or oxygen requirement at 36 weeks' postmenstrual age;
- cerebral palsy at 18 to 24 months' corrected age;
- neurodevelopmental outcome at approximately two years' corrected age (acceptable range 18 months to 28 months) including cerebral palsy, delayed neurodevelopment (Bayley Scales of Infant Development Mental Developmental Index < 70), legal blindness (< 20/200 visual acuity), and hearing deficit (aided or < 60 dB on audiometric testing).

Two review authors would independently assess the quality of the evidence for each of the outcomes above. We planned to consider evidence from RCTs as high quality, but downgrade the evidence one level for serious (or two levels for very serious) limitations based upon the following: design (risk of bias), consistency across studies, directness of the evidence, precision of estimates, and presence of publication bias. We planned to use the GRADEpro Guideline Development Tool to create a 'Summary of findings' table to report the quality of the evidence (GRADEpro GDT).

The GRADE approach results in an assessment of the quality of a body of evidence in one of the following four grades.

1. High: We are very confident that the true effect lies close to that of the estimate of the effect.

2. Moderate: We are moderately confident in the effect estimate: the true effect is likely to be close to the estimate of the effect, but there is a possibility that it was substantially different.

3. Low: Our confidence in the effect estimate is limited: the true effect could be substantially different from the estimate of the effect.

4. Very low: We have very little confidence in the effect estimate: the true effect was likely to be substantially different from the estimate of the effect.

Subgroup analysis and investigation of heterogeneity

- Gestational age: < 26 weeks; 26 to 30 weeks; > 30 weeks
- Source of stem cells: bone marrow; cord blood; Wharton's jelly; placenta; adipose tissue; peripheral blood
- Type of graft: autologous; allogeneic
- Route of administration: intravenous; endotracheal
- MSC dose: 1 to 9 x 10^6/kg; 1 to 9 x 10^7/kg
- Number of doses: multiple or single administration
- Passage: < 3 versus 3 to 6 versus > 6
- Disease severity at time of study entry
    - Prevention: invasive respiratory support with fraction of inspired oxygen (FiO_2) > 0.3 versus invasive respiratory with FiO_2 < 0.3 versus non-invasive respiratory support
    - Treatment of evolving BPD: invasive respiratory support with FiO_2 > 0.3 versus invasive respiratory with FiO_2 < 0.3 versus non-invasive respiratory support
    - Treatment of established BPD: invasive respiratory support with FiO_2 > 0.3 versus invasive respiratory with FiO_2 < 0.3 versus non-invasive respiratory support versus FiO_2 > 0.3 on spontaneous breathing
- Infants who had previously received postnatal steroids for prevention or treatment of chronic lung disease (dexamethasone, hydrocortisone)

Sensitivity analysis

Differences in study design of included trials might affect the results of the systematic review. We planned to perform a sensitivity analysis to compare the effects of stem cells in truly randomized trials as opposed to quasi-randomized trials.

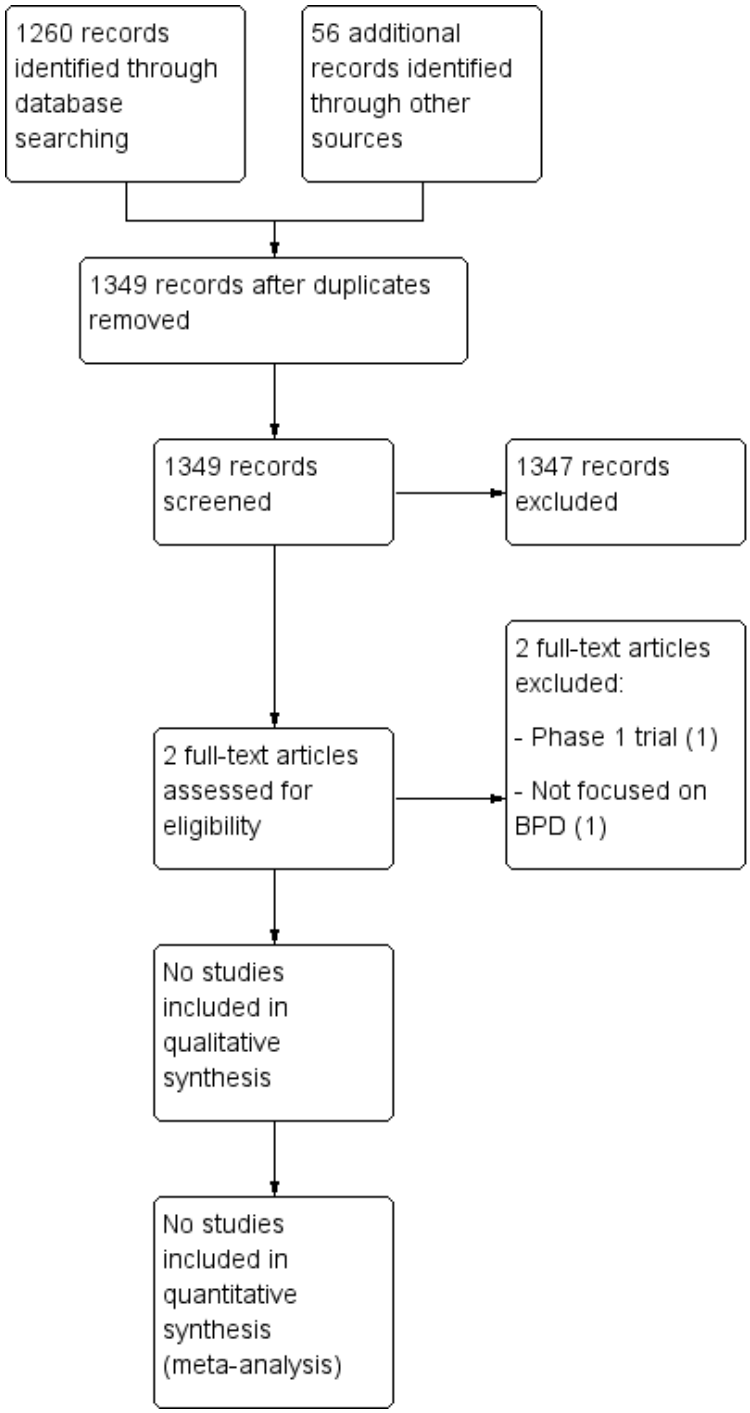
RESULTS

Description of studies

Results of the search

From an initial search of 1349 citations, we extracted 2 studies for further examination. We excluded both studies from the review (see Characteristics of excluded studies table and PRISMA study flow diagram Figure 1).

Figure 1. Study flow diagram.



Our search of ClinicalTrials.gov identified 10 registered studies, of which 2 protocols were randomized trials addressing the use of MSCs for prevention or treatment of BPD in premature infants (see Characteristics of ongoing studies table). Three non-randomized phase 1 trials are also currently registered on our targeted patient group.

Included studies

No studies were identified.

Excluded studies

Two studies were identified but excluded.

Chang 2014 (ClinicalTrials.gov identifier NCT01297205): this is the first published trial on the use of MSCs for BPD in preterm infants with evolving BPD (5 to 14 days requiring continuous ventilatory support). It is dose-escalation trial, assessing safety and feasibility of a single, intratracheal administration of umbilical cord blood-derived MSCs, starting from a low dose of 1 x 10^7 cells/kg for the first three patients, up to the higher dose of 2 x 10^7 cells/kg for the next six patients. Infants were enrolled between February and September 2011. The authors reported no serious adverse effects or dose-limiting toxicity. Levels of pro-inflammatory cytokines (interleukin-6, interleukin-8, matrix metalloproteinase-9, tumor necrosis factor-a, and transforming growth factor-b 1) in tracheal aspirates were reduced after MSC transplantation. The enrolled infants were also compared with historical controls (between January 2009 and November 2011). Bronchopulmonary dysplasia severity appeared to be lower in the transplant recipients, and rates of other adverse outcomes did not differ between the comparison group and transplant recipients. We excluded this study because it was a phase 1 trial and, as such, did not meet the inclusion criteria.

Rudnicki 2015 (ClinicalTrials.gov identifier NCT02050971): this is a prospective single-center study in which preterm infants born below 32 weeks of gestation were randomized to either autologous cord blood transfusion or allogeneic red blood cell transfusion, in order to treat anemia of prematurity. We excluded this study because it was not focused on MSCs and BPD.

Ongoing studies

Our search of PubMed, ClinicalTrials.gov, and the WHO ICTRP revealed two protocols of ongoing randomized trials on our targeted patient group (see Characteristics of ongoing studies table).

NCT01207869: the study 'Intratracheal umbilical cord-derived mesenchymal stem cells for severe bronchopulmonary dysplasia' is a phase 2, double-blind, randomized trial on established BPD. Infants up to six months of age with severe BPD are randomized to an intratracheal administration of either MSCs or normal saline. The status of this Taiwanese study is unknown, having not been verified in the past seven years. We were unable to retrieve information on this study.

NCT01828957: the study 'Efficacy and safety evaluation of Pneumostem versus a control group for treatment of BPD in premature infants' is a double-blind, randomized trial on evolving BPD. Preterm infants less than 29 weeks of gestation are randomized to an intratracheal single dose of allogeneic cord blood-derived MSCs or normal saline, if they are still ventilated (FiO_2 > 0.25) from 5 to 14 days of life. The Korean study includes a registered follow-up to 60 months (NCT01897987). We contacted the researchers, who are currently analyzing the data and have also completed the follow-up enrollment. This study constitutes the phase 2 of the previously published phase 1 trial (Chang 2014).

Two non-randomized trials are also currently registered on our targeted patient group.

NCT02381366: this is a US phase 1 safety study on evolving BPD. In this dose-escalation trial, the researchers are testing two different intratracheal doses (10 and 20 millions of cells) of human allogeneic umbilical cord blood-derived MSCs in preterm infants born between 23 and 28 weeks' gestation, on mechanical ventilation (FiO_2 > 0.25) between 5 and 14 days of life. The cells are frozen and administered within 24 hours from thawing. Enrollment of the study has been completed and the researchers are currently analyzing the data.

NCT02443961: this is a Spanish phase 1 safety trial on infants with evolving BPD. Three doses of 5 million umbilical cord-derived MSCs are administered to infants born below 28 weeks' gestation, if they are still on mechanical ventilation (FiO_2 > 0.4) at 14 ± 2 days of life. Cells are thawed one week before administration, expanded to passage 6 to 10, and administered intravenously in three weekly doses.

Studies in other neonatal populations

NCT01284673: 'Characterization of the cord blood stem cell in situation of neonatal asphyxia'

NCT02274428: 'Phase 1 clinical trial of PNEUMOSTEM treatment in premature infants with intraventricular hemorrhage'

Risk of bias in included studies

We found no studies meeting the inclusion criteria for this review.

Effects of interventions

We found no studies meeting the inclusion criteria for this review.

DISCUSSION

We found no published RCTs or quasi-RCTs addressing the effect of MSC administration for prevention or treatment of BPD in premature infants. However, cell-based therapy is a burgeoning field, and the coming years will likely mark a turning point, since promising animal data have already prompted the clinical translation of MSCs for numerous diseases, including BPD.

The first trial on the use of MSCs in preterm infants was published in 2014 (Chang 2014). This phase 1 dose-escalation trial showed the safety and feasibility of a single, intratracheal administration of allogeneic umbilical cord blood-derived MSCs in premature infants with evolving BPD (5 to 14 days of life with deteriorating respiratory conditions). The authors reported no serious adverse effects or dose-limiting toxicity. We excluded this study from our review because it is a phase 1 trial and, as such, did not meet our inclusion criteria. The follow-up of this trial was recently published, and no significant adverse respiratory, growth, or neurodevelopmental effects were detected in the MSC-treated infants up to 24 months (Ahn 2017). This same group also has completed the enrollment for the first RCT on the same patient population (NCT01828957). The primary outcomes in this phase 2 trial are incidence of BPD (moderate to severe) and mortality at 36 weeks' postmenstrual age. A US phase 1 safety and feasibility study testing the effects of a single intratracheal dose of allogeneic cord blood-derived MSCs in premature infants with evolving BPD has completed participant enrollment, and the results will be available in the next few months (NCT02381366). A Spanish phase 1 trial is expected to start enrollment in 2017. Infants with evolving BPD ($\geq$ 14 days of life) will be treated intravenously with cord-derived MSCs (NCT02443961).

Phase 1 and 2 trials are currently focusing on evolving BPD, which seems at this point to be the most appropriate target population. However, evolving BPD is not a well-defined entity, and it carries a wide range of morbidities and mortality. In the group of infants enrolled in the ongoing studies, previous epidemiological data have reported a mortality rate ranging from 3% to 75% and a risk of moderate to severe BPD from 39% to 100%, depending on the severity of the disease and patient characteristics including sex, race, and birth weight (Lughon 2011). Stratification at randomization, based on outcome predictors or severity of the disease at study entry, would prevent inconsistency in the comparison groups, improving the design of future phase 2 studies.

It must be noted that factors including trial design (i.e. timing and route of administration) and manufacturing procedures greatly affect MSC activity, requiring careful interpretation in data analysis or combining results in meta-analysis. Animal studies have shown that early (as opposed to rescue) (Pierro 2013), intratracheal (as opposed to intravenous) (Chang 2009), MSC administration seems to be most efficient in the treatment of experimental BPD. As a result of the knowledge gained from these preclinical data, all the studies (phase 1 and 2) are currently assessing the administration of allogeneic cord-derived or cord blood-derived MSCs to preterm infants with evolving BPD (early administration). In the Spanish study, umbilical cord-derived cells are thawed one week before administration, expanded to passage 6 to 10, and administered intravenously in three weekly doses. The Korean and US studies are testing intratracheally administered umbilical cord blood-derived MSCs. The products are frozen and administered within 24 hours from thawing, and the cells are passage 3 to 6. These two studies are using the same pharmaceutical product. The similarities in the design of the ongoing studies should theoretically facilitate the combination of study results into a meta-analysis, which would be unreliable if the study conditions were too different. In addition to the trial design, numerous and more complex variables specifically related to the manufacturing of the product need to be taken into account. The manufacturing process of MSCs includes: cell source selection, isolation, expansion/culture conditions, cryostorage, thawing procedure, and lot release. With regard to cell source, perinatal tissue (fetal, placental, umbilical cord, and amniotic) may provide MSCs with greater potency and self renewal than older adult sources (bone marrow, adipose tissue) (Batsali 2013). Among perinatal tissues, cord-derived cells can rely on the most robust protocol of harvesting. However, even when using the same source and complying with good tissue practice (cGTP) and good manufacturing practice (cGMP) requirements (FDA 2011; Sensebe 2011), the protocols currently in use for MSC production are quite dissimilar (Ikebe 2014). Even small variation in the processing methods, such as starting material, plating density, devices used for MSC culture and separation, culture media, media supplements or growth factors, oxygen concentration during culture, passage number, and substances added for cryopreservation, may change the final characteristics and the functionality of the MSC-based product (Panchalingam 2015; Waszak 2012). The use of potency assays may improve the characterization of MSC behavior and the prediction of their clinical efficacy, but these are currently not available. Since one single assay would only marginally ascertain the product peculiarities, the development of multiple complementary assays (assay matrix), may be better suited to describe different biological, immunological, and analytical MSC features (Galipeau 2015). Ideally, the release of an MSC lot should always include disclosure of its 'strength' in terms of tissue repair and immunomodulation based on potency assays. The definition of standardized protocols for MSC production and the expansion of potency optimal requirements are to be considered a prerequisite for scale-up of MSC therapies. Unfortunately, despite the availability of several manufacturing systems, a mutual reference platform does not yet exist. In order to allow for eventual subgroup analysis, all the manufacturing protocols should be detailed in the methods section. The editors and reviewers should possibly ask for comprehensive checklists, available for readers, describing in minute detail each step of MSC manufacturing.

The purity of the MSC lot is another crucial issue. After sample

collection, other cells, in particular fibroblasts, which share some phenotypic features with MSCs, should be removed. One way to obtain a pure MSC product is based on cell selection, conformed to the expression or lack of surface markers (Dominici 2006). However, the definition of MSCs and their characterization is still evolving (Galipeau 2015; Mendicino 2014), with new markers continuing to be identified (Shen 2015). More advanced and specific characterization protocols are needed to improve MSC quality and therapeutic potential. It is possible that MSC lots considered to be pure according to current knowledge will be proven to express different degrees of purity, if newer or more numerous markers are applied. Moreover, although only circumstantial so far, the evidence that donor-related MSC characteristics are likely to have a role on the final product is emerging. As an example, female cord-derived MSCs show better lung protection compared to male cord-derived MSCs in a rodent model of neonatal hyperoxia-induced lung injury (Sammour 2016). Similarly, it is possible that other factors comprising, but not limited to, gestational age at harvesting, placental diseases, chorioamnionitis, and maternal or fetal factors, may alter the efficacy of the cells. It would be ideal to store a sample of each MSC lot and record all possible clinical details on the donor, allowing for subsequent analysis as new knowledge arises.

Safety of MSC is a pivotal issue. Few meta-analyses, either combining the data obtained in any clinical setting ((Lalu 2012) or including studies specific to a certain group of diseases, such as adult respiratory health issues (Zhao 2017), showed no serious adverse effects after MSC administration. However, the level of evidence is low, and the safety of stem cells needs confirmation by more robust and compelling data. Moreover, some cases of adverse events linked to MSC administration have been reported (Ning 2008; Song 2015). The US Food and Drug Administration strongly recommends that scientists and pharmaceutical companies be committed to the principles of adequate evidence generation when dealing with regenerative medicine, in order to ensure that the eventual adoption of this therapy has a favorable and well-defined risk-benefit balance (Marks 2017). Mesenchymal stem cells are thought to sense the pathological signals sent by damaged tissues and act consequently to repair the defect. However, it cannot be excluded that some signals could be misinterpreted by stem cells, leading to unexpected outcomes. As consequence, RCTs and meta-analysis are more likely to provide functional information when addressing the issue of safety in one specific disease.

In conclusion, resources should be organized in order to comprise proper long-term follow-up, possibly up to school age, for safety and efficacy endpoints.

AUTHORS' CONCLUSIONS

Implications for practice

There is insufficient evidence to determine the safety and efficacy of mesenchymal stem cells (MSCs) for the treatment or prevention of BPD in premature infants.

Implications for research

Continuous research and communication between basic science and the pharmaceutical industry are paramount for the future of regenerative medicine. Three distinct research targets are likely to simultaneously and independently change the face of stem cell therapy in the near future: (i) newer insight into characterization of MSCs and standardization of potency assays; (ii) well-designed preclinical studies to provide strong rationale for clinical trials; and (iii) rigorous clinical trials to put the current preclinical evidence to the test and yield the required knowledge. These three separate but interrelated levels of research should continuously influence and redirect each other. While more knowledge is developed, ongoing and future trials will need to provide for possible retrospective re-evaluation (through registries of patients included in MSC trials and storage of MSC lots) of the results based on future unpredictable discoveries.

Long-term follow-up is paramount, and all treated infants should be enrolled in a registry.

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* *Indicates the major publication for the study*

CHARACTERISTICS OF STUDIES

Characteristics of excluded studies *[ordered by study ID]*

Study	Reason for exclusion
Chang 2014	Phase 1 dose-escalation trial to assess the safety and feasibility of a single, intratracheal transplantation of human umbilical cord blood-derived mesenchymal stem cells in 9 preterm infants at high risk for bronchopulmonary dysplasia ClinicalTrials.gov identifier NCT01297205
Rudnicki 2015	Not focused on mesenchymal stem cells and bronchopulmonary dysplasia Single-center, prospective study of the feasibility, safety, and tolerability of autologous whole cord blood transplant in preterm infants born at less than 32 weeks of gestational age who developed anemia of prematurity ClinicalTrials.gov identifier NCT02050971

Characteristics of ongoing studies *[ordered by study ID]*

NCT01207869

Trial name or title	Intratracheal umbilical cord-derived mesenchymal stem cells for severe bronchopulmonary dysplasia
Methods	Randomized double-blind controlled trial Recruitment: active, not recruiting
Participants	Infants affected by severe BPD up to 6 months of life
Interventions	Interventions: umbilical cord-derived MSCs (3×10^6 cells per kg of the infant's weight), instilled through a 6 French end-hole catheter inserted in the endotracheal tube Placebo comparator: normal saline (same amount of MSCs suspension) instilled through a 6 French end-hole catheter inserted in the endotracheal tube
Outcomes	Primary outcome measures: the correlation between the cytokine concentrations in the bronchoalveolar fluid and pulmonary arterial pressure Secondary outcome measures: BPD severity score ranging from 0 to 6 on the serial chest radiographs
Starting date	June 2010
Contact information	Bai-Horng Su, MD, PhD, Chairman of Department of Pediatrics, China Medical University Hospital
Notes	Recruitment: active, not recruiting Study results: no results available Last update: September 2010 clinicaltrials.gov/show/NCT01207869 Study has passed its completion date and status has not been verified in more than 2 years

NCT01284673

Trial name or title	Characterization of the cord blood stem cell in situation of neonatal asphyxia
Methods	Descriptive, bicentre study on 10 cord blood samples from newborn infants with neonatal asphyxia (5) according to predefined criteria, compared to healthy neonates (5). The total duration of the study will be 2 years. Parents will be informed and a signed parental consent will be asked in the hours following birth before the in vitro study
Participants	Conditions: respiratory distress syndrome
Interventions	In vitro characterization of the cord blood stem cell
Outcomes	Biological analysis will include elementary analyses for cell quality control, endothelial progenitor exploration, and investigation of MSC function and of their neuronal differentiation potential (on fresh and frozen samples)
Starting date	Recruitment: completed Study results: no results available
Contact information	Sponsor: Assistance Publique Hopitaux De Marseille
Notes	clinicaltrials.gov/show/NCT01284673 Study has passed its completion date and status has not been verified in more than 2 years

NCT01632475

Trial name or title	Follow-up study of safety and efficacy of Pneumostem in premature infants with bronchopulmonary dysplasia
Methods	
Participants	Conditions: bronchopulmonary dysplasia
Interventions	Interventions: biological: Pneumostem
Outcomes	
Starting date	Recruitment: active, not recruiting Study results: no results available
Contact information	
Notes	clinicaltrials.gov/show/NCT01632475 Study has passed its completion date and status has not been verified in more than 2 years

NCT01828957

Trial name or title	Efficacy and safety evaluation of Pneumostem versus a control group for treatment of BPD in premature infants
Methods	Randomized double-blind controlled trial
Participants	Preterm infants < 29 weeks' gestation with evolving BPD (intubated and ventilated with deteriorating respiratory conditions) from 5 to 14 days of life
Interventions	Interventions: a single intratracheal administration of allogeneic cord blood-derived MSCs. Cells are passage 3 to 6 at a dose of 1 x 10^7 cells/kg. The product is frozen and thawed overnight before administration Placebo comparator: normal saline
Outcomes	Primary outcome measures: incidence of BPD (moderate to severe) or mortality at 36 weeks' postmenstrual age Secondary outcome measures: complications of prematurity, days of oxygen/respiratory support, postnatal steroids, length of stay, body growth
Starting date	April 2013
Contact information	Won-Soon Park, MD, PhD, Department of Pediatrics, Samsung Medical Center Ai-Rhan Kim, Department of Neonatology, Asan Medical Center
Notes	Recruitment: active, not recruiting clinicaltrials.gov/show/NCT01828957 Study has passed its completion date and status has not been verified in more than 2 years

NCT01897987

Trial name or title	Follow-up safety and efficacy evaluation on subjects who completed PNEUMOSTEM phase-II clinical trial
Methods	Recruitment: recruiting Study results: no results available
Participants	Conditions: bronchopulmonary dysplasia
Interventions	Interventions: biological: Pneumostem Placebo comparator: normal saline
Outcomes	
Starting date	
Contact information	
Notes	clinicaltrials.gov/show/NCT01897987 Study has passed its completion date and status has not been verified in more than 2 years

NCT02023788

Trial name or title	Long-term safety and efficacy follow-up study of PNEUMOSTEM in patients who completed PNEUMOSTEM phase-I study
Methods	Recruitment: active, not recruiting Study results: no results available
Participants	Conditions: bronchopulmonary dysplasia; respiratory tract infections; premature birth of newborn
Interventions	Interventions: biological: PNEUMOSTEM
Outcomes	
Starting date	
Contact information	
Notes	clinicaltrials.gov/show/NCT02023788 Study has passed its completion date and status has not been verified in more than 2 years

NCT02274428

Trial name or title	Phase 1 clinical trial of PNEUMOSTEM treatment in premature infants with intraventricular hemorrhage
Methods	Single group assignment; not a randomized controlled trial
Participants	Inclusion criteria: • 23 to 34 weeks • intraventricular hemorrhage grade 3 to 4, confirmed with brain ultrasonogram • within 7 days after intraventricular hemorrhage diagnosis Exclusion criteria: • severe congenital anomaly • intrauterine intracranial bleeding • intracranial infection • severe congenital infection • active and uncontrolled infection, C-reactive protein > 10 mg/dL • Platelet count < 50,000/mL • severe metabolic acidosis (pH < 7.1, Base excess < -20)
Interventions	Drug: Pneumostem
Outcomes	Primary outcome measures: unsuspected death or anaphylactic shock within 6 hours after Pneumostem transplantation. Secondary outcome measures: death or hydrocephalus requiring shunt operation
Starting date	
Contact information	

NCT02274428 (*Continued*)

Notes	clinicaltrials.gov/ct2/show/NCT02274428 Study has passed its completion date and status has not been verified in more than 2 years

NCT02381366

Trial name or title	Safety and efficacy of two dose levels of PNEUMOSTEM in premature infants at high risk for bronchopulmonary dysplasia (BPD) - a US study
Methods	Recruitment: recruiting Study results: no results available
Participants	Conditions: bronchopulmonary dysplasia
Interventions	Interventions: biological: human umbilical cord blood-derived MSCs
Outcomes	
Starting date	
Contact information	
Notes	clinicaltrials.gov/show/NCT02381366 Study has passed its completion date and status has not been verified in more than 2 years

NCT02443961

Trial name or title	Mesenchymal stem cell therapy for bronchopulmonary dysplasia in preterm babies
Methods	Recruitment: not yet recruiting Study results: no results available
Participants	Conditions: bronchopulmonary dysplasia
Interventions	Interventions: biological: mesenchymal stem cell therapy
Outcomes	
Starting date	
Contact information	
Notes	clinicaltrials.gov/show/NCT02443961 Study has passed its completion date and status has not been verified in more than 2 years

NCT02673788

Trial name or title	Follow-up study of safety and efficacy of Pneumostem in premature infants with intraventricular hemorrhage
Methods	
Participants	
Interventions	
Outcomes	
Starting date	
Contact information	
Notes	clinicaltrials.gov/show/NCT02673788 Study has passed its completion date and status has not been verified in more than 2 years

BPD: bronchopulmonary dysplasia
MSC: mesenchymal stem cell

DATA AND ANALYSES

This review has no analyses.

APPENDICES

Appendix 1. Standard search methodology

PubMed: ((randomized controlled trial [pt] OR controlled clinical trial [pt] OR randomized [tiab] OR placebo [tiab] OR drug therapy [sh] OR randomly [tiab] OR trial [tiab] OR groups [tiab]) NOT (animals [mh] NOT humans [mh]))

Cinahl: (randomized controlled trial OR controlled clinical trial OR randomized OR placebo OR clinical trials as topic OR randomly OR trial OR PT clinical trial)

Embase: (human not animal) AND (randomized controlled trial or controlled clinical trial or randomized or placebo or clinical trials as topic or randomly or trial or clinical trial)

Cochrane Library: No additional limiters

CONTRIBUTIONS OF AUTHORS

Maria Pierro (MP), Bernard Thébaud (BT), and Roger Soll (RS) all participated in the conception and drafting of the protocol. Two review authors (MP, BT) independently searched the literature and assessed study eligibility as per inclusion criteria. MP, BT, and RS all participated in the writing of manuscript.

DECLARATIONS OF INTEREST

Maria Pierro is a funded researcher in the field of mesenchymal stem cells.

Bernard Thébaud's work on mesenchymal stem cells is supported by the Canadian Institute for Health Research, the Canadian Stem Cell Network, the Canadian Thoracic Society, the Ottawa Hospital Research Institute and the Children's Hospital of Eastern Ontario Research Institute, and the Ontario Institute of Regenerative Medicine.

Roger Soll is the Co-ordinating Editor of the Cochrane Neonatal Review Group, but played no part in determining if this review was acceptable for publication.

DIFFERENCES BETWEEN PROTOCOL AND REVIEW

We excluded the separate search for long-term neurodevelopmental sequelae, as we believe the initial search is comprehensive.

CHAPTER IV

PROBIOTIC SUPPLEMENTATION IN PRETERM INFANTS DOES NOT AFFECT THE RISK OF BRONCHOPULMONARY DYSPLASIA: A META-ANALYSIS OF RANDOMIZED CONTROLLED TRIALS

Villamor-Martínez E, Pierro M, Cavallaro G, Mosca F, Kramer B, Villamor E

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MDPI

Review

Probiotic Supplementation in Preterm Infants Does Not Affect the Risk of Bronchopulmonary Dysplasia: A Meta-Analysis of Randomized Controlled Trials

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Abstract: Probiotic supplementation reduces the risk of necrotizing enterocolitis (NEC) and late-onset sepsis (LOS) in preterm infants, but it remains to be determined whether this reduction translates into a reduction of other complications. We conducted a systematic review and meta-analysis to evaluate the possible role of probiotics in altering the risk of bronchopulmonary dysplasia (BPD). Fifteen randomized controlled trials (4782 infants; probiotics: 2406) were included. None of the included studies assessed BPD as the primary outcome. Meta-analysis confirmed a significant reduction of NEC (risk ratio (RR) 0.52, 95% confidence interval (CI) 0.33 to 0.81, $p = 0.004$; random effects model), and an almost significant reduction of LOS (RR 0.82, 95% CI 0.65 to 1.03, $p = 0.084$). In contrast, meta-analysis could not demonstrate a significant effect of probiotics on BPD, defined either as oxygen dependency at 28 days of life (RR 1.01, 95% CI 0.91 to 1.11, $p = 0.900$, 6 studies) or at 36 weeks of postmenstrual age (RR 1.07, 95% CI 0.96 to 1.20, $p = 0.203$, 12 studies). Meta-regression did not show any significant association between the RR for NEC or LOS and the RR for BPD. In conclusion, our results suggest that NEC and LOS prevention by probiotics does not affect the risk of developing BPD in preterm infants.

Keywords: probiotics; bronchopulmonary dysplasia; sepsis; necrotizing enterocolitis

1. Introduction

Bronchopulmonary dysplasia (BPD), a chronic lung disease of prematurity, is considered one of the major complications of premature birth [1–4]. The incidence of BPD is inversely proportional to gestational age, with rates reaching up to 60–90% in extremely preterm infants (22–25 weeks gestation). Infants suffering from BPD are at increased risk of death and long-term pulmonary and neurodevelopmental morbidities [5–7].

The pathogenesis of BPD is initiated by the arrest in alveolar and lung vascular development, due to premature birth, and sustained by inflammatory events that play a paramount role in the progression of BPD [3,4,8,9]. The initiation of the inflammatory response can already occur in utero, in the setting of chorioamnionitis [3,4,10,11]. Nevertheless, postnatal stimuli, such as the ex-utero higher oxygen partial pressures, the need for oxygen administration or mechanical ventilation, and the occurrence of postnatal infections (including late onset sepsis (LOS) and necrotizing enterocolitis

(NEC)), perpetuate inflammation and lead to the establishment of BPD [12–14]. A dysregulation of the immune system, toward a sustained status of inflammation which is characteristic of very preterm infants, completes the multifactorial pathophysiological picture [15].

Several treatments, most of which focused on anti-inflammatory or homeostasis-restoring properties, have been attempted in order to prevent or treat BPD [16]. However, meta-analyses could confirm a reduction of BPD only for vitamin A and dexamethasone [16,17]. Moreover, vitamin A showed only a modest effect [17], while the use of dexamethasone is limited in preterm infants by its well-known long- and short-term side effects [18]. Adequate timing, dose, and formulation of steroid therapy is still under investigation in preterm infants at risk for BPD. Lately, regenerative medicine has received a great deal of attention as a promising therapeutic option for complications of prematurity, including BPD [19,20]. However, the knowledge of stem cell function is still incomplete, and further studies are needed to elucidate the impact of several manufacturing aspects that may determine the success or failure of this therapy [19,20]. In summary, despite the continuous advances in neonatal care, BPD remains a significant burden for the premature population, lacking a safe, effective and easily available treatment.

Probiotics are defined as live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host [21,22]. Probiotic supplementation in preterm infants is one of the most studied interventions in neonatal medicine [23–30]. Many randomized controlled trials (RCTs) involving the use of probiotics have been performed in the last years. Several meta-analyses combined these RCTs and demonstrated that probiotic supplementation reduces mortality, NEC, and LOS, as well as the time to achieve full enteral feeding in preterm infants [23–31]. Although until now no study has been performed to analyze the effect of probiotics on BPD as primary outcome, a number of RCTs included BPD as a secondary outcome. There are several hypothetical mechanisms by which probiotics may exert a protective effect against BPD: (1) by reducing postnatal inflammatory processes such as NEC and LOS; (2) by modulating the immune function [32,33]; (3) by improving the nutritional status and growth of the infants [30,31,34]; and (4) through the antioxidant properties of probiotics [35]. Therefore, in the present systematic review we aimed to collect and analyze the current evidence on the effects of probiotic supplementation on the risk of developing BPD in preterm infants.

2. Materials and Methods

A protocol was developed prospectively that detailed the specific objectives, criteria for study selection, the approach to assessing study quality, clinical outcomes, and statistical methodology. The study is reported according to the PRISMA checklist [36].

2.1. Data Sources and Search Strategies

A comprehensive literature search was undertaken using PubMed, EMBASE and CENTRAL (the Cochrane Central Register of Controlled Trials, The Cochrane Library) from their inception to 1 July 2017. Combinations of the following terms (including MeSH terms) were used to search for relevant publications: (probiotic(s) OR lactobacillus OR saccharomyces OR bifidobacterium OR streptococcus) AND ("preterm infant" OR "premature infant" OR "extremely low birth weight infant" OR "very low birth weight infant"). Language was not restricted. Additional strategies to identify studies included manual review of reference lists of key articles that fulfilled our eligibility criteria, use of the "related articles" feature in PubMed, use of the "cited by" tool in Web of Science and Google Scholar, and manual review of reference lists of meta-analyses on probiotics in preterm infants [23–25,27,28,30,34,37–44]. The search method used to identify all relevant articles was discussed and developed by two authors (EV-M and EV) and the final search string was approved by all authors.

2.2. Eligibility Criteria and Study Selection

The initial search was performed by two reviewers (EV-M and EV), who eliminated clearly irrelevant articles based on the title and abstract as defined by the pre-set selection criteria. The final

selection of articles was made by mutual consideration of both authors. Studies were included if they were RCTs involving the use of probiotics in preterm infants (gestational age, GA < 37 weeks) and reported results on BPD. BPD was defined as dependence on supplementary oxygen either at 28 days of life (BPD28) or at a postmenstrual age (PMA) of 36 weeks (BPD36) [2]. However, the use of another BPD definition was not an exclusion criterion.

Studies were reviewed to ensure that study populations did not overlap by checking subject sources and studying time-frame. Where two or more studies reported on the same population, the most recent study was preferentially used (provided it reported data on BPD) to avoid duplicate data.

2.3. Data Extraction and Assessment of Risk of Bias

Two groups of investigators (EV-M/EV and MP/GC) extracted the data independently by using a data collection form designed for this review. Data extracted included: gestational age (GA) and birth weight (BW) of participants, patient inclusion criteria, study design (age at the first day of intervention, duration of intervention, dosage, and type of probiotic), and outcomes of interest (BPD, LOS, NEC, and mortality).

Two reviewers (EV-M and EV) independently assessed risk of bias in each trial by using the Cochrane "Risk of Bias Assessment Tool" [45]. For each domain (allocation sequence, allocation concealment, blinding of participants and outcome assessors, incomplete outcome data, selective outcome reporting, and other potential sources of bias) the risk of bias was assessed as low, high, or unclear. Potential discrepancies during the data extraction process and assessment of risk of bias were resolved by discussion and consensus among all reviewers.

2.4. Statistical Analysis

Studies were combined and analyzed using comprehensive meta-analysis V3.0 software (Biostat Inc., Englewood, NJ, USA). We used a random-effects model to account for anticipated heterogeneity, resulting from the differences in methodology between studies. However, analysis using a fixed-effect model was also carried out to ensure that the model used for the meta-analysis would not affect the results. Effect size was expressed as Mantel–Haenszel risk ratio (RR) and 95% confidence interval (CI). Statistical heterogeneity was assessed with the Cochran's Q statistic and by the I^2 statistic, which is derived from Q and describes the proportion of total variation that is due to heterogeneity beyond chance [45]. An I^2 value of 0% indicates no observed between-study heterogeneity, and large values show increasing between-study heterogeneity. The risk of publication bias was assessed by visual inspection of the funnel plot and using an Egger test. To identify any study that may have exerted a disproportionate influence on the summary effect, we calculated the summary effect excluding studies one at a time. To explore differences between studies that might be expected to influence the effect size, we performed subgroup sensitivity analysis and univariate random-effects meta-regression (method of moments) [46,47]. A potential pitfall with meta-regression analysis is that with few trials and many possible covariates, false positive findings and data dredging can happen [47]. We chose to prespecify NEC, LOS, and mortality as covariates to analyze with meta-regression to protect against this issue. A probability value of less than 0.05 (0.10 for heterogeneity) was considered statistically significant.

3. Results

There was no substantial disagreement between reviewers on articles for inclusion, data extraction, and risk of bias assessment. Based on the titles and abstracts of 1456 citations, we identified 63 potentially relevant studies, of which 15 met the inclusion criteria [48–62] (Figure 1). The main characteristics of the studies are shown in Table 1. The 15 studies included 4782 infants of which 2406 infants received probiotics. Twelve studies [48–51,53–58,60,62] included very preterm (GA < 32 weeks) and/or very low BW (VLBW) infants (<1500 g). One study [48] included extremely low BW preterm infants (<1000 g). Two studies included larger preterm infants; one [52] included infants with GA < 34 weeks and the other [59] included infants with GA < 37 weeks. The included

studies randomized infants to different preparations, times of initiation, and duration of therapy (Table 1). Details of the risk of bias analysis are depicted in Appendix A, Table A1. None of the included studies reported serious adverse events potentially associated with the use of probiotics.

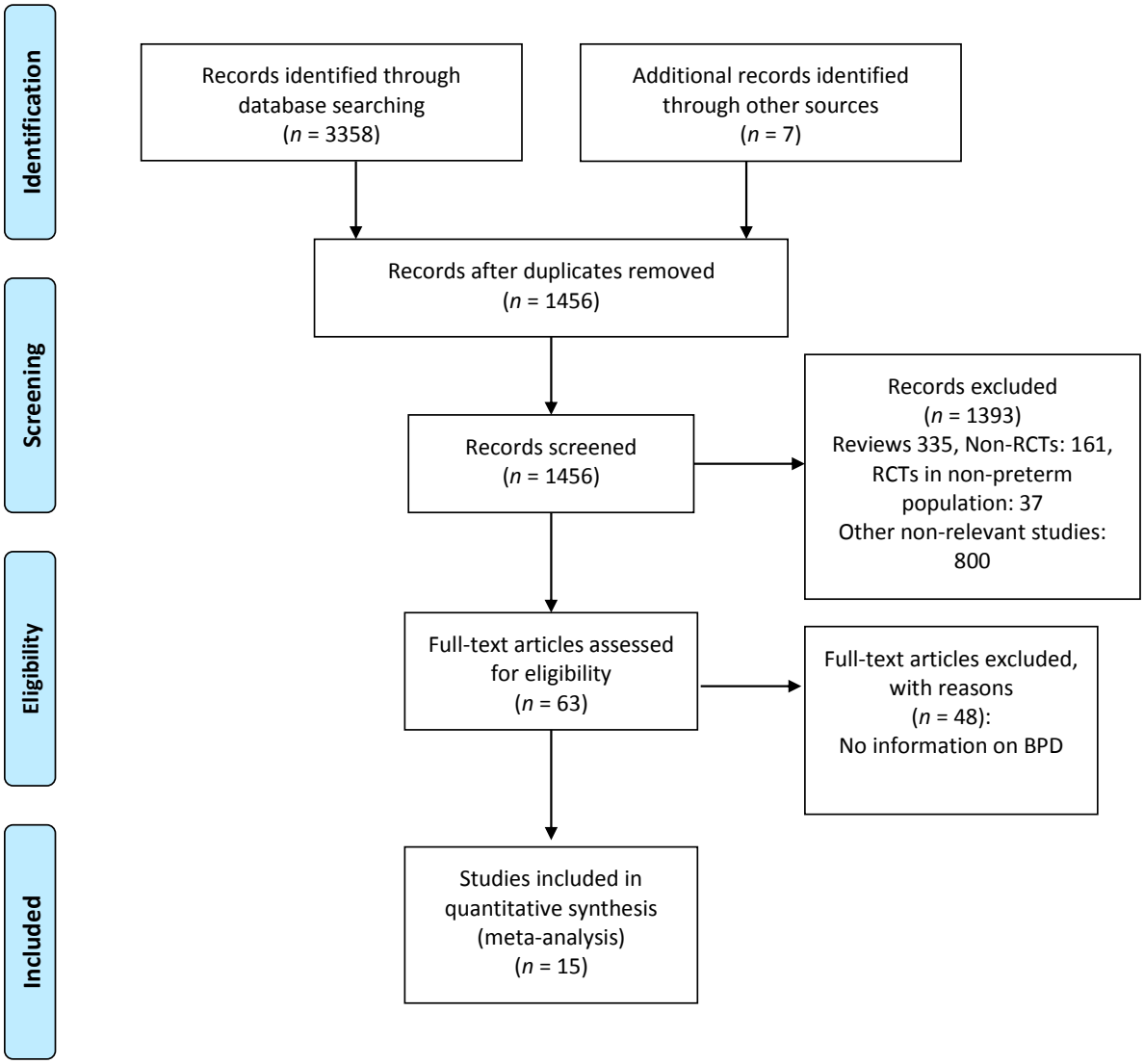

Figure 1. Flow diagram of literature search process. RCTs: randomized controlled trials; BPD: bronchopulmonary dysplasia.

Table 1. Characteristics of the included studies.

Study	Participants	Sample Size, GA (Weeks), BW (g)		Intervention	Duration of Intervention	Primary Outcome	BPD Definition
		Probiotics	Control				
Akar 2017 [49]	GA ≤ 32 weeks or BW ≤ 1500 g	*n* = 124 GA: 28.9 (2.1) BW: 1138 (257)	*n* = 125 GA: 28.6 (2.5) BW: 1142 (267)	*Lactobacillus reuteri* vs. no probiotics	From first feed until discharge	Neurodevelopmental outcome	BPD36
Al Hosni 2012 [48]	BW 501–1000 g	*n* = 50 GA: 25.7 (1.4) BW: 778 (138)	*n* = 51 GA: 25.7 (1.4) BW: 779 (126)	*Lactobacillus rhamnosus* + *Bifidobacterium infantis* vs. no probiotics	Once daily from the time of initiation of enteral feeds, until discharge or 34 weeks PMA	% infants <10th centile at 34 weeks PMA	BPD36
Costeloe 2016 [62]	GA < 31 weeks	*n* = 650 GA (median): 28.0 (IQR: 26.1–29.4) BW: 1039 (312)	*n* = 660 GA (median): 28.0 (IQR: 26.1–29.6) BW: 1043 (317)	*Bifidobacterium breve* BBG-001 vs. placebo	Commenced within 48 hours of birth, until 36 weeks PMA or discharge	NEC ≥ stage 2, LOS, death	BPD36, Severe BPD
Demirel 2013 [50]	GA ≤ 32 weeks and BW ≤ 1500 g	*n* = 135 GA: 29.4 (2.3) BW: 1164 (261)	*n* = 136 GA: 29.2 (2.5) BW: 1131 (284)	Saccharomyces *boulardii* vs. no probiotics	Once daily from the time of initiation of enteral feeds, until discharge	NEC ≥ stage 2 or death	BPD28
Dilli 2015 [51]	GA < 32 weeks and BW < 1500 g	*n* = 100 GA: 28.8 (1.9) BW: 1236 (212)	*n* = 100 GA: 28.2 (2.2) BW: 1147 (271)	*Bifidobacterium lactis* vs. placebo	From day 8 of life, once daily until discharge or a maximum of 8 weeks	NEC ≥ stage 2	BPD28, BPD36
Fujii 2006 [52]	GA < 34 weeks	*n* = 11 GA: 31.3 (3.2) BW: 1378 (365)	*n* = 8 GA: 31.2 (2.0) BW: 1496 (245)	*B. breve* M-16V vs. placebo	From several hours after birth until discharge	Serum cytokine levels and expression of Transforming growth factor beta signaling Smad molecules	BPD28
Jacobs 2013 [53]	GA < 32 weeks and BW < 1500 g	*n* = 548 GA: 27.9 (2.0) BW: 1063 (259)	*n* = 551 GA: 27.8 (2.0) BW: 1048 (260)	*B. infantis* + Saccharomyces *thermophilus* + *B. lactis* vs. placebo	From enteral feed ≥ 6 mL/day until discharge or term corrected age.	LOS	BPD28, BPD36
Lin 2008 [54]	GA <34 weeks and BW < 1500 g	*n* = 217 BW: 1029 (246)	*n* = 217 BW: 1077 (214)	*Lactobacillus acidophilus* + *Bifidobacterium* bifidum vs. no probiotics	From first feeding, for 6 weeks.	Death or NEC ≥ Stage 2	BPD36
Manzoni 2009 [55]	BW < 1500 g	*n* = 151 GA: 29.8 (2.8) BW: 1138 (253)	*n* = 168 GA: 29.5 (3.2) BW: 1109 (269)	*L. rhamnosus* GG + lactoferrin vs. placebo	From day 3 of life, for 6 weeks or until discharge	LOS	BPD36
Saengtawesin 2014 [56]	GA ≤ 34 weeks and BW ≤ 1500 g	*n* = 31 GA: 31.0 (1.8) BW: 1250 (179)	*n* = 29 GA: 30.6 (1.8) BW: 1208 (199)	*L. acidophilus* + *B. bifidum* vs. no probiotics	From first enteral feed until 6 weeks of age or discharge	NEC ≥ Stage 2	BPD28

Table 1. *Cont.*

Study	Participants	Sample Size, GA (Weeks), BW (g)		Intervention	Duration of Intervention	Primary Outcome	BPD Definition
		Probiotics	Control				
Sari 2012 [57]	GA <33 or BW < 1500 g	*n* = 86 GA: 29.7 (2.5) BW: 1241 (264)	*n* = 88 GA: 29.8 (2.3) BW: 1278 (273)	*Lactobacillus sporogenes* vs. no probiotics	From first enteral feed until discharge	Growth and neurodevelopment at 18–22 months	BPD36
Serce 2013 [58]	GA ≤ 32 weeks and BW ≤ 1500 g	*n* = 104 GA: 28.8 (2.2) BW: 1126 (232)	*n* = 104 GA: 28.7 (2.1) BW: 1162 (216)	*S. boulardii* vs. placebo	From first enteral feed until discharge	NEC ≥ Stage 2 or death or LOS	BPD36
Stratiki 2007 [59]	GA 27–37 weeks	*n* = 41 GA (median): 31 (range: 27–37) BW (median): 1500 (range: 900–1780)	*n* = 36 GA (median): 30.5 (range: 26–37) BW (median): 1500 (range: 700–1900)	*B. lactis* vs. no probiotics	From day 2 to discharge	Intestinal permeability by the sugar absorption test	Undefined
Totsu 2014 [60]	BW < 1500 g	*n* = 153 GA: 28.6 (2.9) BW: 1016 (289)	*n* = 130 GA: 28.5 (3.3) BW: 998 (281)	*B. bifidum* vs. placebo	Commenced within 48 h of birth and continued until discharge	Postnatal day when enteral feed exceeding 100 mL/kg/day	BPD28, BPD36
Underwood 2009 (CUL) [61] [1]	GA < 35 weeks and BW 750–2000 g	*n* = 30 GA: 29.5 (2.6) BW: 1394 (356)	*n* = 29 GA: 29.3 (2.6) BW: 1393 (363)	*L. rhamnosus* GG + inulin vs. placebo	From first feed until 28 days or discharge	Weight gain	BPD36
Underwood 2009 (PBP) [61] [1]	GA < 35 weeks and BW 750–2000 g	*n* = 31 GA: 30.2 (2.4) BW: 1461 (372)	*n* = 29 GA: 29.3 (2.6) BW: 1393 (363)	*L. acidophilus* + *Bifidobacterium longum* + *B. bifidum* + *B. infantis* + inulin vs. placebo	From first feed until 28 days or discharge	Weight gain	BPD36

[1] Culturelle® (CUL) and ProBioPlus DDS® (PBP) were the names assigned by the authors to the probiotic preparations. BPD: bronchopulmonary dysplasia; BPD28: bronchopulmonary dysplasia, defined as oxygen dependence at 28 days of life; BPD36: bronchopulmonary dysplasia, defined as oxygen dependence at 36 weeks post-menstrual age; Severe BPD: defined as any baby at 36 weeks PMA still receiving mechanical ventilator support or in at least 30% oxygen or more than 0.1 L/min of low flow oxygen. BW: birth weight; GA: gestational age; IQR: interquartile range; NEC: necrotizing enterocolitis; PMA: postmenstrual age; LOS: late-onset sepsis. Data for GA and BW given in mean (standard deviation), unless noted otherwise.

BPD was not the primary outcome in any of the included studies. Six studies [53,55,58,60–62] clearly defined BPD as BPD28 and/or BPD36, whereas nine studies did not [48–52,54,56,57,59]. A clarification on BPD definition was kindly provided by the authors of eight studies [48–52,54,56,57]. After these clarifications, data on BPD28 were available from six studies [50–53,56,60]. We decided to pool the study of Stratiki et al. [59] that did not specify a BPD definition, with studies reporting BPD28. Neither the individual studies nor the meta-analysis could detect a significant effect of probiotic supplementation on BPD28 (RR 1.01, 95% CI 0.91 to 1.11, $p = 0.900$, Figure 2). The use of a fixed effect model instead of a random effects model did not significantly affect the results of the meta-analysis (RR 1.00, 95% CI 0.91 to 1.10, $p = 0.999$). In sensitivity analyses, excluding one study at a time, the summary RR ranged from 0.99 (95% CI 0.89–1.10, $p = 0.900$), when the study of Totsu et al. [60] was excluded, to 1.04 (95% CI 0.86–1.25, $p = 0.703$), when the study of Jacobs et al. [53] was excluded (Appendix A, Table A2). The study of Fujii et al. [52] included larger infants than the other five studies (Table 1). However, when this study was excluded, overall results were not substantially affected (RR 1.01, 95% CI 0.91–1.11, $p = 0.983$). Exclusion of the study by Stratiki et al. [59], in which BPD was not clearly defined, did not significantly affect results (RR 1.01 95% CI 0.91–1.11, $p = 0.829$). Further sensitivity analysis and assessment of publication bias were not performed for BPD28 due to the low number of studies.

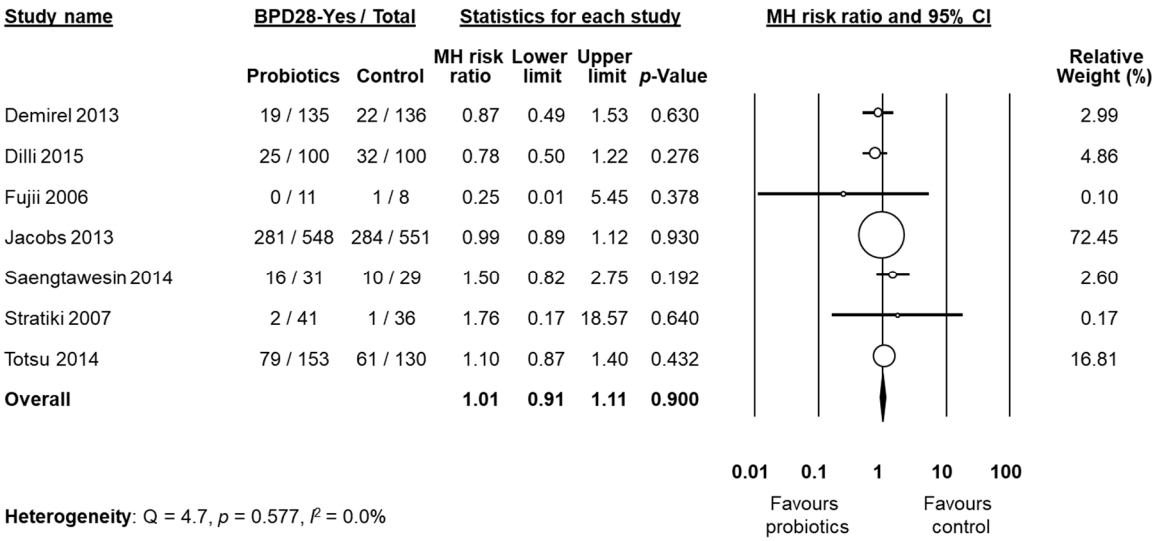

Figure 2. Random effects meta-analysis: Probiotic supplementation and risk of BPD28 (bronchopulmonary dysplasia, defined as oxygen dependence at 28 days of life). MH: Mantel–Haenszel; CI: confidence interval.

Data on BPD36 were available from 11 studies [48,49,51,53–55,57,58,60–62]. The study of Underwood et al. [61] randomized infants into three different groups: a placebo group and two treatment groups based on different probiotic preparations (Table 1). For the purposes of this analysis, the two treatment groups of the trial of Underwood et al. [61] were considered as two separate studies. The study of Lin et al. [54], showed a significant increase of the BPD36 risk in the infants receiving probiotics (RR 1.38, 95% CI 1.01 to 1.88, $p = 0.043$). In contrast, neither the other individual studies nor the meta-analysis could detect a significant effect of probiotic supplementation on BPD36 (RR 1.07, 95% CI 0.96 to 1.20, $p = 0.203$, Figure 3). Although some degree of asymmetry was observed by visual inspection of the funnel plot, Egger's test could not show any evidence of publication bias (Figure 4). The use of a fixed effect model instead of a random effects model did not significantly affect the results of the meta-analysis (RR 1.08, 95% CI 0.98 to 1.18, $p = 0.123$). In sensitivity analyses, excluding one study at a time, the summary RR ranged from 1.04 (95% CI 0.93–1.17, $p = 0.488$), when the study of

Lin et al. [54] was excluded, to 1.09 (95% CI 0.97–1.23, $p = 0.138$), when the study of Al Hosni et al. [48] was excluded (Appendix A Table A3).

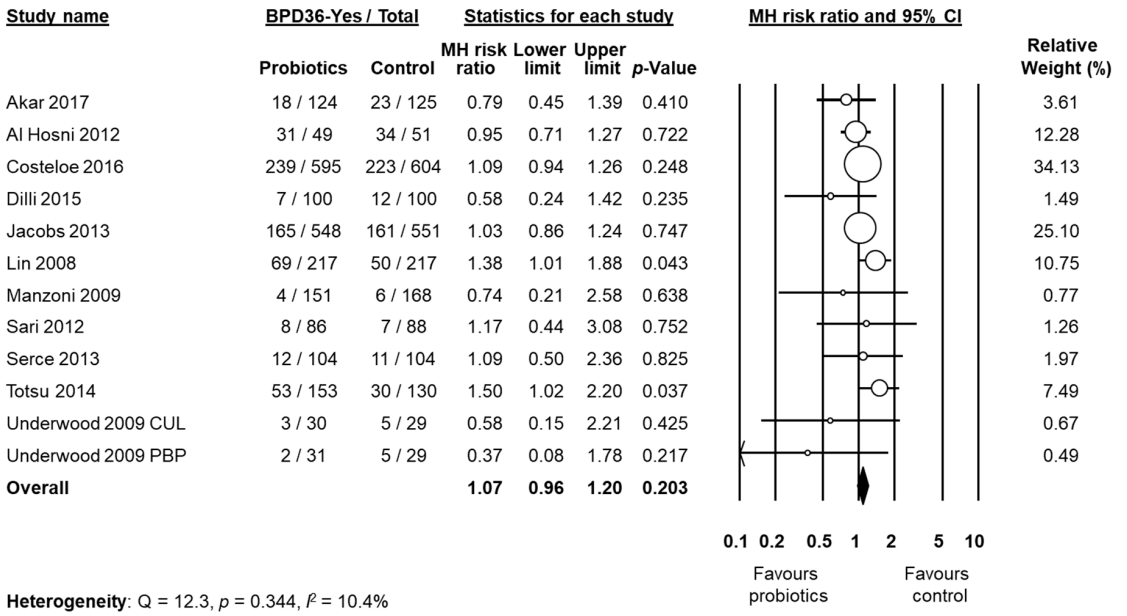

Figure 3. Random effects meta-analysis: Probiotic supplementation and risk of BPD36 (bronchopulmonary dysplasia, defined as oxygen dependence at 36 weeks post-menstrual age). MH: Mantel–Haenszel; CI: confidence interval. CUL: Culturelle preparation; PBP: ProBioPlus DDS preparation.

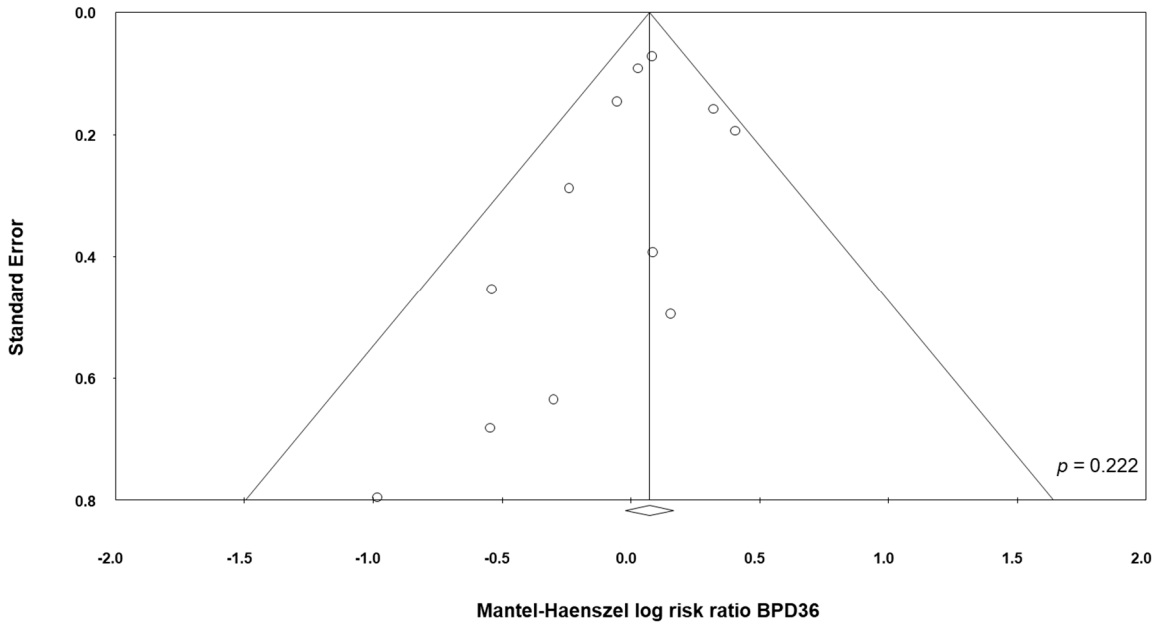

Figure 4. Funnel plot assessing publication bias for BPD36 (bronchopulmonary dysplasia, defined as oxygen dependence at 36 weeks post-menstrual age).

One study [62] also included, besides BPD36, the category severe BPD (defined as any baby at 36 weeks PMA still receiving mechanical ventilator support or in at least 30% oxygen or more than 0.1 L/min of low flow oxygen) (Table 2). They report that the probiotics group did not have a significantly different risk of severe BPD compared to the control group (RR 1.21, 95% CI 0.90 to 1.62, $p = 0.200$).

Table 2. Subgroup analysis of probiotics and risk of BPD.

Subgroup	*k*	BPD Definition	Sample Size	MH RR	95% CI	*p*
Studies where *Lactobacillus* was part of the supplementation	6	BPD36	1335	1.01	0.80–1.29	0.904
Studies where *Bifidobacterium* was part of the supplementation	5	BPD28	1601	1.00	0.90–1.11	0.999
	4	BPD36	2781	1.10	0.90–1.33	0.346
Single-strain supplementation	4	BPD28	773	0.97	0.79–1.18	0.763
	7	BPD36	2372	1.08	0.88–1.32	0.480
Multiple-strain supplementation	2	BPD28	1159	1.01	0.90–1.13	0.829
	5	BPD36	2012	1.06	0.87–1.29	0.574
Studies with infants mean BW < 250 g	5	BPD28	1913	1.01	0.91–1.11	0.893
	9	BPD36	4091	1.08	0.96–1.22	0.195
Studies with low risk of bias on random sequence generation and allocation concealment	9	BPD36	3752	1.08	0.97–1.19	0.155
Studies with low risk of bias on incomplete outcome data	10	BPD36	3927	1.09	0.96–1.23	0.188
Studies with low risk of bias on selective reporting	9	BPD36	3493	1.06	0.94–1.18	0.344

BPD: bronchopulmonary dysplasia; BPD28: bronchopulmonary dysplasia, defined as oxygen dependence at 28 days of life; BPD36: bronchopulmonary dysplasia, defined as oxygen dependence at 36 weeks post-menstrual age; CI: confidence interval; *k*: number of studies included; MH RR: Mantel–Haenszel risk ratio.

For the outcome BPD36, we conducted additional sensitivity analysis by excluding studies that had uncertain/high risk of bias in the different domains. In addition, we carried out subgroup analyses of studies where *Bifidobacterium* was part of the supplementation, studies where *Lactobacillus* was part of the supplementation, studies where multiple-strain supplements were used, studies where single-strain supplements were used, and studies where infants had a mean BW < 1250 g. No subgroup analysis could demonstrate a significant effect of probiotics on BPD36 (Table 2).

All the included studies reported data on NEC (Table 3) and, when pooled, we observed that probiotics significantly reduced the risk of developing NEC (RR 0.52, 95% CI 0.33–0.81, $p = 0.004$, Table 4). This significant reduction of NEC was also observed when we pooled the studies that reported BPD28 (RR 0.40, 95% CI 0.18–0.88, $p = 0.022$), and when we pooled the studies that reported BPD36 (RR 0.48, 95% CI 0.29–0.81, $p = 0.006$, Table 4). We performed meta-regression analyses (methods of moments) to investigate the possible correlation between the effect size for NEC and the effect size for BPD. As shown in Figure 5, meta-regression could not detect a statistically significant correlation between the reduction in NEC produced by the probiotics and the effect size for BPD36.

All the included studies reported data on LOS (Table 3), and meta-analysis demonstrated a close to significant reduction of LOS in the probiotics group (RR 0.82, 95% CI 0.65–1.03, $p = 0.084$, Table 4). Similarly, the meta-analysis of studies that reported BPD28 found a close to significant effect of probiotics on LOS (RR 0.79, 95% CI 0.63–1.00, $p = 0.054$), and the meta-analysis of studies that reported BPD36 found a close to significant reduction in LOS (RR 0.80, 95% CI 0.62–1.04, $p = 0.090$, Table 4). We performed meta-regression analyses (methods of moments) to investigate the possible correlation between the effect size for LOS and the effect size for BPD36. As shown in Figure 6, meta-regression could not detect a statistically significant correlation between the reduction in LOS produced by the probiotics and the effect size for BPD36.

Table 3. NEC, LOS and mortality in the included studies.

Study	NEC (Affected/Total)		NEC Definition	LOS (Affected/Total)		LOS Definition	Mortality (Affected/Total)		Mortality Definition
	Probiotics	Control		Probiotics	Control		Probiotics	Control	
Akar 2017 [49]	1/124	6/125	NEC stage ≥ 2	8/124	19/125	Culture-proven sepsis	14/200	16/200	Death before 18–24 month follow-up
Al Hosni 2012 [48]	2/50	2/51	NEC stage ≥ 2	13/50	16/51	Culture-proven sepsis	3/50	4/51	Death before 34 weeks PMA
Costeloe 2016 [62]	61/650	66/660	NEC stage ≥ 2	73/650	77/660	Culture-proven sepsis > 72 h	54/650	56/660	Death during primary hospitalization
Demirel 2013 [50]	6/135	7/136	NEC stage ≥ 2	20/135	21/136	Culture-proven sepsis	5/135	5/136	Death after 7 days of life
Dilli 2015 [51]	2/100	18/100	NEC stage ≥ 2	8/100	13/100	Culture-proven sepsis > 72 h	3/100	12/100	Not defined
Fujii 2006 [52]	0/11	0/8	Not defined	1/11	1/8	Not defined	0/11	0/8	Death during primary hospitalization
Jacobs 2013 [53]	11/548	24/551	NEC stage ≥ 2	72/548	89/551	Culture-proven sepsis > 48 h	30/548	31/551	Death during primary hospitalization
Lin 2008 [54]	4/217	14/217	NEC stage ≥ 2	40/217	24/217	Culture-proven > 72h	2/217	9/217	Death during intervention (6 weeks)
Manzoni 2009 [55]	0/151	10/168	NEC stage ≥ 2	7/151	29/168	Culture-proven sepsis > 72 h	6/153	12/168	Death during primary hospitalization
Saengtawesin 2014 [56]	1/31	1/29	NEC stage ≥ 2	2/31	1/29	Not defined	0/31	0/29	Death during primary hospitalization
Sari 2012 [57]	3/86	7/88	NEC stage ≥ 2	24/86	19/88	Not defined	5/110	8/111	Death before 18 to 22 months of age
Serce 2013 [58]	7/104	7/104	NEC stage ≥ 2	19/104	25/104	Culture-proven sepsis	5/104	4/104	Death during primary hospitalization
Stratiki 2007 [59]	0/41	3/36	NEC stage ≥ 2	0/41	3/36	Culture-proven sepsis	0/41	0/36	Not defined
Totsu 2014 [60]	0/153	0/130	NEC stage ≥ 2	6/153	10/130	Culture-proven sepsis ≥ 1 week	2/153	0/130	Death during primary hospitalization
Underwood 2009 (CUL) [61] [1]	1/30	1/29	NEC stage ≥ 2	4/30	4/29	Culture-proven sepsis > 72 h	0/30	0/29	Death during primary hospitalization
Underwood 2009 (PBP) [61] [1]	1/31	1/29	NEC stage ≥ 2	2/31	4/29	Culture-proven sepsis > 72 h	0/31	0/29	Death during primary hospitalization

[1] Culturelle (CUL) and ProBioPlus DDS (PBP) were the names assigned by the authors to the probiotic preparations. LOS: late-onset sepsis; NEC: necrotizing enterocolitis.

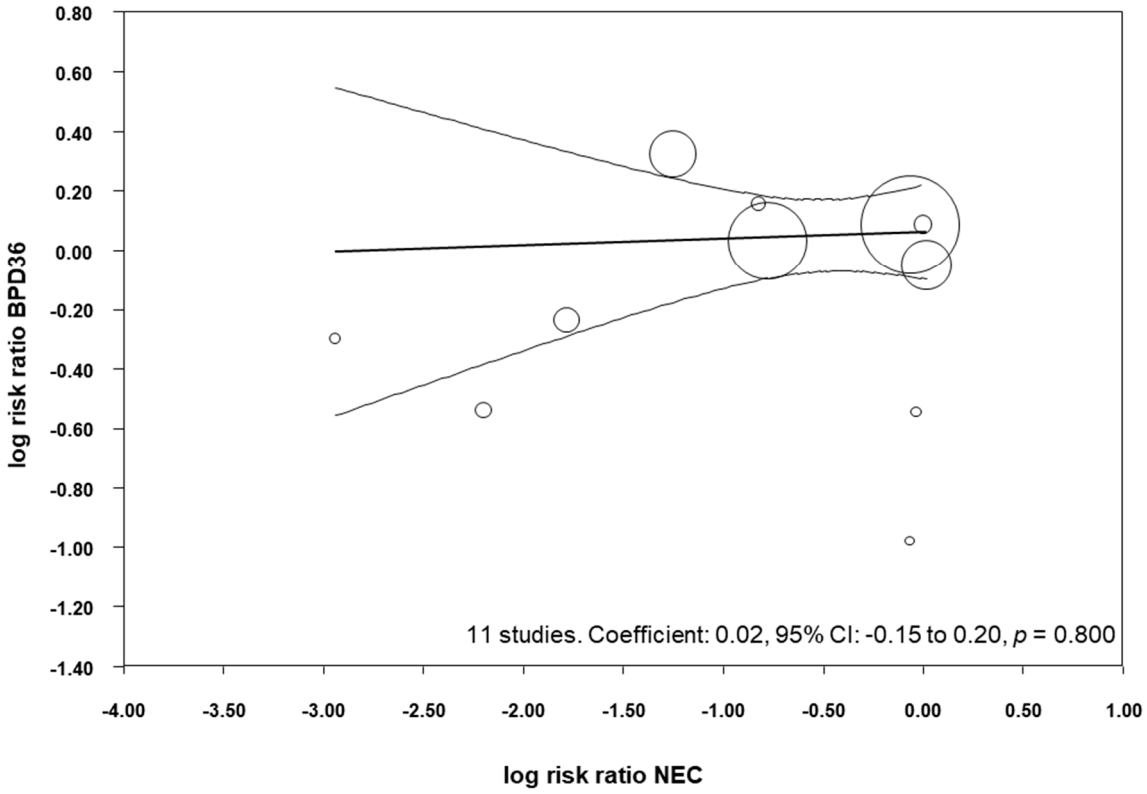

Figure 5. Meta-regression plot of probiotics and risk of BPD36 (bronchopulmonary dysplasia, defined as oxygen dependence at 36 weeks post-menstrual age) and probiotics and risk of necrotizing enterocolitis (NEC), CI: confidence interval.

Table 4. Random effects meta-analysis of probiotics and LOS, NEC and mortality.

Meta-Analysis	*k*	BPD Definition	MH Risk Ratio	95% CI	Z	*p*	Heterogeneity		
							Q	*p*	I^2
Probiotics NEC	15	All	0.52	0.33 to 0.81	−2.88	0.004	22.0	0.055	40.9%
	7	BPD28	0.40	0.18 to 0.88	−2.29	0.022	6.3	0.175	37.0%
	12	BPD36	0.48	0.29 to 0.81	−2.73	0.006	20.4	0.025	51.1%
Probiotics LOS	15	All	0.82	0.65 to 1.03	−1.73	0.084	26.8	0.031	44.0%
	7	BPD28	0.79	0.63 to 1.00	−1.93	0.054	3.6	0.72	0.0%
	12	BPD36	0.80	0.62 to 1.04	−1.70	0.090	24.5	0.011	55.1%
Probiotics mortality	11	All	0.84	0.66 to 1.07	−1.38	0.169	10.4	0.410	3.4%
	4	BPD28	0.78	0.37 to 1.66	−0.65	0.518	5.2	0.155	42.8%
	10	BPD36	0.82	0.62 to 1.07	−1.45	0.146	10.3	0.328	12.5%

BPD: bronchopulmonary dysplasia; BPD28: bronchopulmonary dysplasia, defined as oxygen dependence at 28 days of life; BPD36: bronchopulmonary dysplasia, defined as oxygen dependence at 36 weeks post-menstrual age; CI: confidence interval; *k*: number of studies included; LOS: late onset-sepsis; MH: Mantel–Haenszel; NEC: necrotizing enterocolitis.

All the included studies reported data on mortality (Table 3), but meta-analysis could not demonstrate a significant reduction of mortality in the probiotics group (RR 0.80, 95% CI 0.60–1.06, $p = 0.114$, Table 4). Moreover, the meta-analysis of studies that reported BPD28 could not find a significant effect of probiotics on mortality (RR 0.78, 95% CI 0.37 to 1.66, $p = 0.518$), and neither could the meta-analysis of studies that reported BPD36 (RR 0.77, 95% CI 0.56 to 1.05, $p = 0.101$). We performed meta-regression analyses (methods of moments) to investigate the possible correlation between the effect size for mortality and the effect size for BPD36. This meta-regression could not detect a statistically significant correlation between the changes in mortality produced by the probiotics and the effect size for BPD36 (coefficient 0.04, 95% CI −0.13 to 0.21, $p = 0.638$).

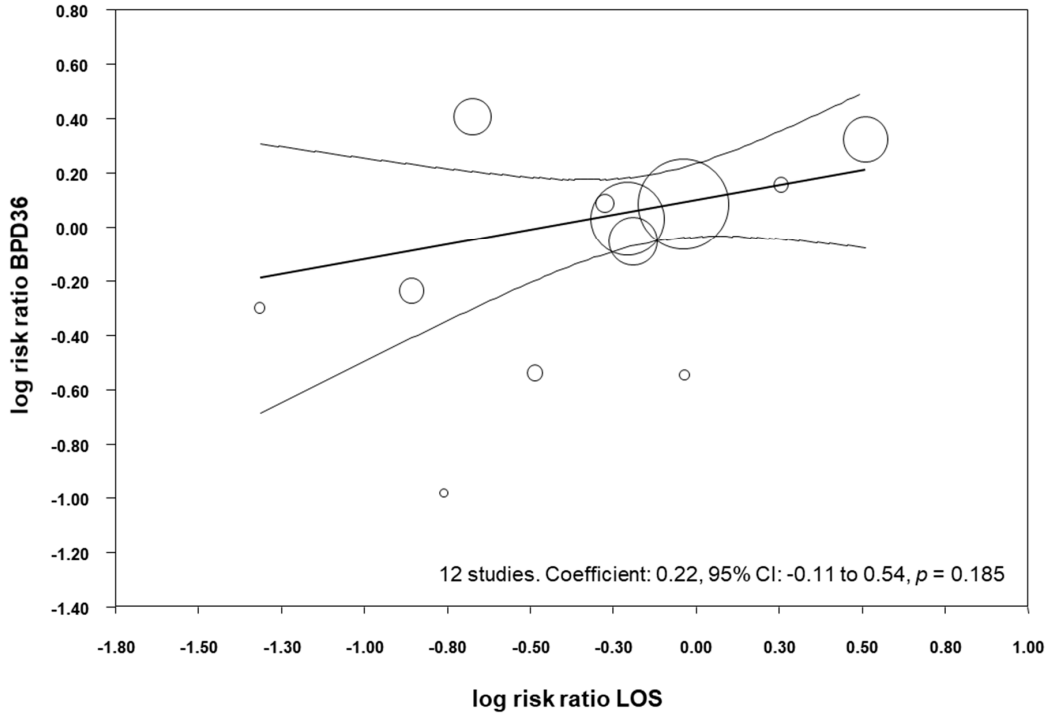

Figure 6. Meta-regression plot of probiotics and risk of BPD36 (bronchopulmonary dysplasia, defined as oxygen dependence at 36 weeks post-menstrual age) and probiotics and risk of late-onset sepsis (LOS), CI: confidence interval.

4. Discussion

Inflammatory events, such as NEC and LOS, are not only life-threatening for (very) preterm infants but also may mediate major short- and long-term adverse outcomes [63,64]. Current evidence indicates that probiotic supplementation significantly reduces NEC and LOS in preterm infants, but our data suggest that this decrease is not accompanied by a concomitant reduction in BPD. The present meta-analysis could not demonstrate any significant effect of probiotic supplementation on the risk of developing of BPD. Similarly, in a recent meta-analysis we found that probiotics did not significantly affect the risk of retinopathy of prematurity (ROP) [44]. However, our results should be interpreted with caution since the included RCTs showed relevant methodological differences in terms of enrolment criteria, timing, dose, and formulation of the probiotics used. Moreover, BPD was not the primary outcome in any of the studies and the number of RCTs of probiotics reporting on BPD as secondary outcome was relatively small. In addition, none of the included studies specifically targeted the most vulnerable population for BPD (infants < 28 weeks GA).

Inflammatory processes such as NEC and LOS may increase the risk of developing BPD through direct and indirect mechanisms. Proinflammatory cytokines may exert a direct effect on lung development or sensitize the lung to the effects of oxygen, mechanical ventilation, or other stressors [8,9,15,65]. On the other hand, infants suffering from NEC and LOS often require more aggressive and prolonged mechanical ventilation, that may lead to increased lung injury [8,9,15,65]. It has been suggested that avoiding postnatal infection is more important than avoiding invasive mechanical ventilation to decrease the inflammatory response in developing lungs [65]. Studies directed at evaluating the impact of quality improvement efforts to reduce LOS in preterm infants showed that a reduction in LOS is accompanied by decreased rates of BPD [66,67]. However, BPD is a multifactorial condition in which genetic predisposition, as well as prenatal and prenatal conditions all play a role [1–4]. In an interesting study, Lapcharoensap et al. showed a positive relationship between the reduction in LOS and the reduction in BPD with a coefficient of determination (r^2) of 0.08,

suggesting that only the 8% of the reduction of BPD is attributable to the reduction in nosocomial infection rates [67].

The 15 studies included in our meta-analysis represent a subset of the larger number of RCTs included in the meta-analyses on probiotics for NEC and LOS prevention. Therefore, we analysed whether the protective effects of probiotics on NEC and/or LOS were also present in the RCTs included in our study. Pooling the 15 studies showed a significant reduction of NEC (RR 0.52, 95% CI 0.33 to 0.81) and a close to significant reduction of LOS (RR 0.82 95% CI 0.65 to 1.03) in the probiotics group. We speculated that studies with higher protective effects against NEC and/or LOS would show more effect on the development of BPD. However, meta-regression did not show a significant correlation between the RR for NEC and LOS and the RR for BPD. This suggests that the reduction in postnatal inflammatory events did not translate into a reduction of BPD.

Several meta-analyses showed that probiotics reduce mortality among VLBW infants [23,25,38]. It has been suggested that improved survival of VLBW infants may result in increased numbers of patients with BPD [68]. In the group of studies included in our meta-analysis, we could not observe a significant effect of probiotics on mortality (RR 0.80, 95% CI 0.60 to 1.06). In addition, meta-regression could not show a significant correlation between the RR for mortality and the RR for BPD. Therefore, our data suggest that the effect of probiotics on mortality did not affect the rate of BPD in the RCTs. Nevertheless, a robust conclusion from meta-regression would require a larger number of included studies [46,47].

One important limitation inherent to any meta-analysis on BPD is the heterogeneity of the definition of the condition [16,69,70]. In a systematic review which included 47 RCTs of drugs for BPD, 34% did not identify the definition of BPD that was used. Of the trials that defined BPD, 22 used oxygen dependency at 36 weeks PMA, with two trials refining that definition with a test of oxygen need [16]. Fourteen trials provided data on oxygen requirement and four trials used both oxygen supplementation at 28 days and oxygen supplementation at 36 weeks PMA [16]. Similarly, in our meta-analysis only six out of 15 RCTs reported a definition of BPD. Upon request, the authors of eight studies kindly clarified their definition. Even after clarification, there was marked heterogeneity in BPD definition. As pointed out by Jobe and Bancalari [69], current definitions of BPD lack precision and do not have good predictive values for later pulmonary and neurodevelopmental outcomes. There are substantial efforts being made to develop better diagnostic criteria for BPD [69], but it will take time before these improved definitions of BPD are reflected in RCTs and meta-analyses.

As mentioned above, the RCTs included in our analysis had important differences in the type, amount, and timing of probiotic supplementation. The choice of probiotic strain(s) is crucial and meta-analyses on probiotics have been criticized because, in most of them, probiotics administered for treatment/prevention of a specific disease or condition were all evaluated together [26,71–73]. It is now generally accepted that different bacterial strains of the same genus and species, verified also by genomic information, may exert completely different effects on the host [72]. Separate meta-analyses analysing the effects of well-defined individual, single-strain or multiple-strain probiotic preparations appear to be more appropriate, but the important heterogeneity of the RCTs makes this approach very difficult [26,71–73]. We attempted to explore whether the studies using *Lactobacillus* or *Bifidobacterium* species showed a different effect on BPD. We also performed a separate analysis for multi-strain probiotics because recent meta-analyses suggest that the use of more than one strain has a stronger effect in the prevention of NEC [74]. None of these subgroup analyses suggested a significant preventive effect of probiotics on BPD. However, the number of studies included in the subgroup analysis was low, making the results inconclusive.

Besides their effect on NEC and LOS prevention, there are some other mechanisms of action ascribed to probiotics which may directly counteract the disruption of lung development prompting to BPD [26,75]. In the first place, the immature immune system of premature infants is unable to balance pro-inflammatory responses, leading to a sustained status of inflammation that contributes significantly to several neonatal diseases, including BPD [15]. A decreased number of T regulatory

cells (Tregs), which constitute the anti-inflammatory lymphocytic subset, and higher proportions of activated pro-inflammatory T cells have been related with the development of BPD [76,77]. Probiotics seem to have a role in improving Treg generation, expansion and activity, while decreasing activation/proliferation of the pro-inflammatory lymphocytic subsets. These effects may result in the recovery of the immune homeostasis with polarization of the immune system toward an anti-inflammatory phenotype [78,79]. Secondly, it has been suggested that each additional day of antibiotic therapy in the first 2 weeks of life in VLBW infants may be associated with an increased BPD rate and severity [80]. This could be explained by the antibiotic-induced decrease in diversity of lung microbiota which has been linked to BPD development [81]. Probiotics are known to restore intestinal microbiota after antibiotic therapy [82] and to produce a strong suppressive effect on airway inflammation [83]. Lastly, poor nutrition is associated with lung underdevelopment and the occurrence of BPD [84]. In experimental NEC, probiotic supplementation reversed the detrimental effects of combined hyperoxia and suboptimal nutrition on lung vascular endothelial growth factor (VEGF) levels, suggesting that this strategy may help improve lung vasculogenesis [85].

In conclusion, our study could not demonstrate any significant effect of probiotic supplementation on the risk of developing of BPD. Given the remarkable theoretical benefits of probiotics supplementation in ameliorating several aspects of BPD pathogenesis and the limitations of the analysis, our data should be seen as a starting point rather than definitive results. The main merit of our study was to collect, for the first time, the available data on the role of probiotic supplementation in the prevention of BPD, and to revise the possible specific mechanisms of action. Nevertheless, further experimental and clinical data are needed to draw more solid conclusions. Particularly, more studies designed to select the optimal probiotic preparation, dosing, and duration of therapy are still needed [29]. These studies should compare probiotic strains that have been reported to be safe and effective in previous trials [73] and include outcomes, such as BPD, which can be indirectly affected by the changes in immunity and nutritional status induced by probiotic supplementation.

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Author Contributions: E.V.-M. carried out the search and selected studies for inclusion, collected data, contributed to statistical analysis and interpretation of results, and drafted the initial manuscript. M.P. collected data, contributed to statistical analysis and interpretation of results, and drafted the initial manuscript. G.C. collected data, contributed to statistical analysis and interpretation of results, and reviewed and revised the manuscript. F.M. supervised data collection, contributed to interpretation of results, and reviewed and revised the manuscript. B.K. contributed to interpretation of results and reviewed and revised the manuscript. E.V. conceptualized and designed the study, carried out the search and selected studies for inclusion, supervised data collection, carried out statistical analyses, contributed to interpretation of results, and reviewed and revised the manuscript. All authors approved the final manuscript as submitted.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Risk of bias assessment of studies included in meta-analysis.

Study	Random Sequence Generation	Allocation Concealment	Blinding of Participants and Personnel	Blinding of Outcome Assessment	Incomplete Outcome Data	Selective Reporting	Other Bias
Akar 2017 [49]	LR	UR	LR	LR	UR	UR	UR
Al Hosni 2011 [48]	UR	UR	LR	LR	LR	LR	LR
Costeloe 2016 [62]	LR	LR	LR	LR	LR	LR	LR
Demirel 2013 [50]	LR	LR	LR	LR	LR	LR	LR
Dilli 2015 [51]	LR	LR	LR	LR	LR	LR	LR
Fujii 2006 [52]	UR	UR	HR	UR	LR	UR	UR
Jacobs 2013 [53]	LR	LR	LR	LR	LR	LR	LR
Lin 2008 [54]	LR	LR	LR	LR	LR	UR	LR

Table A1. *Cont.*

Study	Random Sequence Generation	Allocation Concealment	Blinding of Participants and Personnel	Blinding of Outcome Assessment	Incomplete Outcome Data	Selective Reporting	Other Bias
Manzoni 2009 [55]	LR	UR	LR	LR	LR	LR	LR
Saengtawesin 2014 [56]	UR	UR	HR	HR	UR	UR	UR
Sari 2012 [57]	LR	LR	LR	LR	LR	LR	LR
Serce 2013 [58]	LR	LR	LR	LR	UR	UR	LR
Stratiki 2007 [59]	UR	UR	LR	LR	UR	UR	LR
Totsu 2014 [60]	LR	UR	LR	LR	LR	LR	LR
Underwood 2009 [61]	LR	LR	LR	LR	LR	LR	LR

HR: High risk of bias; LR: Low risk of bias; UR: Unclear risk of bias.

Table A2. Sensitivity analyses for BPD28: results of random effects meta-analyses when removing one study.

Removed Study	Statistics with Study Removed				
	MH RR	Lower Limit 95% CI	Upper Limit 95% CI	*Z*-Score	*p*
Demirel 2013 [50]	1.01	0.92	1.12	0.21	0.832
Dilli 2015 [51]	1.02	0.92	1.13	0.37	0.708
Fujii 2006 [52]	1.01	0.91	1.11	0.15	0.878
Jacobs 2013 [53]	1.04	0.86	1.25	0.38	0.703
Saengtawesin 2014 [56]	1.00	0.90	1.10	−0.09	0.931
Totsu 2014 [60]	0.99	0.89	1.10	−0.22	0.829
Stratiki 2007 [59]	1.01	0.91	1.11	0.11	0.916

BPD28: bronchopulmonary dysplasia, defined as oxygen dependence at 28 days of life; CI: confidence interval; MH RR: Mantel–Haenszel risk ratio.

Table A3. Sensitivity analyses for BPD36: results of random effects meta-analyses when removing one study.

Removed Study	Statistics with Study Removed				
	MH RR	Lower Limit 95% CI	Upper Limit 95% CI	*Z*-Score	*p*
Akar 2017 [49]	1.09	0.97	1.21	1.45	0.146
Al Hosni 2012 [48]	1.09	0.97	1.23	1.48	0.138
Costeloe 2016 [62]	1.07	0.93	1.22	0.94	0.350
Dilli 2015 [51]	1.08	0.97	1.21	1.43	0.153
Jacobs 2013 [53]	1.09	0.96	1.24	1.32	0.188
Lin 2008 [54]	1.04	0.93	1.17	0.69	0.488
Manzoni 2009 [55]	1.08	0.96	1.20	1.32	0.187
Sari 2012 [57]	1.07	0.96	1.20	1.25	0.213
Serce 2013 [58]	1.07	0.96	1.20	1.26	0.209
Totsu 2014 [60]	1.05	0.93	1.17	0.76	0.448
Underwood 2009 (CUL) [61] [1]	1.08	0.97	1.20	1.34	0.179
Underwood 2009 (PBP) [61] [1]	1.08	0.97	1.20	1.36	0.173

[1] Culturelle (CUL) and ProBioPlus DDS (PBP) were the names assigned by the authors to the probiotic preparations. BPD36: bronchopulmonary dysplasia, defined as oxygen dependence at 36 weeks post-menstrual age; CI: confidence interval; MH RR: Mantel–Haenszel risk ratio.

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CHAPTER V

MOTHER'S OWN MILK AND BRONCHOPULMONARY DYSPLASIA: A SYSTEMATIC REVIEW AND META-ANALYSIS

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Mother's Own Milk and Bronchopulmonary Dysplasia: A Systematic Review and Meta-Analysis

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Background: Bronchopulmonary dysplasia (BPD) is the most common complication of very preterm birth and can lead to lifelong health consequences. Optimal nutrition is a cornerstone in the prevention and treatment of BPD. In very preterm infants, mother's own milk (MOM) feeding is associated with lower risks of necrotizing enterocolitis, retinopathy of prematurity, and sepsis. Although several studies have shown that MOM may protect against BPD, a systematic analysis of the evidence has not been performed to date.

Methods: A comprehensive literature search was conducted using PubMed/MEDLINE and EMBASE, from their inception to 1 December 2017. Longitudinal studies comparing the incidence of BPD in preterm infants fed with exclusive MOM, MOM supplemented with preterm formula (PF), and/or exclusively fed with PF were selected. A random-effects model was used to calculate the Mantel Haenszel risk ratio (RR) and 95% confidence interval (CI).

Results: Fifteen studies met the inclusion criteria (4,984 infants, 1,416 BPD cases). Use of exclusive MOM feedings was associated with a significant reduction in the risk of BPD (RR 0.74, 95% CI 0.57–0.96, 5 studies). In contrast, meta-analysis could not demonstrate a significant effect on BPD risk when infants fed with more than 50% MOM were compared with infants fed with <50% MOM (RR 0.98, 95% CI 0.77–1.23, 10 studies) or when infants fed with MOM supplemented with PF were compared with infants fed with exclusive PF (RR 1.00, 95% CI 0.78–1.27, 6 studies). Meta-regression showed that differences in gestational age were a significant confounder of the effect of MOM.

Conclusion: To our knowledge, this is the first systematic review and meta-analysis that specifically evaluates the role of MOM on BPD. Our data indicate that MOM may reduce the incidence of BPD when used as an exclusive diet, but this result needs to

be interpreted with caution. We did not find the same difference in analyses with other dosages of MOM. Further studies adequately powered to detect changes in BPD rates and that adjust for confounders are needed to confirm the beneficial effects of MOM on BPD.

Keywords: mother's own milk, human milk, bronchopulmonary dysplasia, preterm formula, meta-analysis, systematic review, meta-regression

INTRODUCTION

Mother's own milk (MOM), fresh or frozen, is the normative standard for preterm infant feeding and nutrition (1–4). If MOM is unavailable despite significant lactation support, pasteurized donor human milk (DHM) is the recommended alternative over the use of bovine milk-based preterm formula (PF) (1–4). However, it is increasingly recognized that numerous MOM components which could contribute to its protective effects against adverse outcomes of prematurity are reduced or absent in DHM (5).

Bronchopulmonary dysplasia (BPD) is one of the most common complications of prematurity, and it predicts multiple adverse outcomes including chronic respiratory impairment and neurodevelopmental delay (6, 7). Optimal nutritional support is considered a cornerstone in the treatment/prevention of BPD (8). Recently, we performed a systematic review and meta-analysis on the effects of DHM on BPD (9). Meta-analysis of randomized controlled trials (RCTs) could not demonstrate that supplementation of MOM with DHM had a significant effect on BPD risk when compared to supplementation with PF. However, meta-analysis of observational studies showed a protective effect of DHM supplementation on BPD (9). Two very recent systematic reviews confirmed that the protective effects of human milk (i.e., MOM and/or DHM) on BPD are only observed in meta-analysis of observational studies (10, 11). Using the GRADE-system (12), the authors of these meta-analyses consider the evidence to be inconclusive.

Despite the important differences between DHM and MOM, the umbrella term "human milk" is frequently used to encompass both MOM and DHM, implying that the beneficial effects of MOM can be directly extrapolated to DHM (5, 13). Moreover, many of the studies and meta-analyses have compared PF feedings with various combinations of PF, MOM, and DHM. A recent meta-analysis evaluated the effects of MOM on retinopathy of prematurity (ROP) (14). This analysis excluded data on DHM and showed that the overall incidence of ROP was reduced among infants fed MOM compared with those fed PF. To the best of our knowledge, no systematic review has focused on the role of MOM in the development of BPD. The analysis of exclusive MOM vs. PF was beyond the scope of our previous study (9), and Miller et al. and Huang et al. did not study the effect of MOM separately from that of DHM (10, 11). Therefore, we aimed to conduct a systematic review and meta-analysis on the association between MOM/PF feeding and BPD development. The present meta-analysis does not include data on DHM.

METHODS

This study is a continuation of our previous review on DHM and BPD (9), and shares much of the same methodology. We expanded on the protocol of our earlier study, and specified the objectives, criteria for study inclusion, method for evaluating study quality, outcomes and statistical methodology. We report this study according to the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) (15). The PRISMA checklist for this report can be found in the **Supplementary Material**.

Data Sources and Search Strategy

We modified and expanded on the search strategy of our earlier review (9). We carried out a comprehensive literature search using PubMed/Medline and EMBASE, from their inception to March 1, 2018. The search strategy for PubMed used the following terms, including Mesh terms: (breast milk OR infant feeding OR mother's own milk) AND (preterm infant OR very low birth weight infant) AND (outcome OR bronchopulmonary dysplasia OR BPD) AND (observational study OR cohort study OR case-control). We used a similar strategy in EMBASE. We applied no language restrictions. We translated articles when needed. We included cohort and case-control studies in this review, as well as RCTs with an observational arm. Other types of studies were excluded, but when considered relevant, they were read to identify additional studies to include. We also used the "cited by" tool in Google Scholar and Web of Science to identify studies for inclusion. Moreover, we included articles which we came across in the elaboration of our earlier review (9).

Eligibility Criteria and Study Selection

We included studies if they were original cohort or case-control studies, which examined very preterm (gestational age, GA <32 weeks) or very low birth weight (VLBW, BW <1,500 g) infants receiving either MOM or PF, and which included at least two groups divided according to feeding policy. Only full-length published studies were considered for inclusion. Studies were included if they reported results on the incidence of BPD. We defined BPD as oxygen dependence at 28 days of life (BPD28) or as oxygen dependence at 36 weeks adjusted gestational age (BPD36). Studies were excluded if the group receiving MOM or PF also received DHM. Two reviewers (EV-M, EV) independently screened the results of the searches, and included studies according to the inclusion criteria using EndNote (RRID:SCR_014001), using the methodology of Bramer et al. (16). Studies on which reviewers disagreed for inclusion were

identified, and discrepancies were resolved through discussion or by consulting the other authors.

Data Extraction

We collected the following information per study: citation information, study design, number of patients, number of centers, location of study, inclusion and exclusion criteria, patient characteristics (GA, BW), type of feeding received (MOM, PF, combination of MOM and PF, and type of fortifier), and incidence of BPD per group. Two researchers (EV-M, EV) extracted the data using an Excel sheet designed for this review. We resolved discrepancies in data extraction through discussion, or by consulting the other authors. Another researcher (MP) independently validated the accuracy of the data extracted.

Assessment Risk of Bias

Two researchers (EV-M, MP) assessed the risk of bias in included studies. We used the Newcastle-Ottawa Scale (NOS) for quality assessment of cohort and case-control studies. The NOS is used to assign a score to studies on selection (0–4 points), comparability (0–2 points), and outcome/exposure (0–3 points), for a total score of up to 9 points. Discrepancies were resolved through discussion.

Statistical Analysis

We used Comprehensive Meta-Analysis V3.0 software (RRID:SCR_012779) to combine and analyze studies. The Mantel Haenszel (MH) risk ratio (RR) for BPD with 95% confidence interval (CI) was calculated in each study. Due to anticipated heterogeneity, we used a random-effects model to combine studies. This model accounts for heterogeneity between and within studies and it does not assume that "true" effect sizes are identical across studies. Subgroup analyses were conducted according to the mixed-effects model (17). In this model a random-effects analysis is used to combine studies within each subgroup, and a fixed-effect model is used to combine subgroups and yield the overall effect. The model does not assume study-to-study variance (tau-squared) to be the same for all subgroups. We assessed statistical heterogeneity using the Cochran's Q statistic, and the I^2 statistic which is derived from it. We planned to evaluate the risk of publication bias through visual inspection of the funnel plot and with Egger's regression test (18). We decided *a priori* to analyze the effect of GA as a confounding factor, by analyzing the mean difference in this covariate between groups, and through subgroup analysis, by removing studies with large differences in GA from analysis. We decided to use the group with the higher MOM intake as the reference group in all our analyses. We carried out sensitivity analyses by removing one study from analyses at a time. We used an $\alpha = 0.05$ for statistical significance ($\alpha = 0.10$ for statistical heterogeneity).

RESULTS

After removing duplicates, our comprehensive search found 965 articles, of which we identified 84 as potentially relevant, and 15 met our inclusion criteria (19–33) after full-text review. The PRISMA search diagram is shown in **Figure 1**. The characteristics of the included studies are shown in **Supplementary Table 1**. Fourteen included studies were observational cohorts, of which seven were prospective (20, 21, 23, 25, 30, 31, 33) and seven were retrospective (19, 22, 24, 26, 28, 29, 32), and one study was a retrospective case-control (27). One study was excluded from meta-analysis because it did not group by type of feeding (22). We divided studies according to the proportion of feeding that was MOM or PF in each group, and we made three comparisons for analysis: (1) Exclusive MOM vs. Any PF; (2) Mainly MOM vs. Mainly PF; (3) Any MOM vs. Exclusive PF.

Quality Assessment

Three studies scored six points on the NOS, 10 studies scored seven points, and two studies scored the maximum of 9 points. We downgraded studies in quality for not adjusting for confounders ($k = 13$), for excluding infants who were lost to follow-up ($k = 2$) and for not defining BPD clearly ($k = 1$).

Exclusive MOM vs. Any PF

Five studies (19–21, 27, 30) compared infants who received a diet of exclusive MOM to infants who received MOM and any supplementation with PF. Meta-analysis of these studies found that the exclusive MOM group had a reduced risk of BPD (RR 0.74, 95% CI 0.57–0.96, $p = 0.021$, **Figure 2**). When we excluded the study of Fewtrell et al. which used a different definition of BPD (BPD28), the effect of MOM on BPD remained significant (RR 0.71, 95% CI 0.54–0.93 $p = 0.014$). Sensitivity analysis showed that removing the study of Madore et al. (27) or the study of Schanler et al. (30) made the overall association no longer significant (**Supplementary Figure 1**).

When we analyzed the difference in GA between groups, meta-analysis did not find a significant difference (MD GA −0.06 weeks, 95% CI −0.38–0.25, $p = 0.689$, **Supplementary Figure 2**). None of the included studies had a mean difference in GA between groups which was larger than 0.3 weeks.

Out of the five studies which had an exclusive MOM group, three studies (19–21) also provided data on a group of infants receiving exclusive PF. We carried out a subgroup analysis of these studies. When pooled, meta-analysis could not find a significant difference in BPD risk between groups (RR 1.08, 95% CI 0.63–1.87, $p = 0.770$ **Figure 3**). When we excluded the study of Fewtrell et al., which defined BPD as BPD28 instead of BPD36, the effect of MOM did not change in significance (RR 1.10, 95% CI 0.58–2.09, $p = 0.777$). When we removed the study of Assad et al. for having a MD in GA (of 1.5 weeks) between groups ≥0.5 weeks, the results did not change in significance (RR 0.88, 95% CI 0.57–1.37, $p = 0.583$). An analysis on the difference in GA between groups did not find a significant difference overall (MD −0.41 weeks, 95% CI −1.19–0.36, $p = 0.295$, **Supplementary Figure 3**).

Mainly MOM vs. Mainly PF

Ten studies compared infants receiving mainly MOM vs. infants receiving mainly PF. We included studies which had stricter criteria for comparison (i.e., exclusive MOM vs. exclusive PF) in this analysis as well. Meta-analysis could not find a significant difference in risk of BPD between the mainly MOM and the

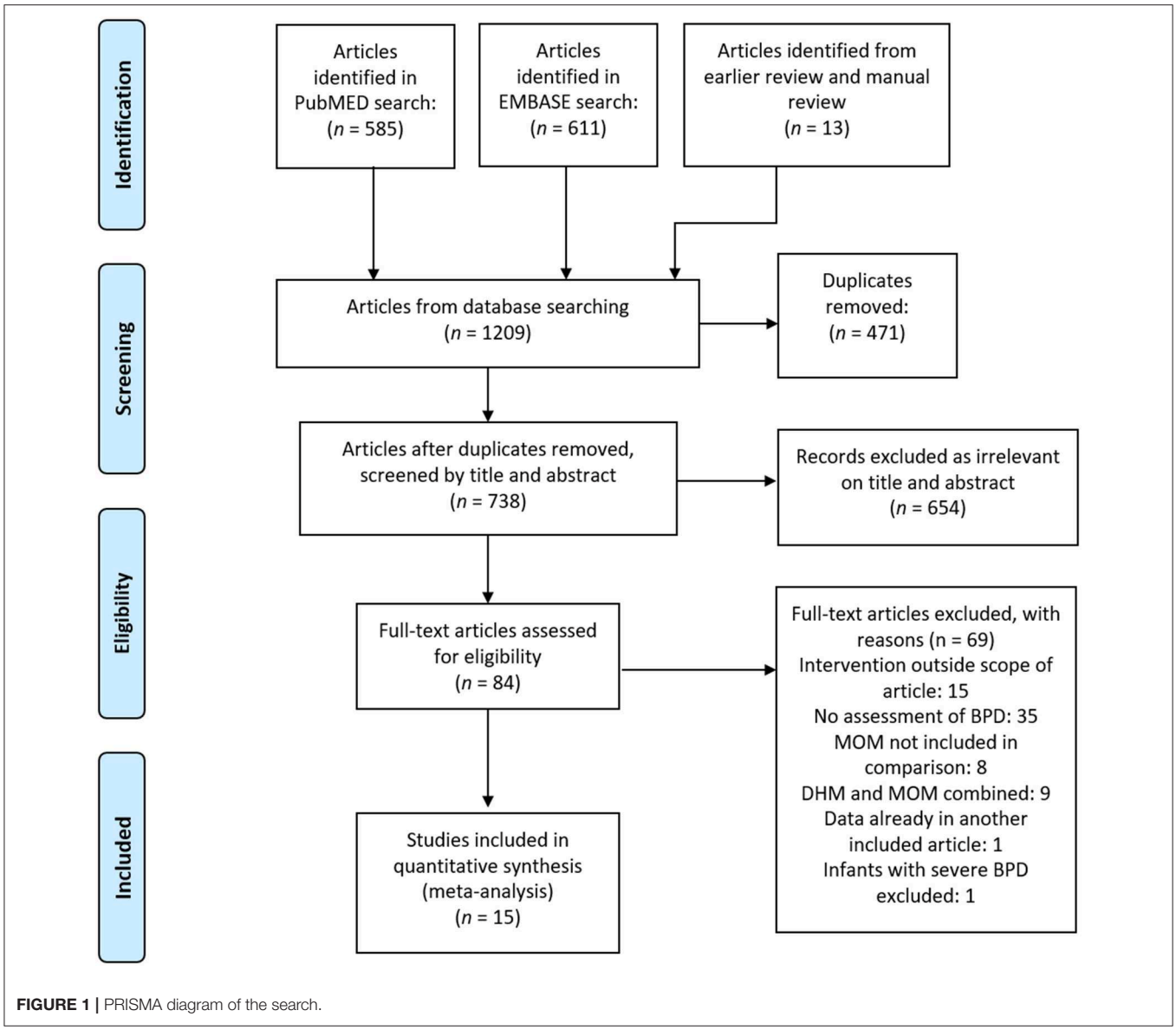

FIGURE 1 | PRISMA diagram of the search.

mainly PF group (RR 0.98, 95% CI 0.77–1.23, $p = 0.833$, **Figure 4**). When we excluded the study of O'Connor et al. for using a definition of BPD at 28 days of life instead of at 36 weeks PMA, the effect of MOM on BPD development remained non-significant (RR 0.99, 95% CI 0.75–1.31, $p = 0.938$). Excluding any study one at a time did not change the significance of the effect of MOM on BPD (**Supplementary Figure 4**).

We used meta-analysis to study the differences in GA between the MOM and PF groups in each study. Meta-analysis found no significant MD in GA when pooling all studies together (MD −0.31 weeks, 95% CI −0.78–0.17, $p = 0.204$, **Supplementary Figure 5**). However, individual studies showed significant differences in GA between groups, and the heterogeneity was very high ($p < 0.001$, $I^2 = 92.1\%$), which indicated that GA could be a significant confounder. When we used subgroup analysis to exclude studies where the difference in GA between groups was larger than 0.5 weeks, we were left with 6 studies, but the effect of MOM on BPD did not change significantly (RR 0.99, 95% CI 0.82–1.18, $p = 0.890$).

We used meta-regression to explore the role of GA in potentially modifying the effect of MOM on BPD development. Meta-regression found a significant association between MD in GA and the risk of BPD in the MOM group (Coefficient: −0.59, 95% CI −0.95 to −0.23, $p = 0.001$, $R^2 = 1.00$, **Figure 5**). This indicates that in studies where the MOM group had a higher risk of BPD, this group was also more premature than the PF group, and in studies where the MOM group had a lower risk of BPD, this group was also more mature than the PF group.

Any MOM vs. Exclusive PF

Six studies (19, 20, 23–25, 33) compared infants who received any MOM to infants who received exclusive PF. We also included

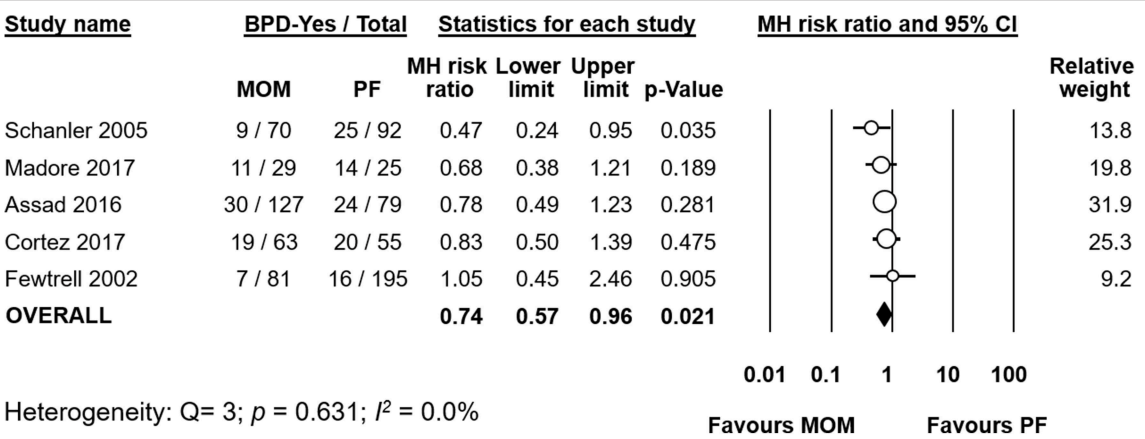

FIGURE 2 | Meta-analysis of exclusive MOM vs. any PF and risk of BPD. MOM, mother's own milk; PF, preterm formula; BPD, bronchopulmonary dysplasia; CI, confidence interval; MH, Mantel-Haenszel.

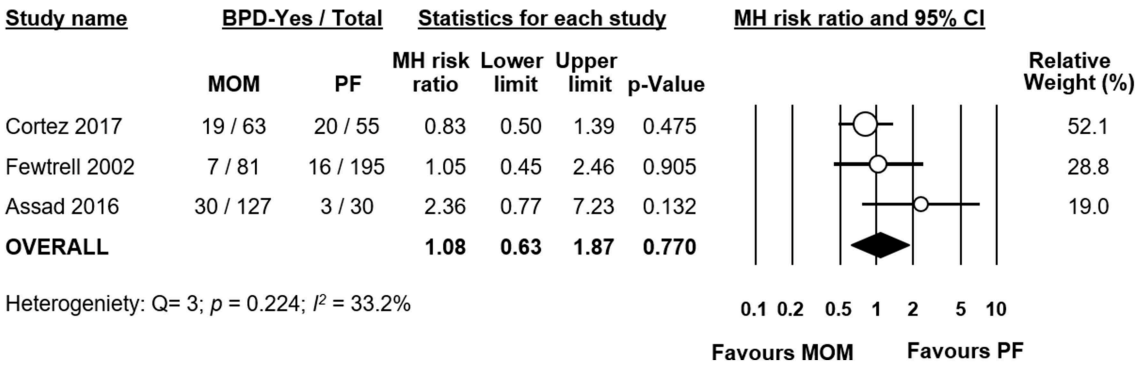

FIGURE 3 | Meta-analysis of exclusive MOM vs. exclusive PF and risk of BPD. MOM, mother's own milk; PF, preterm formula; BPD, bronchopulmonary dysplasia; CI, confidence interval; MH, Mantel-Haenszel.

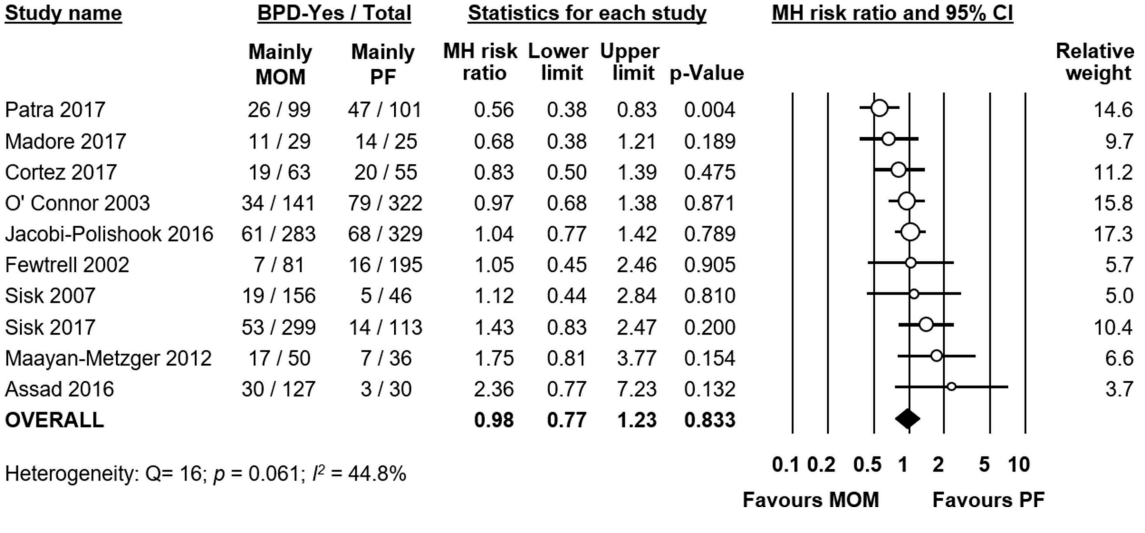

FIGURE 4 | Meta-analysis of mainly MOM vs. mainly PF and risk of BPD. MOM, mother's own milk; PF, preterm formula; BPD, bronchopulmonary dysplasia; CI, confidence interval; MH, Mantel-Haenszel.

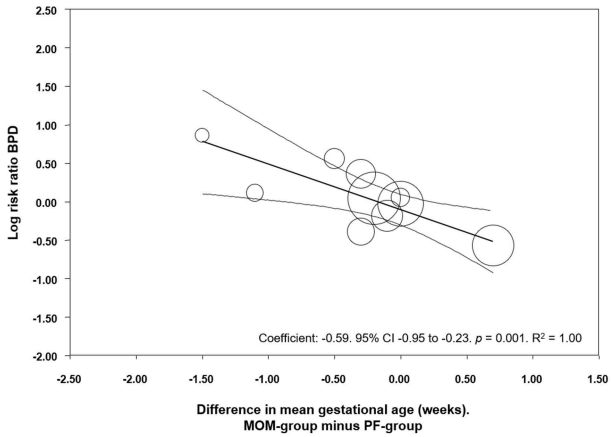

FIGURE 5 | Meta-regression of MD in GA between the MOM and PF group, and risk of BPD. CI, confidence interval; BPD, bronchopulmonary dysplasia.

studies in this comparison where the infants of the MOM group received larger proportions of MOM (e.g., infants receiving mainly MOM or exclusive MOM). Meta-analysis could not find a significant effect of any MOM on the risk of developing BPD (RR 1.00, 95% CI 0.78–1.27, $p = 0.975$, **Figure 6**). When we removed the study of Hylander et al. from the analysis, which did not clarify their definition of BPD, the effect of any MOM remained non-significant (RR 1.07, 95% CI 0.80–1.42, $p = 0.665$). Removal of any one study did not affect the significance of the results (**Supplementary Figure 6**).

Meta-analysis found that there was a significant difference in mean GA between the any MOM group and the exclusive PF group, with the infants receiving any MOM being born earlier (MD −0.50 weeks, 95% CI −0.99 to −0.01, $p = 0.045$, **Figure 7**). Removing studies where the groups differed by more than 0.5 weeks in GA left us with three studies (20, 24, 33), but the effect of MOM on BPD remained non-significant (RR 0.93, 95% CI 0.82–1.05, $p = 0.236$, **Supplementary Figure 7**).

Publication Bias

We tested the three comparisons for publication bias (**Supplementary Figure 8**), but neither visual inspection of the funnel plot nor Egger's regression test could find significant evidence of publication bias. A small number of studies made the analyses on "Exclusive MOM vs. Any PF" and "Any MOM vs. Exclusive PF" inconclusive (**Supplementary Figure 8**).

Adjusted Data

Two studies (23, 26) reported data on BPD incidence that was adjusted for confounders. Furman et al. (23) reported incidence of BPD by amount of maternal milk received, and they adjusted for several confounders (BW, sex, and ethnicity). They found no significant difference in BPD risk for varying levels of MOM intake, compared to receiving exclusive PF. Maayan-Metzger et al. (26) used logistic regression to adjust for confounders including GA, BW and sex. They found that receiving only or mainly MOM, compared to receiving only or mainly PF, did not significantly affect the risk of developing BPD. They found the same result in the subgroup of infants with GA 24–28 weeks.

Other Studies

One study (22) did not group according to MOM or PF intake. Instead they compared infants with BPD to infants without BPD and studied median intake of MOM in the first 6 weeks of life. In their study infants were given MOM, supplemented by PF when necessary. They found infants with BPD had a significantly lower median daily MOM intake compared to infants without BPD (2.3 mL/kg/d vs. 10.8). The protective effect on BPD of a higher MOM intake at 42 days remained after adjustment for confounding factors (RR 0.98, 95% CI 0.96–0.99, $p = 0.030$).

DISCUSSION

RCTs are widely regarded to provide the highest degree of evidence (34). However, the random allocation of infants to a group receiving PF instead of MOM is not ethical and, therefore, evidence must be based on observational studies (14, 35). To our knowledge, this is the first systematic review and meta-analysis that specifically evaluated the role of MOM on BPD. We found that MOM reduced the risk of developing BPD but only when used as an exclusive diet. In contrast, meta-analysis could not find significant changes in BPD risk when comparing infants fed mainly with MOM with those fed mainly with PF, or when comparing any MOM vs. exclusive PF.

The reduction of BPD rates when MOM is used as an exclusive diet may have various explanations. The major pathogenetic clue of BPD is the arrest in the alveologenesis and vasculogenesis of the lung due to very preterm birth (36). Superimposed inflammatory events complete this detrimental picture (37, 38). Prenatally, in the setting of chorioamnionitis, the overwhelming inflammatory cascade may interfere with lung development (37). Postnatally, the intensive care support needed by very preterm infants, including resuscitation, mechanical ventilation, and oxygen administration, carries a high grade of inflammation to the immature lung, leading to the establishment of BPD (38). When postnatal infections occur, the incidence of BPD sharply increases (39–41). Finally, inadequate nutrition can further worsen BPD (42). MOM may reduce the incidence of BPD thanks to nutritional and bioactive components, counteracting oxidative stress (43), inflammation (44, 45), and nutritional flaws involved in the BPD pathogenesis (46, 47). In addition, MOM may also impact the risk of BPD indirectly by reducing the incidence of necrotizing enterocolitis (NEC) and late-onset sepsis (LOS).

Due to the observational character of the studies included in the meta-analysis, the MOM and PF groups may differ in a number of maternal and infant characteristics which may affect the development of BPD. Previous studies have shown associations between characteristics such as ethnicity, socioeconomic status, maternal education, pregnancy hypertensive disorders, smoking during pregnancy, GA, BW, infant sex, Apgar score, or respiratory distress syndrome, and rate of MOM feeding in preterm infants (32, 48–54). We evaluated one possible major confounder: difference in GA between groups. GA played a role in modifying the association

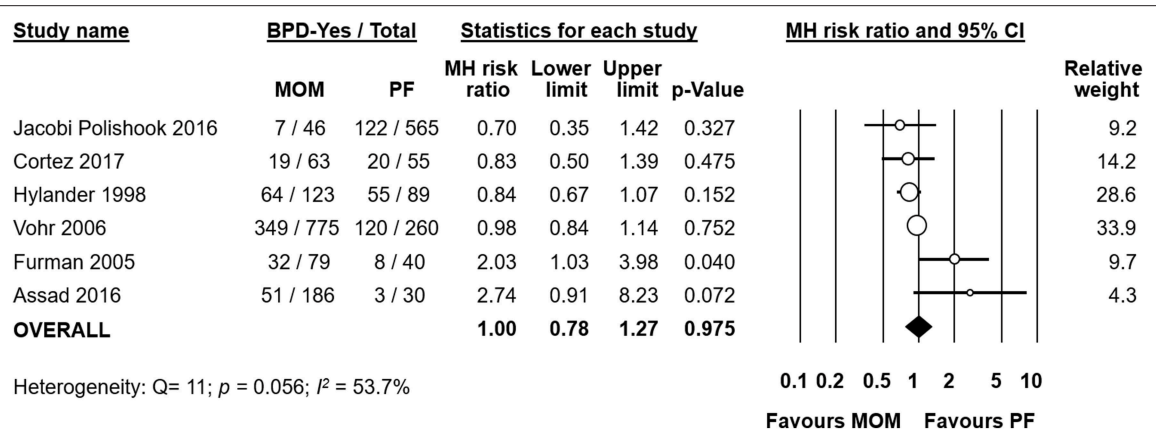

FIGURE 6 | Meta-analysis of Any MOM vs. Exclusive PF and risk of BPD. MOM, mother's own milk; PF, preterm formula; BPD, bronchopulmonary dysplasia; CI, confidence interval; MH, Mantel-Haenszel.

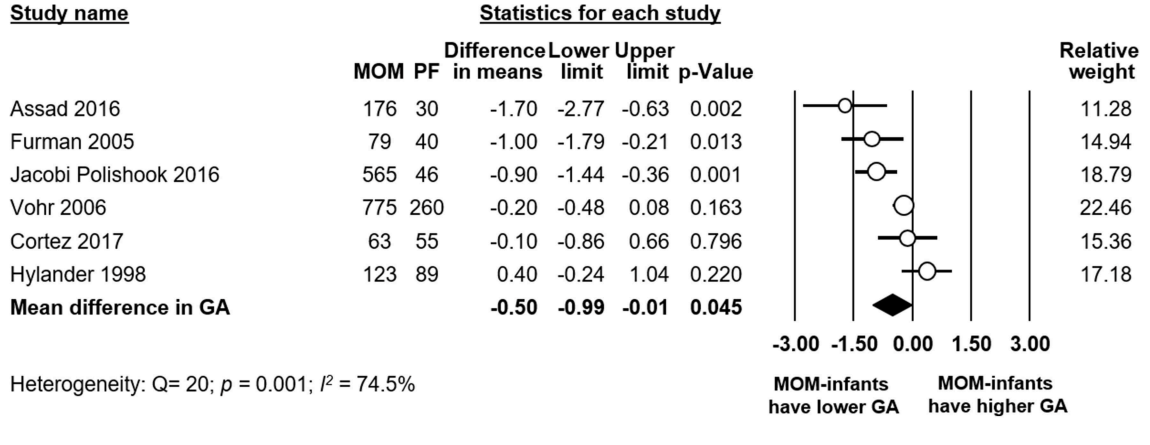

FIGURE 7 | Meta-analysis of Any MOM vs. Exclusive PF, mean difference (MD) in gestational age (GA) between groups. MOM, mother's own milk; PF, preterm formula.

between MOM and BPD, as we have shown through meta-regression and sub-group analyses. This is relevant since the incidence of all the complications of prematurity, including NEC, LOS, and BPD, is inversely related to GA. In studies which compared mainly MOM vs. mainly PF, the comparison that included the highest number of studies, meta-regression showed a significant correlation between difference in GA and the protective effect of MOM on BPD (**Figure 5**). In other words, in the studies where the mainly MOM group had a higher BPD risk, this group was also more premature than the mainly PF group. Interestingly, in the comparison where we found a significant positive result (exclusive MOM vs. any PF), the differences in GA between groups were small. This suggests that the protective effects of exclusive MOM are not affected by GA as confounder in this analysis.

Several studies have reported that the effects of human milk in reducing the incidence of adverse outcomes of prematurity are dose-dependent (14, 23, 31, 55–58). It has been suggested that at least 50% of the infant's total enteral intake should be MOM in order to achieve a decreased incidence of NEC (13). With regards to BPD, Patel et al. have shown a 9.5% reduction in the risk of BPD for each 10% increase in MOM received from birth to 36 weeks PMA. This may generate a reduction in BPD risk up to 63% when an exclusive MOM diet is compared with an exclusive PF diet (55). Surprisingly, the present meta-analysis could not demonstrate a different rate of BPD in infants fed exclusive MOM when compared with infants fed exclusive PF. However, this analysis was based on only three studies (**Figure 3**). Moreover, in one of the studies the infants in the PF group had a markedly higher GA (1.5 weeks) than the infants of the MOM group. To date, there are no exact limits set in the amount of MOM that would produce benefits in terms of BPD reduction (59). The studies that we analyzed documented a high variability of MOM amount in their study groups. Since the relation between MOM and BPD, may not be as direct as for NEC and LOS, it is possible that higher minimum amounts of MOM may be needed to detect significant differences. In addition, the conditions of storage and the use of fresh, refrigerated, frozen, or deep-frozen MOM may affect the antioxidant as well as other biological properties of MOM (60).

CONCLUSION

Our data indicate that MOM may reduce the incidence of BPD when used as an exclusive diet, but this result needs to be interpreted with caution. We did not find the same difference in analyses with other dosages of MOM, which may be related to the high variability in the available studies and the dose-dependent beneficial effects of MOM. It may also be due to differences in GA between infants who receive MOM and infants who receive PF, which we found had modified the protective effects of MOM against BPD. Moreover, there may be other differences in infant and maternal characteristics that play a role and which we could not account for. Further studies, adequately powered to detect changes in BPD rates, and that adjust for the different characteristics of infants who receive MOM and PF are needed to confirm the beneficial effects of MOM on BPD.

AUTHOR CONTRIBUTIONS

EV-M designed the study, performed the search, selected studies for inclusion, collected data, performed the statistical analyses, contributed to the interpretation of the results, and drafted the initial manuscript. MP revised collected data, contributed to statistical analysis and interpretation of the results, and reviewed and revised the manuscript. GC contributed to interpretation of results and reviewed and revised the manuscript. FM contributed to interpretation of results and reviewed and revised the manuscript. EV conceptualized and designed the study, performed the search, selected the studies for inclusion, supervised data collection, contributed to the statistical analyses and interpretation of the results, and reviewed and revised the manuscript. All authors approved the final manuscript as submitted.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2019.00224/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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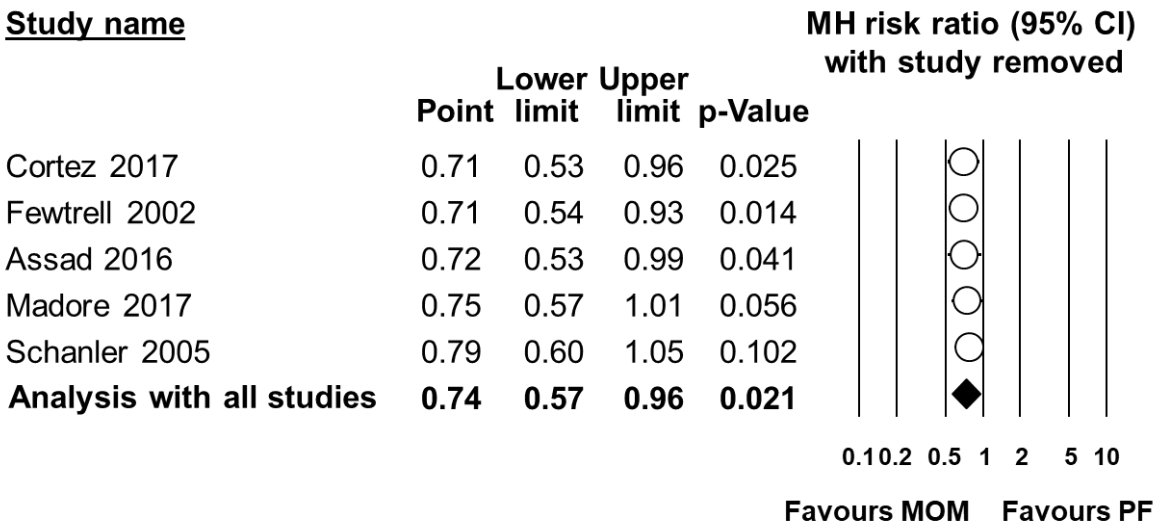
Supplementary Material

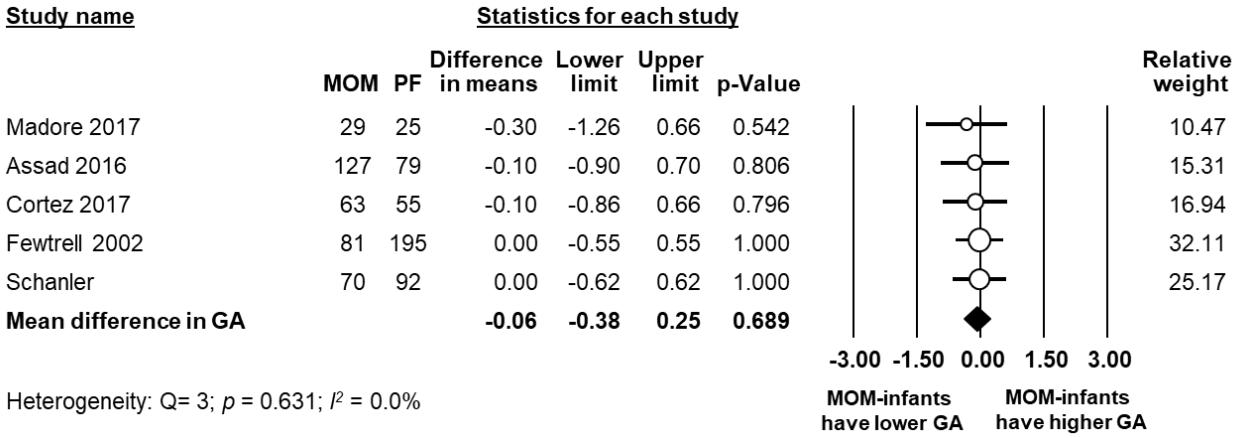
Mother's Own Milk and Bronchopulmonary Dysplasia: A Systematic Review and Meta-Analysis

Eduardo Villamor-Martínez, Maria Pierro, Giacomo Cavallaro, Fabio Mosca, Eduardo Villamor

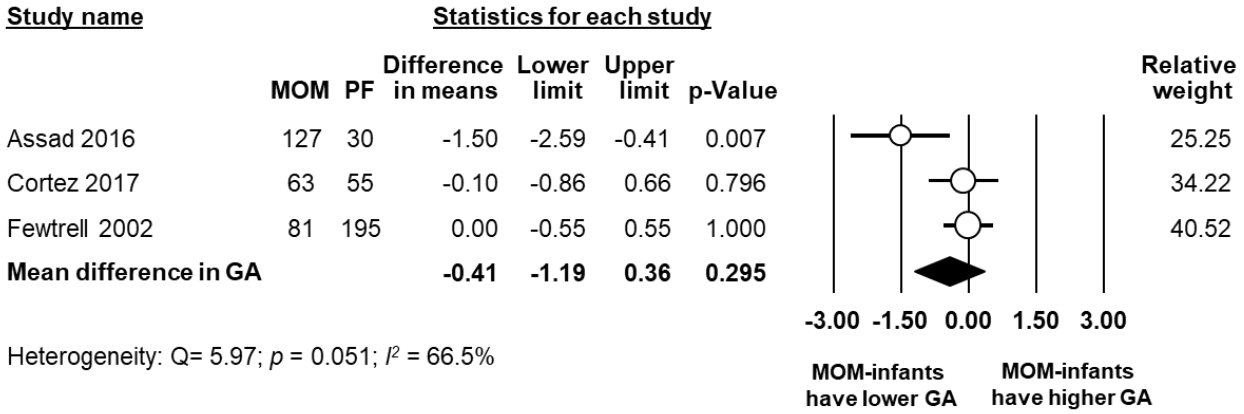
1 Supplementary Figures and Tables



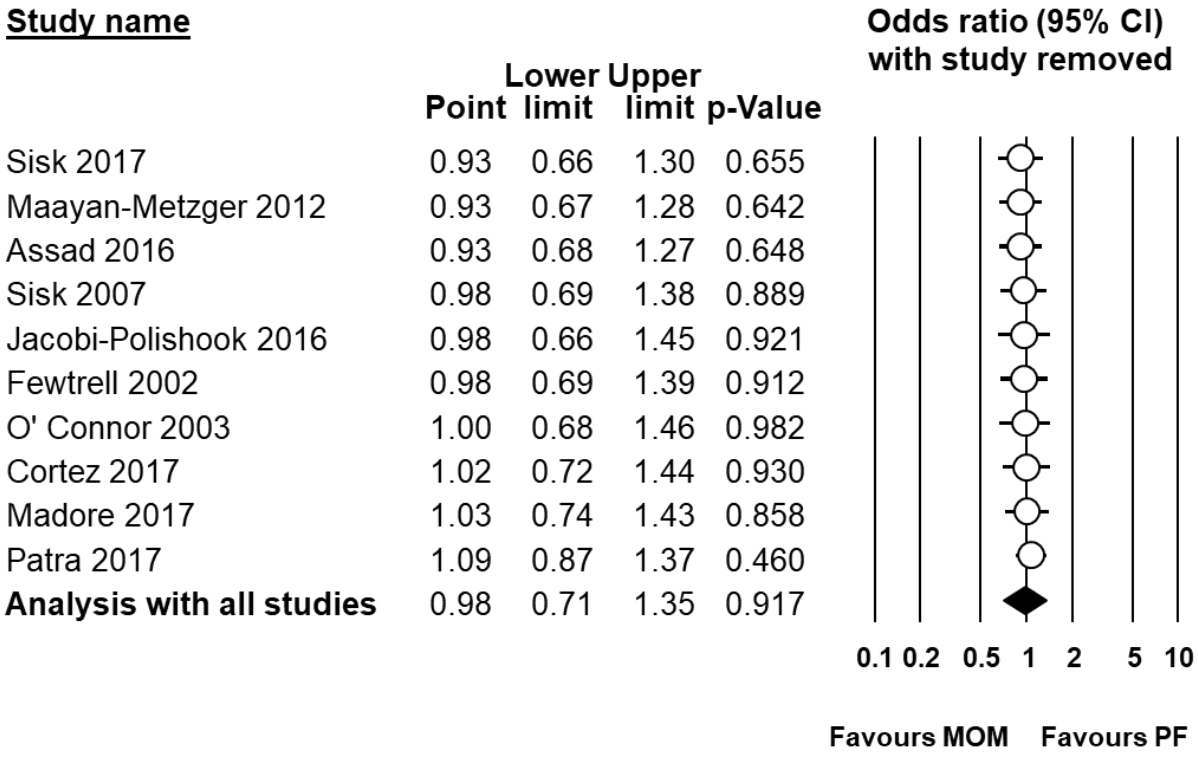
Supplementary Figure 1. Meta-analysis of Exclusive MOM vs. Any PF, effect of removing one study each time. MOM: mother's own milk; PF: preterm formula; MH: Mantel-Haenszel.



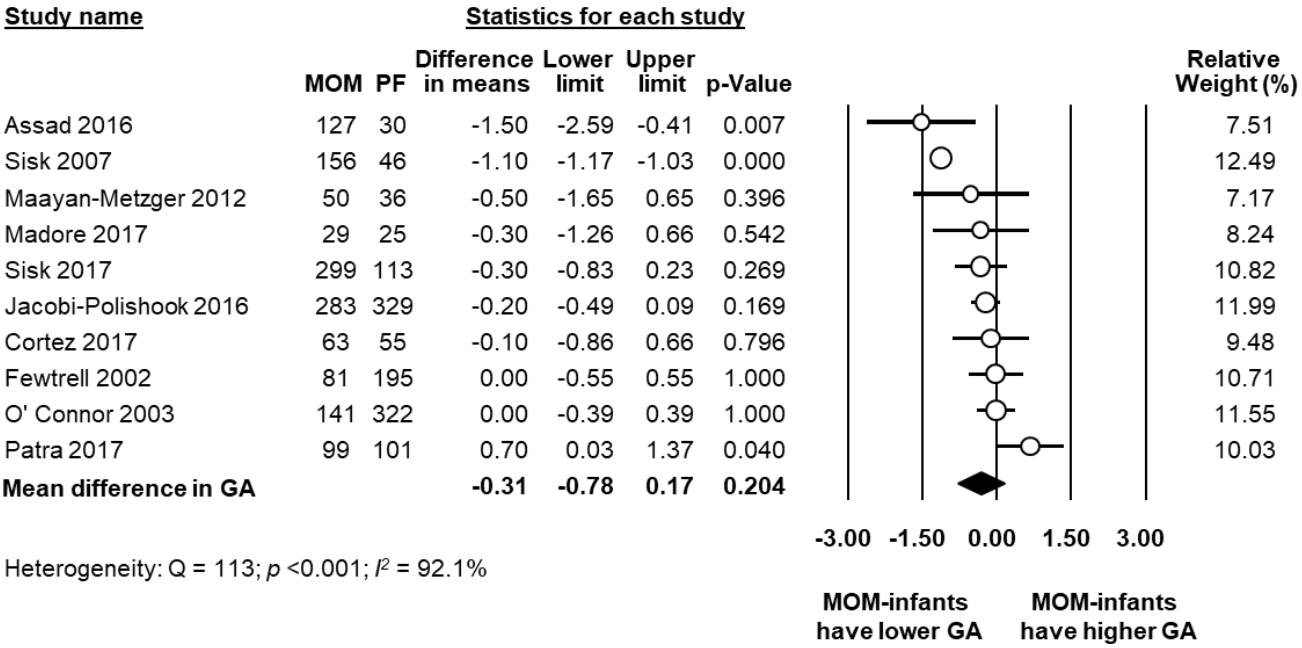
Supplementary Figure 2. Meta-analysis of Exclusive MOM vs. Any PF, mean difference (MD) in gestational age (GA) between groups. MOM: mother's own milk; PF: preterm formula.



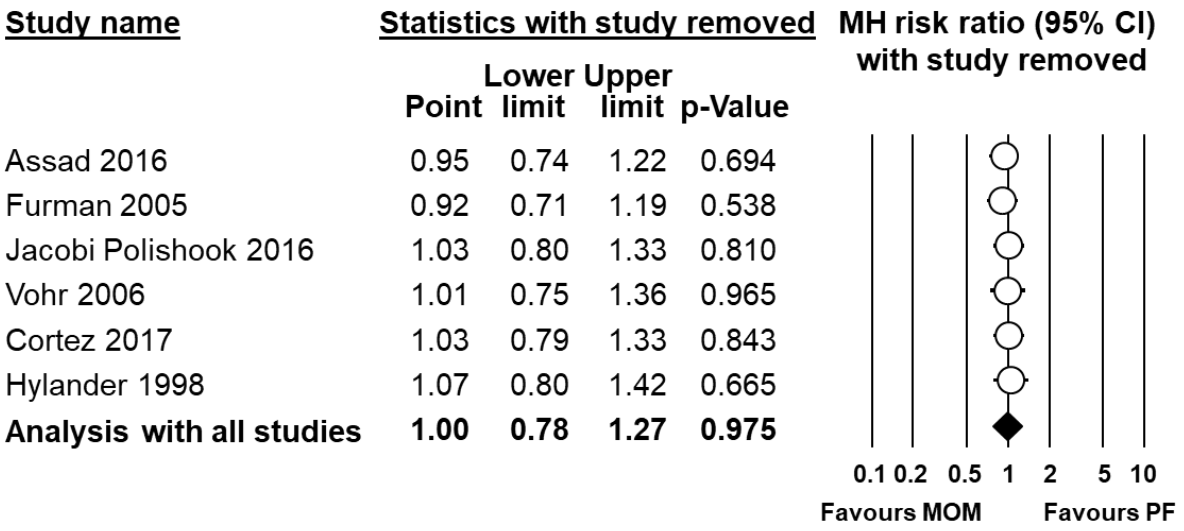
Supplementary Figure 3. Meta-analysis of Exclusive MOM vs. Exclusive PF, mean difference (MD) in gestational age (GA) between groups. MOM: mother's own milk; PF: preterm formula.



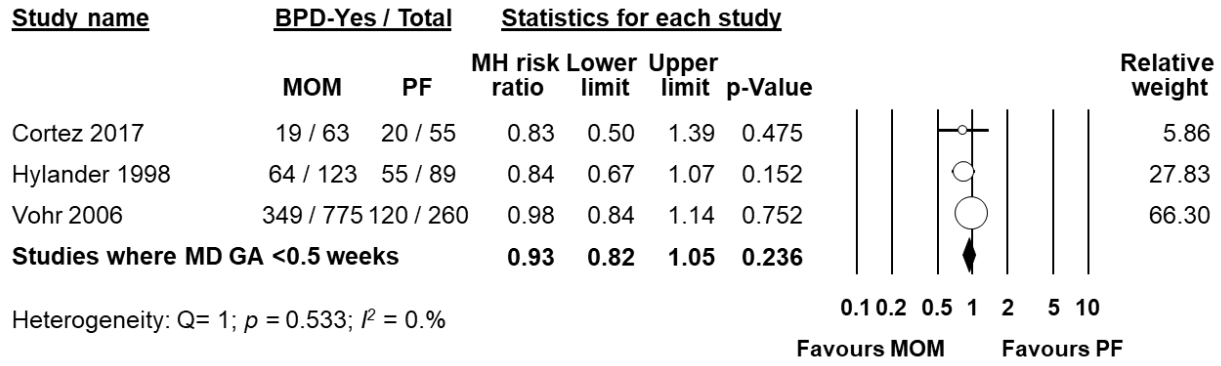
Supplementary Figure 4. Meta-analysis of Mainly MOM vs. Mainly PF, effect of removing one study each time. MOM: mother's own milk; PF: preterm formula.



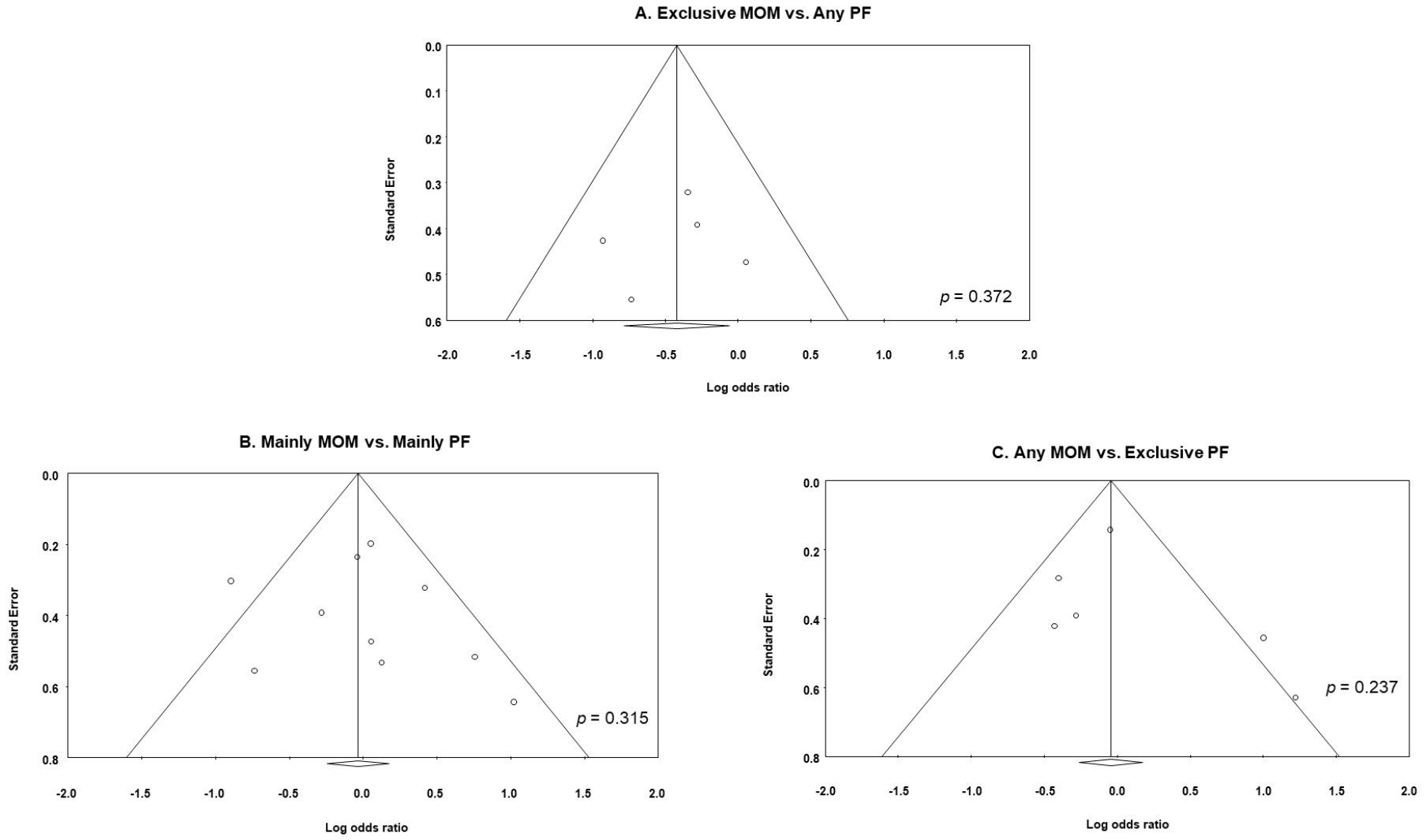
Supplementary Figure 5. Meta-analysis of Meta-analysis of Mainly MOM vs. Mainly PF, mean difference (MD) in gestational age (GA) between groups. MOM: mother's own milk; PF; preterm formula.



Supplementary Figure 6. Meta-analysis of Any MOM vs. Exclusive PF, effect of removing one study each time. MOM: mother's own milk; PF: preterm formula.



Supplementary Figure 7. Meta-analysis of Any MOM vs. Exclusive PF and risk of BPD, only including studies where the mean difference (MD) in gestational age (GA) was <0.50 weeks. MOM: mother's own milk; PF: preterm formula; BPD: bronchopulmonary dysplasia; CI: confidence interval.



Supplementary Figure 8. Funnel plot analysis of publication bias. MOM: mother's own milk; PF: preterm formula.

Supplementary Table 1. Synoptic table of characteristics of all included studies.

First author, year	Location	Study design	Primary outcome(s)	Respiratory outcome(s)	Groups	Inclusion criteria	Study duration	Fortified	Patients (centers)	Comments
Assad et al. 2016	Baltimore, MD, USA	Retrospective cohort	Feeding intolerance, time to full feeds, NEC, length of stay, weight gain, cost of hospitalization	BPD36	- MOM + DHM + DHM-based fortifier; - MOM + bovine fortifier; - MOM + bovine fortifier + PF; - Exclusive PF	GA <29 weeks and/or BW ≤1500g	Until discharge	DHM fortifier in exclusive human diet, bovine fortifier in bovine groups	293 (1)	
Cortez et al. 2017	Jacksonville, FL, USA	Prospective cohort	NEC, infection	BPD36	- Received >95% MOM - Received >95% PF	GA <33 weeks	36h of age until discharge	Bovine fortifier added to MOM	118 (3)	
Fewtrell et al. 2002	London, UK	RCT (for our exposure: prospective cohort)	MDI and PDI at 18 months, Passamanick and Sherrard's Developmental Screening Inventory at 9 months PMA	BPD28	- Exclusive MOM - Exclusive PF	BW <1750g, GA <37 weeks	Until discharge	Not specified	283 (3)	
Fonseca et al. 2017	Porto Alegre, Brazil	Retrospective cohort	Amount of MOM received by BPD patients vs. non-BPD infants	BPD28	MOM + PF in varying amounts, study compares MOM-intake by BPD patients vs. non-BPD patients	GA <32 weeks and/or BW <1500g	6 weeks or discharge	Bovine fortifier added to MOM	425 (1)	BPD was inversely associated with amount of MOM, even after controlling for confounders
Furman et al. 2003	Cleveland, OH, USA	Prospective cohort	Neonatal morbidity, length of hospitalization	BPD36	- Exclusive PF - 1-24 mL/kg/d MOM + PF - 25-49 mL/kg/d MOM + PF - ≥50 mL/kg/d MOM + PF	GA <33 weeks, BW <1500g	4 weeks	Bovine fortifier added to MOM	119 (1)	MOM-intake divided over 4 groups by volume of MOM (in mL/kg/d), 0, 1-24, 25-49 and ≥50.
Hylander et al. 1998	USA	Retrospective cohort	Infection (culture proven sepsis, NEC and/or pneumonia)	BPD?	- Any MOM - Exclusive PF	BW <1500g.	Until discharge	Bovine fortifier added to MOM	212 (1)	
Jacobi-Polishook et al. 2016	Boston, MA, USA	Prospective cohort	Neurodevelopmental outcome	BPD36	- Exclusive PF	GA ≤33 weeks	40 weeks corrected age	Bovine fortifier added to MOM	611 (5)	

First author, year	Location	Study design	Primary outcome(s)	Respiratory outcome(s)	Groups	Inclusion criteria	Study duration	Fortified	Patients (centers)	Comments
			(Bayley II) at 18 months		- MOM + PF, divided into four quartiles based on MOM-intake					
Maayan-Metzger et al. 2012	Tel Aviv, Israel	Retrospective cohort	Short-term neonatal outcomes	BPD28	- Only and mainly MOM - Only and mainly PF	GA ≤32 weeks	Until discharge	Bovine fortifier added to MOM	360 (1)	Data taken from large prospective, randomized controlled trial, designed to assess possible benefits of supplementing formula with arachidonic and docosahexeanoic acid
Madore et al. 2017	Boston, MA, USA	Retrospective case-control	Growth, neurodevelopment	BPD36	- Exclusive MOM; - PF>50%	BW <1000g	First month of life	Bovine fortifier added to MOM	81 (1)	
O' Connor et al. 2003	Toronto, Canada	Retrospective cohort	Growth and development outcomes	BPD28	- >80% MOM + PF - ≥50% MOM + PF - <50% MOM +PF - MOM + >80% PF	GA <33 weeks	Until term corrected GA.	Bovine fortifier added to MOM	463 (9)	
Patra et al. 2017	Chicago, IL, USA	Retrospective cohort	Neurodevelopmental outcome	BPD36	MOM + PF, split into 5 quintiles based on proportion of MOM as total intake	GA <35 weeks, BW <1500g	Until 20 months corrected GA	Bovine fortifier added to MOM	251 (1)	Study uses same sample as Patel et al. 2017. We used the data from this article for meta-analysis.
Schanler et al. 2005	Houston, TX, USA	RCT (for our exposure: prospective cohort)	Late onset sepsis and/or NEC	BPD36	- Exclusive MOM - MOM + PF	GA ≤29 weeks	19 days or discharge	Bovine fortifier added to MOM	243 (1)	
Sisk et al. 2007	Winston-Salem, NC, USA	Prospective cohort	NEC	BPD36	- MOM >50% - PF >50%	BW 700-1500g	Until discharge	Bovine fortifier added to MOM	202 (1)	
Sisk et al. 2017	Winston-Salem, NC, USA	Retrospective cohort	NEC stage ≥2	BPD36	- MOM ≥50% - DHM ≥50% - PF ≥50%	GA ≤32w and BW ≤1500g	Within 2 hours of birth until 34 weeks PMA	Bovine fortifier added to MOM and to DHM	563 (1)	
Vohr et al. 2006	15 centers, USA	Prospective cohort	Neurodevelopmental outcome at 18 months	BPD36	- Any MOM + PF - Exclusive PF	BW ≤1000g	Until discharge	Bovine fortifier added to MOM (varied per center)	1035 (15)	

BPD28: Defined as supplemental oxygen after day 28 of life. BPD36: Defined as supplemental oxygen at 36 weeks corrected GA. BPD28-36: defined as supplemental oxygen after day 28 of life, or at 36 weeks corrected GA age. BPD?: No definition of bronchopulmonary dysplasia given. MOM: Mother's own milk. PF: Preterm formula. DHM: Donor human milk

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	1
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	2
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	2
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	2,3
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	3
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	3
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	3
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	3
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	3
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	3
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	3
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	4
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	4

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	4
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	4
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	4
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	4, Supplementary Table 1
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	4
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	4-7
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	4-7, Supplementary Figures
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	7
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	4-7
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	7
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	8
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	8
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	9

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

CHAPTER VI

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

GENERAL DISCUSSION

1. Mesenchymal stem cells and perivascular cells can prevent and rescue hyperoxia-induced lung injury.

The first aim of the thesis was to investigate the possible option of a novel therapy for BPD. Stem cells, in particular mesenchymal stem cells (MSCs), may represent a promising choice for several diseases so far considered untreatable. Perivascular cells (PCs), which are considered the precursors of MSCs had never been investigated in models of neonatal diseases. We proved that systemic and endotracheal prophylactic administration of MSCs and PCs (before exposure to hyperoxia) improves survival, prevents lung injury, reduces inflammation and attenuates pulmonary hypertension in experimental BPD. Lung compliance and exercise tolerance were also significantly improved by MSC and PC administration (1). As a prove of concept, in our results PCs showed the same results as MSCs. However, MSCs have been much more deeply investigated as they have been known for decades. Chang et al, showed that MSC administration seemed to be most beneficial by endotracheal (ET), compared with intravenous (IV) or intraperitoneal administration (2). They also observed a dose-dependent effect with no benefit at 5×10^3 (approximately 5×10^2/g body weight) and increased benefit from 5×10^4 (approximately 5×10^3 cells/g) to 5×10^5 cells (approximately 5×10^4 cells/g) (3). Intrapulmonary delivery of MSCs on day 4 of life was safe up to 6 months, as assessed by lung morphometry and exercise capacity (1). In our data neonatal prophylactic endotracheal MSC treatment significantly improved exercise capacity and almost normalized alveolar architecture up to 6 months of age in rodents exposed to hyperoxia in the neonatal period. Total body computerized tomography (CT scans) showed no signs of tumor formation (1). These data need to be confirmed by systematic sampling of tissue histology.

Rescue administration of MSCs (after exposure to hyperoxia), have led to inconsistent results, with no (4) or negligible (5) improvement. However, in both of these rescue studies, the same absolute dose as the prophylactic experiments was used, without adjusting for the increase in body weight of the older animals. When we adjusted the dose per body weight, although the prophylactic treatment still elicited a superior response, the rescue administration of MSCs and PCs was able to ameliorate lung injury (1). No studies reported on the effects of rescue MSC administration on the vascular component of the lung injury. Rescue administration of MSCs shows a milder improvement in terms of inflammation and oxidation as compared to early administration (5). Taken together these data suggest a possible beneficial effect of a rescue MSC treatment, but early timing seems to be more efficient.

The evidence of low engraftment of both, MSCs and PCs, despite exciting therapeutic results, strongly confirms the stem cell paracrine activity as their major mechanism of action. This notion has paved the way to test novel cell-free derived products. Several studies have

documented the efficacy of MSC conditioned media (CdM) in experimental models of different diseases. In our experiments, MSC CdM could effectively prevent and treat the hyperoxia-induced lung injury. In vitro, cell-free bone marrow-derived MSC CdM prevented hyperoxia-induced alveolar epithelial cell apoptosis, accelerated wound healing of alveolar epithelial cells and preserved endothelial cord formation under hyperoxic conditions (6). In vivo, we showed that daily intraperitoneal administration of MSC and PC CdM prevents oxygen-induced alveolar simplification, improves lung function and reduces pulmonary hypertension (1). The beneficial effects in terms of lung architecture and exercise capacity of the 2 week course of CdM administration in the neonatal period were documented up to 6 months of life, with no evident side effects (1).

Although the therapeutic benefit of CdM is remarkable, the downside of this strategy is the lack of cell homing and cell adaptation to the local injurious environment. Waszak et al. exposed bone marrow derived MSCs ex vivo to hyperoxia for 24 hours. This preconditioning strategy enhanced the production of the antioxidant stanniocalcin-1 in the CdM. The beneficial effects of the CdM collected from preconditioned cells and injected into hyperoxic rats were more pronounced as compared to non-preconditioned media (7).

In the setting of hyperoxic injury, a single prophylactic injection of cell-free bone marrow-derived MSC CdM seems to be even more effective on alveolar and lung vascular growth than cell therapy itself (4), which has been documented up to 3 months (8). A single rescue dose of CdM, after exposure to hyperoxia, is also able to reverse hyperoxia-induced parenchymal fibrosis, partially reverse alveolar injury, fully reverse pulmonary hypertension and normalize lung function (9). In addition to the pleiotropic effects on lung structure, inflammation and oxidative stress, MSC CdM may also boost local repair activity such as the proliferation of resident lung progenitor cells (10). The compelling and persistent results of a single dose of CdM may be explained by the presence of microparticles and exosomes in the CdM. These particles act as therapeutic packages carrying combinations of bioactive molecules and miRNA, which have the ability to incorporate in resident cells and transfer functional RNA (11). In a variety of animal models, the use of MSC-derived exosomes, purified by ultracentrifugation and size exclusion (12), is strongly associated with improved organ function following experimental injury (13). Lee et al. demonstrated that exosomes from bone marrow-derived MSCs, in the setting of hypoxia-induced pulmonary hypertension, prevent pulmonary hypertension, right ventricular hypertrophy and hypoxia-activated inflammation, whereas exosome-depleted CdM had no effect (14). Exosomes from bone marrow-derived MSCs significantly reduce lung edema, lung protein permeability and lung inflammation in LPS-induced acute lung injury (15).
In summary, evidence from animal models show that cells and cell CdM ameliorate many pathologic components leading to BPD. Early, intratracheal administration seems to be most efficient. MSCs and PCs also display long-term safety and efficacy in rodents

Although the option of a cell free product is appealing, the administration of the entire cocktail entails a risk of unanticipated side effects, due to presence of yet undetermined

active molecules. Technological advances are still required for the isolation, characterization, quality control, and large-scale manufacturing of exosomes. At the present, regenerative medicine seems to favour cell therapy.

2. Mesenchymal stem cells for BPD: from bench to bedside

Although there are still gaps in knowledge about MSCs, the promising animal data have prompted the clinical translation of cell therapies for yet untreatable diseases. While PCs have not been yet tested in the clinical setting, preliminary trials investigating the potential of MSCs to treat BPD have already been started.

The search for currently registered clinical trials, testing MSCs as intervention, retrieved 935 results (https://clinicaltrials.gov/ct2/results?cond=&term=mesenchymal+stem+cells&cntry=&state=&city=&dist=). Safety of MSC administration in the adult (16) and pediatric (17) population has been widely documented, while efficacy is still under investigation for several diseases.

With regards to premature infants, Chang et al showed that the endotracheal transplantation of two different doses of human cord-derived MSCs in preterm infants at high risk for BPD is feasible and apparently safe (18). The treatment was well tolerated, without dose-limiting toxicity or immediate (up to 6 hours) complications after transplantation. Six severe adverse events were recorded. However, they were typical complications of extreme prematurity and not attributed to the MSC therapy. This trial was designed to test safety and was not randomized and powered to investigate efficacy measures. The long-term follow-up study was recently published and showed no concerns (19).

To determine if MSCs, administered intravenously or endotracheally, are safe and effective in preventing and/or treating BPD, in preterm infants, we performed a Cochrane review, searching for RCTs and quasi-RCTs. We found no published studies addressing the effect of MSC administration for prevention or treatment of BPD in premature infants that fulfilled the inclusion criteria. However, nine studies are currently investigating the effects of the MSC administration in patients at risk for BPD (4 RCTs and 5 phase 1-2 single arm studies) (https://clinicaltrials.gov/ct2/results?cond=Bronchopulmonary+Dysplasia&term=mesenchymal+stem+cells&cntry=&state=&city=&dist=) and results will be available soon.
It should be mentioned that pulling the data from studies on stem cells will likely be more challenging than other drugs, because the efficacy of this treatment can be hampered by several product and patient related factors. Dosage, timing, and route of administration greatly affect MSC homing, regeneration, and paracrine capacity (20). In addition to these elements, numerous and more complicated variables, altering the manufacturing of the final MSC product, need to be considered when approaching the design and the results of clinical trials assessing MSCs as intervention. The most important elements are outlined below.

1. *Timing*

Treatment of established BPD requires, by definition, waiting 28 days of life for mild BPD and 36 weeks postmenstrual age (PMA) for moderate and severe BPD. Although this target may be appropriate to prove safety, confirmation of efficacy could be hampered by the delayed timing. Severely injured lungs may be too compromised to be significantly renewed. In addition, the signals, needed to recruit and modulate the effects of MSCs, change with the course of the disease, becoming minimal or absent at a chronic phase of injury (21).

Prevention of BPD at birth (prophylactic) and up to 5 days (early administration) is particularly appealing because more than 50% of infants born before 26 weeks' gestation will develop BPD (22). In addition, based on animal data, prophylactic administration seems the most effective option. However, the major limitation to this approach is the risk of overtreatment.

The best therapeutic target is likely to be the evolving BPD. Although evolving BPD is not an entirely defined entity, it refers to oxygen-dependent and ventilator-dependent extremely preterm infants, between 7 and 27 days of life (23), when the respiratory disease represents the leading cause of death (24). This timeframe may represent the optimal therapeutic window for MSCs. In this population, overtreatment could be easily avoided by selecting the most compromised patients at the highest risk for death and/or severe BPD (https://neonatal.rti.org/index.cfm?fuseaction5tools.main).

2. *Route of administration*

Efficacy, bioavailability, and functionality of any pharmacologic drug depend on the route of administration. Regarding lung diseases, the 2 most relevant routes of administration are IV and ET. Compared with traditional medications, systemic MSC infusion has the advantage of preferential migration and homing into damaged tissues (21).

When considering pulmonary diseases, the localization into the lung is also facilitated by the natural biodistribution of MSCs. After IV transfusion, MSCs initially concentrate into the lung. Afterward, some cells move gradually either to the liver, spleen, kidney, or bone marrow, in non-injurious models; or to the injury sites in various experimental disease models (25, 26). Because several organs may be damaged in severely ill premature infants, MSCs may migrate toward those sites of injury, after the first passage through the lung, eventually improving the overall outcome. The IV route may also be less invasive than the ET route in spontaneously breathing patients. Despite the obvious benefits of the IV route, evidence from animal studies has shown that the ET administration provides superior therapeutic benefit at least in the experimental settings. The ET route allows delivery of MSCs at the alveolar interface and this may alter the interactions and responses of MSCs. In addition, co-administration with surfactant may facilitate the distribution of MSCs. The choice of the most convenient route of administration may be influenced by the timing of intervention

because survivors at 36 weeks PMA are usually supported by non-invasive ventilation, whereas patients with severe evolving BPD are still mechanically ventilated.

3. *Dose*

The conversion of the effective preclinical doses, according to the body mass index of the premature infants (27), results in a theoretic effective dose ranging from 8 x 10^5/kg up to 8 x 10^6/kg (3) ET and 1.6 x 10^6/kg (4) up to 3.2 x 10^7/kg (2) IV. In a recent phase 1 trial, Chang and colleagues (18) did not report dose-limiting toxicity up to a dose of 2 x 10^7/kg. The high-dose treatment seemed to be associated with better outcomes, although this study was not designed to show efficacy. These results need to be confirmed in randomized phase 2 trials that are currently on-going (NCT01828957; NCT01207869). The need for several culture passages and the volumes required to obtain adequate cell concentrations (a crucial variable for the ET route) may influence the quality of the cell product and be limiting factors to the efficacy of higher doses.

4. *Source of cells*

The source of MSCs can greatly affect their efficacy. Although MSCs have been isolated from any tissue (28), some sources are more clinically relevant than others. Bone marrow is the first described and best-known source of MSCs. However, it may not be optimal for patient treatment due to the aging of the cell with the donor aging (29), the paucity of the MSC among the other cells present in the bone marrow aspirate (30), and the painful and invasive procedure needed to obtain the cells. Adipose tissue is an emerging source of MSCs. It offers a greater number of cells and it is more accessible than bone marrow, although aging of the cells with donor aging is still an issue (31). Human extra embryonic tissues (chorion, amniotic membranes, and umbilical cord) as well as human placental fluids (amniotic fluid and umbilical cord blood) are an appealing source of MSCs. A significant advantage of these perinatal tissues is their availability without invasive and painful harvesting procedures (32). Even more interestingly, MSCs from these neonatal tissues may be superior to MSCs derived from adult sources. Perinatal MSCs display superior cell biological properties, such as stronger immunomodulatory and immunosuppressive potential (33), improved proliferative capacity, life span (34) and stemness (32), and higher trophic (35) and anti-inflammatory (34) activity, compared with adult MSCs. To date, among the different perinatal sources, the most used and practical is the umbilical cord. In particular, robust and reproducible techniques for harvesting and expansion are available for Wharton Jelly, rather than cord blood or perivascular tissues.

5. *Practical aspects of mesenchymal stromal cell manufacturing*

Passage number is one of the major determinants of the MSC product function. A passage is the process of removing cells from a culture flask and plating them into more culture flasks. Passaging is necessary to obtain a sufficient number of cells for transplantation. The number of passages is inversely proportional to the efficiency of homing, with freshly isolated cells

performing superior results to cultivated cells (36). Moreover, MSCs tend to show genotypic and phenotypic variation when cultured for extended periods of time (37), although cord-derived cells seem to be less sensitive to aging in culture (34). Early-passage cells may have superior homing and therapeutic potential and thus improve the chances of success of the stem cell therapy in clinical trials. Culture conditions Several factors, including isolation and characterization techniques, production to scale-up, cryopreservation, and banking, can affect the efficacy of MSC therapy and have been reviewed elsewhere (38). These details, however, will be important in designing and interpreting the results of clinical trials.

6. Type of transplantation

Autologous transplantation of cord MSCs (from the cord of the patient) is particularly appealing in neonates (39) but has some shortcomings: (i) impracticality of the prophylactic or early administration due to lead time, (ii) need for on-site good manufacturing practice facilities to handle clinical-grade cell products, and (iii) yet to be explored possible differences between the term and preterm cord MSCs.

Considering the low immunogenicity of MSCs, although immune rejection is still possible (20), allogeneic transplantation (from a donor) may be the most suitable option for preterm infants at risk for BPD (39) by providing a readily available, quality-controlled, off-the-shelf product.

In conclusion, MSCs offer very promising therapeutic options for the prevention and treatment of the complications of extreme prematurity, including BPD. Although the knowledge of their function and manufacturing process is still incomplete, the documented safety of MSCs in adults and children, and the dramatic benefits observed in experimental models, has prompted the clinical translation of this promising therapy. Selection of patients, timing and route of administration, source of cells, and protocols for MSC manufacturing can determine success or failure of this therapy and indelibly influence further research. During the next 5 years, well-designed trials will indicate whether MSC therapy can become a novel breakthrough in neonatal medicine.

3. Current evidence does not support beneficial effects of probiotics in preventing BPD

While the field of regenerative medicine for the treatment of BPD will gathered the necessary information to know if MSCs may be a definitive option, some strategies could help improving the conditions of BPD patients and alter the course of the disease.

Probiotics have attracted much attention as a potential weapon to improve overall human health thanks to the alterations of the microflora of the host. The most accepted definition of probiotics of probiotics is: “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (40). Probiotic bacteria can be delivered and ingested separately as medicinals or supplements, can also be mixed with or naturally exist in functional foods. Once delivered, probiotic bacteria can exert their beneficial actions

through different mechanisms: enhancement of the epithelial barrier, increased adhesion to intestinal mucosa, and concomitant inhibition of pathogen adhesion, competitive exclusion of pathogenic microorganisms (41). Moreover, probiotics are believed to produce active molecules that generate beneficial effects on host biological functions (41). These metabolic by products are sometimes referred to as “postbiotics” and may function biologically as immune modulators (42-44).

Considering this broad-spectrum biochemical interactions, probiotics have been tested in a variety of adult and paediatric diseases (45, 46). However, the only clinical conditions in which strains of whole live probiotics have been definitively shown to be effective so far are acute gastroenteritis and antibiotic-associated diarrhoea (45, 46) . In children, these effects are strain- and dose-dependent, more effective if started at the onset of the disease, and more evident in children living in developed countries (47, 48).
However, the significance of the results of RCTs and meta-analysis testing the efficacy of probiotics are biased by a great variability administration regimens and study design. A very large number of probiotic strains have been used in clinical studies for the treatment of the same clinical condition, and the same strain of probiotics has been used to treat different diseases. Various dose ranges and types of administration (capsules, powders, tablets, drops or yogurts) have been chosen in clinical trials. Study designs of the clinical trials are also very inhomogeneous in endpoints and outcomes, limiting the validity of meta-analysis. This heterogeneity makes almost impossible to draw definitive conclusions.
With regards to preterm infants, prophylactic administration of enteral probiotics has been shown to decrease the incidence of severe necrotizing enterocolitis (NEC), mortality, late-onset sepsis (LOS) and time to achieve full enteral feeding (49-51)...As a consequence, many NICUs are currently using probiotics as standard practice for prevention of NEC in preterm neonates (52-56).

The beneficial effects of probiotics in diminishing inflammatory events in preterm infants, may pave the way to a possible role of probiotics in preventing BPD. However, so far, the effects of probiotics in lowering BPD rates, either as a direct effect or as a consequence of LOS and NEC reduction, have not been tested. Therefore, we performed a meta-analysis to investigate the possible link between probiotic administration and lower BPD rates. Surprisingly, despite the convincing theoretical benefits, probiotics seemed not involved in prevention of moderate and severe BPD. However, the possible role of probiotics in preventing BPD is compelling. A reduction of postnatal systemic infections may decrease the inflammatory response in developing lungs (57) and may be accompanied by decreased BPD rates (58, 59). Besides their effect on NEC and LOS prevention, there are some other mechanisms of action ascribed to probiotics which may directly counteract the disruption of lung development prompting to BPD (60). Probiotics may re-equilibrate the immature immune system of premature infants which is unable to balance pro-inflammatory responses, leading to a sustained status of inflammation, that contributes significantly to

several neonatal diseases, including BPD (61). In second place, probiotics may restore intestinal microbiota, whose modification given by the frequent antimicrobial therapy may predispose to BPD development (62-64). Probiotics have also been shown to produce a strong suppressive effect on airway inflammation characterized by reduced inflammatory cell infiltration and levels of Th2 cytokines in lung tissues (65). Moreover, our results should be interpreted with caution since the included RCTs, as for adult and paediatric RCTs, showed relevant methodological differences in terms of enrolment criteria, timing, dose, and formulation of probiotic used. Moreover, BPD was never the primary outcome and the number of RCTs reporting on BPD as secondary outcome is relatively small.

In conclusion, our study could not demonstrate any significant effect of probiotic supplementation on the risk of developing of BPD. Given the remarkable theoretical benefits of probiotics supplementation in ameliorating several aspects of BPD pathogenesis and the limitations of the analysis, our data should be seen as a starting point rather than definitive results. Well-designed RCTs selecting the optimal probiotic preparation, dosing, and duration of therapy and including BPD among primary outcomes are necessary.

4. Mother's own milk diet potentially prevents BPD

Nutritional flaws are an important issue for the development of BPD (66). Choosing the optimal nutrition for preterm infants may decrease the risk of developing complications of prematurity, including BPD.

A human milk diet has been proven to provide significant health benefits to preterm infants. However, since mothers own milk is not always available, the first alternative to feed preterm infants should be pasteurized donor human milk (DHM) (67-70).

DHM was shown to reduce the incidence of NEC in several studies (71) and it was confirmed by a recent Cochrane review (72), while effects on mortality and other outcomes are not fully determined yet (72, 73). We documented in a recent meta-analysis (74) a protective effect of DHM on BPD, when compared to supplementation with formula milk in observational studies. However, pasteurization may have detrimental effects on maternal milk, causing a great reduction in vitamins (C, D and B6) and bile salt stimulated lipase (75). Using raw mother's own milk (MOM) allows avoiding human milk treatments. Besides pasteurization, refrigeration and freezing may have some consequences on the integrity of the milk. Although storing human milk at 4–6°C for up to 96 hours had no major effects on the nutritional content, enzymes and osmolality (76), it altered milk antioxidant capacities and induced lipolysis, increasing free fatty acid concentrations and decreasing pH (76, 77). Freezing human milk at −20°C and thawing it after two months has been shown to reduce vitamin C by two-thirds (77). In a previous meta-analysis, we found no differences between DHM and MOM on BPD reduction, while the meta-analysis of three observational studies showed that pasteurization of MOM seems to reduce the beneficial effects of the raw MOM (74). However, our previous meta-analysis did not investigate specifically the effects of

MOM on BPD and no meta-analysis to our knowledge was designed to do so. Consequently, we decided to investigate the role of MOM as a prevention strategy for BPD.

The main result of our analysis is that MOM can reduce the incidence of BPD, when used as an exclusive diet. To our knowledge this is the first meta-analysis focusing specifically on MOM and BPD. A predominantly MOM diet may reduce the incidence of BPD through different mechanisms put in place by the nutritional and bioactive components of the human milk that may counteract several mechanisms leading to BPD. These mechanisms include oxidative stress (78), inflammation (79, 80) and nutritional flaws (81, 82). In addition, MOM may also impact the risk of BPD indirectly by reducing the incidence of NEC and sepsis (72, 75).

As a secondary result, our analysis revealed that, in case any amount of infant formula is added to MOM, the beneficial effects of MOM could not be detected anymore. This could be related to the intrinsic biases of our analysis, which include the limited number of available studies, the absence of RCTs, and the heterogeneity of the design of the available observational studies. However, this result may be due to the fact that the beneficial effects of human milk are dose dependent (83-88). Most of the studies set the minimum amount of MOM to define a diet as “mainly MOM” at 50 ml/kg, based on the evidence gathered from the minimum effective dose in preventing NEC and LOS. However, since the relation between MOM and BPD, may not be as direct as for NEC and LOS, it is possible that higher minimum amounts of MOM may be needed to detect significant differences.

In conclusion our data confirm that MOM reduces the incidence of BPD when used as an exclusive diet, reinforcing the need to strongly support breastfeeding in the NICU. The donor human milk programmes should be accompanied by a more global strategy in the NICU which includes strong and specific breastfeeding support for mothers who deliver preterm and healthcare organisations should optimise how they collect, store and handle the administration of fresh mother's own milk.

5. Future perspectives

5.1 FUNCTION AND THERAPEUTIC POTENTIAL OF CORD-DERIVED MSCS AND PLACENTA-DERIVED-MSCS OBTAINED UNDER DIFFERENT PERINATAL CONDITIONS

Clinical trials investigating the therapeutic effects of exogenously administered MSCs have yield conflicting results (89). Although the cause of this inconsistency has yet to be fully established, the evidence that donor-related MSC characteristics are likely to have a role on the final product is emerging. Female cord-derived MSCs show better lung protection compared to male cord-derived MSCs in a rodent model of neonatal hyperoxia-induced lung injury (90). In-vitro studies showed that cord-derived MSC proliferation, stemness markers, telomerase, osteogenic and chondrogenic differentiation, antioxidant enzymes and gene expression for mitochondrial function were significantly lower in diabetic mothers as compared to non-diabetic mothers (91). According to this preliminary evidence, it is possible

that several factors comprising, but not limited to, gestational age at harvesting, placental diseases, chorioamnionitis, and maternal or foetal factors, may alter the efficacy of the cells. Moreover, the cell environment has been proven to modify the paracrine effect of MSCs in order to respond to the specific stimuli they are exposed to (7). Accordingly, the perinatal environment may "program" placenta-derived and cord-derived MSCs in order to counter each specific feto-maternal pathological condition. Studying the effects of perinatal conditions on MSC efficacy and therapeutic potential may not only help to choose the best donor in terms of efficiency of the cell product, but also target the most appropriate product for each specific foetal and neonatal condition, including the different BPD phenotypes.

Therefore, we will aim to explore MSC potency, function, stemness, aging, differentiation capacity and extracellular vesicles production under different perinatal conditions (i.e. chorioamnionitis, pregnancy induced hypertension, diabetes, IUGR) as compared to healthy pregnancy at different gestational ages.

5.2 GESTATIONAL HYPERTENSION AND BPD

It has recently been suggested that the BPD diagnosis may include a group of diseases with different pathogenesis and different phenotypes that need to be defined more specifically (92). The different pathophysiological clues leading to BPD, including altered breathing patterns, underdeveloped lung vasculature, altered alveolar surface may coexist or be the peculiar feature of each specific phenotype (92). The pathological condition leading to premature birth and its downstream signalling pathways may be a major determinant of the BPD phenotype, differently impairing the delicate balance of lung development. In most cases, premature birth is due to one of two major pathological factors: intrauterine inflammation (preterm labour, preterm membrane rupture, and cervical insufficiency) or placental vascular disorders (gestational hypertensive disorders (GHD) and foetal growth restriction) (93). Deepening the link between each mechanisms and BPD development may help the understanding of the BPD pathogenesis. A recent systematic review, examining the effects of pre-eclampsia on neonatal outcomes in premature infants, found that, although GHD did not increase the risk for BPD in the entire cohort of premature infants, it was significantly associated to BPD in infants below 29 weeks' gestation (94). However, the study was not specifically focused on BPD and it included a limited number of articles for the BPD outcome (9 studies) (94). Therefore, we are currently conducting a more extensive meta-analysis to advance the understanding of the antecedents of BPD, in particular the vascular disorders. The analysis of antenatal conditions related to BPD, such as GHD, may confirm the hypothesis that BPD is not a univocal disease, but rather a group of different pathological entities that may benefit from tailored treatments.

5.3 CONNECTING THE DOTS BETWEEN INFANT RESPIRATORY DISTRESS SYNDROME AND BRONCHOPULMONARY DYPSLASIA

Despite being the most common diseases related to prematurity, both, the acute *infant* Respiratory Distress Syndrome (iRDS), due the lack of the tensioactive lung surfactant and the chronic form of lung disease, BPD, cannot count on well-established definitions. The lack of exact definitions generates considerable epidemiological and therapeutic uncertainty when approaching neonatal lung diseases. Although, chest X-Ray can give a clue of the underlying disease, it is neither specific nor sensitive for iRDS and BPD (95, 96).

From a practical standpoint, acute respiratory distress in premature infants is classified as iRDS in case surfactant replacement therapy is required (97), although criteria for surfactant administration are not universal (97). After surfactant therapy, half of the patients below 28 weeks' gestation will rapidly solve the acute phase of their lung injury (98). Of these, approximately 50% will subsequently develop a chronic form of lung disease (98). On the contrary, in case surfactant does not provide a significant benefit during the acute phase, BPD develops in 70% of the patients uninterruptedly from iRDS (98). In a smaller subset of patients BPD develops after a silent acute phase (98). These patterns of lung disease are likely to be different and specific entities that need to be fully characterized.

At the present, BPD can only be diagnosed starting from 36 weeks post-menstrual age (PMA), as the most widely accepted definition for BPD is "oxygen dependency at 36 weeks PMA" (99). However, although it is not known exactly when iRDS turns into BPD, the lack of surfactant alone is inadequate to explain ventilator/oxygen-dependency after the first week of life (97, 99). Therefore, we aim to study the entire respiratory course of preterm infants in order to identify the chronic patterns as early as possible.

We will prospectively analyse and correlate **antenatal determinants** (maternal history, placental pathology), **proteomic and metabolomic analysis** of cord blood, bronchoalveolar lavage and saliva samples, the patient clinical parameters and data of **serial lung ultrasound (LUS) measurements** from birth to discharge**.** LUS has been recently introduced in neonatal care as a novel, simple and reliable, radiation free tool for the diagnosis of various neonatal lung diseases. LUS is able to detect iRDS with an extremely high specificity and sensitivity (6). Two small pilot studies found that the i-RDS pattern progress towards a BPD pattern within the first weeks of life (100). LUS can be repeated numerous times without any risk to the patient, with low cost and immediate turnaround time to obtain results, making it the perfect tool to follow-up the evolution of i-RDS towards CLD.

We aim at integrating LUS imaging, biological and clinical data though the use of big data analysis. Multi-parametric physiological data acquired by each monitor at bedside (e.g., HRC index, respiratory rate, blood oxygen saturation, perfusion index, blood pressure, body temperature matched with incubator temperature), in addition to clinical data (e.g., ventilation parameters, intravenous drugs such as xanthines, vasoactive drugs, analgesic drugs,) obtained from electronic medical records could be used to develop a model that identifies each different pulmonary phenotype. However, the huge amount of data collected from multiparameter monitoring system, electronic medical charts and

metabolomics/proteomic analysis are difficult to analyze and handle when using traditional database tools. Recently, the application of data-driven, machine learning techniques to medical data seemed to provide a valid alternative analytic approach in health-care settings (101, 102). Databases that include detailed patient information analyzed by big data analytic software provide researchers with an opportunity to discover novel patient subpopulations (102). Therefore, we planned to apply a Big Data-driven, machine learning approach to define and predict different neonatal pulmonary patterns. We will link the different lung patterns with in-hospital and post-discharge mortality, oxygen dependency at home, pulmonary hypertension, readmission to the hospital for respiratory reasons, episodes of wheezing up to 2 years of age.

We believe that we will be able to identify the onset of the chronic lung disease of prematurity and characterize each respiratory pattern in order to eventually target optimal and well-timed therapies.

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