School of Allied Health

Characterising Biomarkers of Chronic Lung Disease in Survivors of Preterm Birth

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Human Ethics

The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) – updated March 2014. The proposed research study received human research ethics approval from the Curtin University Human Research Ethics Committee (EC00262), Approval Numbers #HRE2020-0097; HRE2018-0407; HRE2018-0385; HRE2019-0723 and other relevant Human Research Ethics Committee approvals as detailed in the Methodology chapter.

Signature:

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Date: 8/9/2021

Acknowledgement of Country

We acknowledge that Curtin University works across hundreds of traditional lands and custodial groups in Australia, and with First Nations people around the globe. We wish to pay our deepest respects to their ancestors and members of their communities, past, present, and to their emerging leaders. Our passion and commitment to work with all Australians and peoples from across the world, including our First Nations peoples are at the core of the work we do, reflective of our institutions' values and commitment to our role as leaders in the Reconciliation space in Australia.

Abstract

Introduction

Survivors of preterm birth exhibit increased respiratory morbidity, with impaired lung structure and function which may worsen over time. Many pro-inflammatory events take place in the neonatal stage following preterm birth, with inflammation and oxidative stress playing a key role in the development of chronic lung disease of prematurity. Emerging evidence suggest inflammation and oxidative stress remains elevated in preterm-born children compared to their term-born counterparts throughout childhood into adolescence. These processes may be an important contributor to persistently poorer respiratory outcomes in this cohort and may be a potential target for intervention.

This thesis aimed to:

- Better understand the phenotype of respiratory function in preterm-born children
- Identify the presence and extent of inflammation and oxidative stress biomarkers present in preterm-born children at different ages across infancy and later childhood and young adulthood
- Use metabolomics methods to discover altered metabolic pathways in preterm-born infants and children that are associated with worse respiratory outcomes in childhood.

Methods

Infants, children and young adults who were born very prematurely and healthy controls provided exhaled breath condensate (EBC) and urine samples. EBC and urine samples were analysed for known inflammatory biomarkers including cysteinyl leukotrienes, leukotriene B4, 8-isoprostane, interleukin-8 (IL-8) and matrix metalloproteinase-9 (MMP-9). Urine was also analysed for metabolomic profile using liquid chromatography/mass spectrometry. Fractional exhaled nitric oxide (FeNO), respiratory oscillometry, spirometry and bronchodilator responsiveness were assessed in participants aged 6-23 years.

Results

Preterm-born participants had poorer spirometry outcomes and altered peripheral respiratory mechanics on oscillometry when compared to term-born controls ($p<0.05$), however fractional exhaled nitric oxide levels and rates of bronchodilator responsiveness were not different between these groups overall. Worse spirometry and oscillometry outcomes were associated with increased prematurity and more days of mechanical ventilation and oxygen supplementation in the neonatal period.

Preterm-born infants at 12-16 months of age exhibited higher levels of leukotriene B4 and 8-isoprostane in EBC than term-born controls ($p<0.05$). In older children, cysteinyl leukotrienes in EBC and urine were not different between term and preterm participants. IL-8 was elevated in the exhaled breath of term-born participants when compared to those born preterm ($p<0.05$). IL-8 and MMP-9 levels in urine

did not differ between groups, however urinary leukotriene B4 and 8-isoprostane was elevated in those born preterm ($p<0.05$). Biomarker levels did not differ between preterm-born participants with and without a diagnosis of bronchopulmonary dysplasia.

Metabolomics analysis of urine collected from preterm-born neonates at 36 weeks postmenstrual age identified several amino acids associated with a more severe neonatal course, although metabolomic profiles did not significantly differ between those with and without a diagnosis of bronchopulmonary dysplasia. Analysis of urine from participants in later childhood and young adulthood identified differing metabolomic profiles between those born preterm and those born at term.

Conclusion

Preterm-born children displayed poorer respiratory function outcomes than those born at term. Those born preterm exhibited evidence of increased neutrophilic inflammation and oxidative stress, rather than the eosinophilic profile associated with "typical" childhood asthma. Additionally, metabolomic analysis shows preterm-born individuals with an altered metabolome from those born at term, which may provide insight into other pathways of disease contributing to poorer respiratory outcomes in this population.

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List of Abbreviations

AChE	Acetylcholinesterase
ATS	American Thoracic Society
AX	Area under reactance curve
BPD	Bronchopulmonary dysplasia
CPAP	Continuous positive airway pressure
CT	Computed tomography
COPD	Chronic Obstructive Pulmonary Disease
DLCO	Diffusing capacity of the lung for carbon monoxide
EBC	Exhaled breath condensate
ELISA	Enzyme-linked immunosorbent-assay
ERS	European Respiratory Society
FDR	False Discovery Rate
FEF_{25-75}	Forced mid-expiratory flow
FeNO	Fraction of exhaled nitric oxide
FEV_1	Forced expiratory volume in 1 second
FOT	Forced Oscillation Technique
Fres	Resonant frequency
FVC	Forced vital capacity
GA	Gestational age
^{3}HeMR	Helium-3 magnetic resonance
HREC	Human Research Ethics Committee
ICS	Inhaled corticosteroids
IL-8	Interleukin-8
IQR	Interquartile range
ISAAC	International Study of Asthma and Allergies in Childhood
KEMH	King Edward Memorial Hospital
LC/MS	Liquid chromatography/mass spectrometry
LTB4	Leukotriene B4
MMP-9	Matrix metallo-proteinase 9

NICU	Neonatal Intensive Care Unit
O_2	Oxygen
PC-CVA	Principal component-canonical variate analysis
PCH	Perth Children's Hospital
P_IO_2	Partial pressure of inspired oxygen
PMA	Postmenstrual age
QC-RSD	Quality Control-Relative Standard Deviation
R_5	Resistance at 5 Hz
ROS	Reactive oxygen species
RV	Residual volume
SpO_2	Peripheral oxyhaemoglobin saturation
TLC	Total lung capacity
X_5	Reactance at 5 Hz
$R_{10.in-ex}$	Change in end-inspiration and end-expiration resistance at 10 Hz
$X_{10.in-ex}$	Change in end-inspiration and end-expiration reactance at 10 Hz

Chapter 1: Literature review

This literature review has been adapted from a published review with updated sources. Peer-reviewed but unedited manuscript version available in Appendix B (2).

PRETERM BIRTH AND THE LUNGS

Lung development in utero

Prematurity is associated with adverse neonatal outcomes, particularly respiratory outcomes as a result of interrupted lung development (3). Very premature infants are particularly vulnerable as they are born during the canalicular (16–26 weeks gestational age (GA)) and saccular (26–36 weeks GA) stages of lung development (4) and consequently forced to exchange gas with an immature lung, with lung development occurring in a relatively hyperoxic *ex utero* environment compared to the *in utero* milieu (3).

The cannalicular period commences around 16 weeks GA with the formation of the final generations of the bronchial tree and the early development of lung parenchyma (3, 5) (Figure 1). The distal airspace widens, causing cuboidal epithelial cells to flatten once they come into close contact with vasculature, resulting in the formation of an air-blood barrier which is crucial for gas exchange (5, 6). Flattening of the cuboidal epithelial cells is evident by 20 weeks, and marks the differentiation of these cells into type-I and type-II epithelial cells (7). Type-I epithelial cells are gas-exchanging and line most of the airway internal surface (3). Type-II epithelial cells contain lamellar bodies, and produce small amounts of surfactant by 22 to 24 weeks GA which reduces the surface tension of the lungs and prevents alveolar collapse (3).

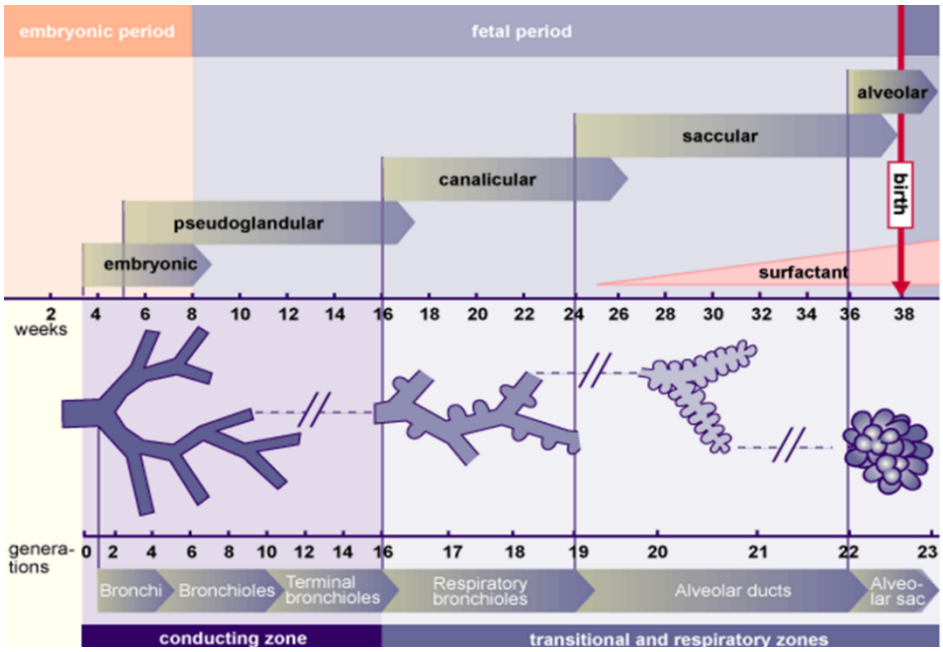

Figure 1: Developmental stages of the lung *in utero*. The development of the conducting, transitional and respiratory zones of the lung occurs during the embryonic, pseudoglandular, cannalicular, saccular and alveolar stages in the embryonic and fetal periods of gestation. (1)

The saccular stage of development occurs from 24 weeks GA, and is characterised by the continued expansion and development of the lung parenchyma, with airways ending in small, thin-walled saccules in clusters (3). Within the saccular stage, a natural increase in circulating cortisol occurs, which is critical in lung maturation in readiness for gas exchange (3). Tissue remodelling, alveolar cell differentiation, reabsorption of lung liquid and stimulation of the surfactant system all occur as a result of the increase in cortisol (3).

The alveolar period is the fifth stage of lung development and begins prenatally around 36 weeks GA, continuing into the postnatal period (6). Within the alveolar period, saccules develop into fully-formed alveoli, and this alveolarisation process occurs mostly in the postnatal period, with 85 % of alveolarisation occurring after birth continuing throughout childhood and even into adulthood (6, 8). The microvascular period begins at birth and involves the growth and maturation of the pulmonary vasculature supply to airways (9).

The lungs are one of the last organs to develop *in utero* with alveolarization and microvascular development only beginning at towards the end of the fetal period or at birth. However, preterm birth disrupts normal lung development which results in preterm-born infants requiring respiratory support with some needing invasive mechanical ventilation and exogenous surfactant amongst a number of other medical interventions to survive (3).

Preterm birth and chronic lung disease

The chronic lung disease of prematurity, bronchopulmonary dysplasia (BPD), was first described over 50 years ago in infants with an average gestational age (GA) of 34 weeks (w) and prolonged exposure to high oxygen concentrations (10). Significant improvements in neonatal critical care – including routine use of surfactant therapy – occurred during the 1990s (11), such that approximately half of babies born at 25 w GA now survive in high-middle income countries (12). In Western Australia, the survival rates for babies born at 25 weeks exceeds 80 % (13). With more infants surviving at earlier gestational ages, the clinical and pathological characteristics of prematurity and BPD have changed profoundly. "New" BPD is defined as the requirement for supplemental oxygen for at least 28 days, and is characterised by fewer and larger alveoli, decreased and damaged pulmonary vasculature (14), inflammation and variable smooth muscle hyperplasia (15). Prenatal factors such as *in utero* inflammation (16), intrauterine growth restriction (17), maternal smoking (18), male sex (19), Caucasian race (20) and genetic factors (21) increase the risk of poor respiratory outcomes and BPD (Figure 2). Lower gestational age (22) and longer mechanical ventilation are associated with supplemental oxygen duration and therefore more severe BPD (22). Postnatal pulmonary inflammation and oxidative stress play a key role in the pathogenesis of BPD (16, 23) and are discussed in more detail in the context of initiating a pathological process that may indeed persist beyond the neonatal intensive care unit (NICU).

Inflammation and oxidative stress in the neonatal period

Pro-inflammatory cytokines, adhesion molecules, selectins, and chemokines are found in high levels in infants with BPD and are associated with endothelial interactions leading to decreased vascularisation and simplified alveoli (16). The inflammatory pathway involved in BPD can be initiated in the prenatal period, for example chorioamnionitis (inflammation of the foetal membranes) elicits a pulmonary inflammatory response in the neonate (16). Inflammation is then exacerbated by neonatal events including supplemental oxygen therapy, prolonged mechanical ventilation and pulmonary and systemic infections following preterm birth, which decrease alveolar number, internal lung surface area and in turn, lung function (24-26).

Oxidative stress results from the production of reactive oxygen species (ROS) exceeding the capacity of antioxidant defences (27). Preterm infants are often supplemented with oxygen at high concentrations to ensure adequate tissue oxygenation, which can produce ROS, particularly in the presence of inflammation in the lung (23). Subsequent lung injury is of particular consequence to preterm infants, who have decreased intracellular antioxidant defences, such as reduced levels of superoxide dismutase and catalase, compared to term infants (23). Additionally, many preterm neonates have detectable free iron in their airway surface liquid, which is associated with increased risk of oxidative injury (28) due to the production of toxic hydroxyl radicals (23). Several studies observe associations between high levels of oxidative stress markers – including *o*-tyrosine, super-oxide proteinases, uric acid, ascorbic acid, surfactant lipid peroxidation and hydroxyl radicals – with prematurity and greater risk of BPD (29, 30).

Interplay between inflammation, oxidative stress and lung injury in prematurity

During pulmonary inflammation, activated neutrophils release ROS, which functionally deactivate protease inhibitors, leading to excess proteases (31), which may contribute to BPD pathogenesis (16). The release of ROS not only favours tissue damage, but increases capillary permeability, facilitating the passage of cytokines and contributing to a further increase in inflammation (32). In this way, inflammation and oxidative stress occur in a complex simultaneous and circular manner. Inflammation-induced oxidative stress is evident in infants with BPD, whose alveolar macrophages produce larger amounts of hydrogen peroxide than controls (33).

In summary, pulmonary inflammation and oxidative stress play a key role in lung injury and BPD pathogenesis. We hypothesise that consequences of these factors may persist beyond the neonatal period and contribute to the poor long-term respiratory outcomes seen in survivors of preterm birth. Certainly, as the oldest survivors of this "new" BPD reach their 20s, it is becoming clear that contemporary survivors of preterm birth are at increased risk of significant and persistent respiratory disease (34).

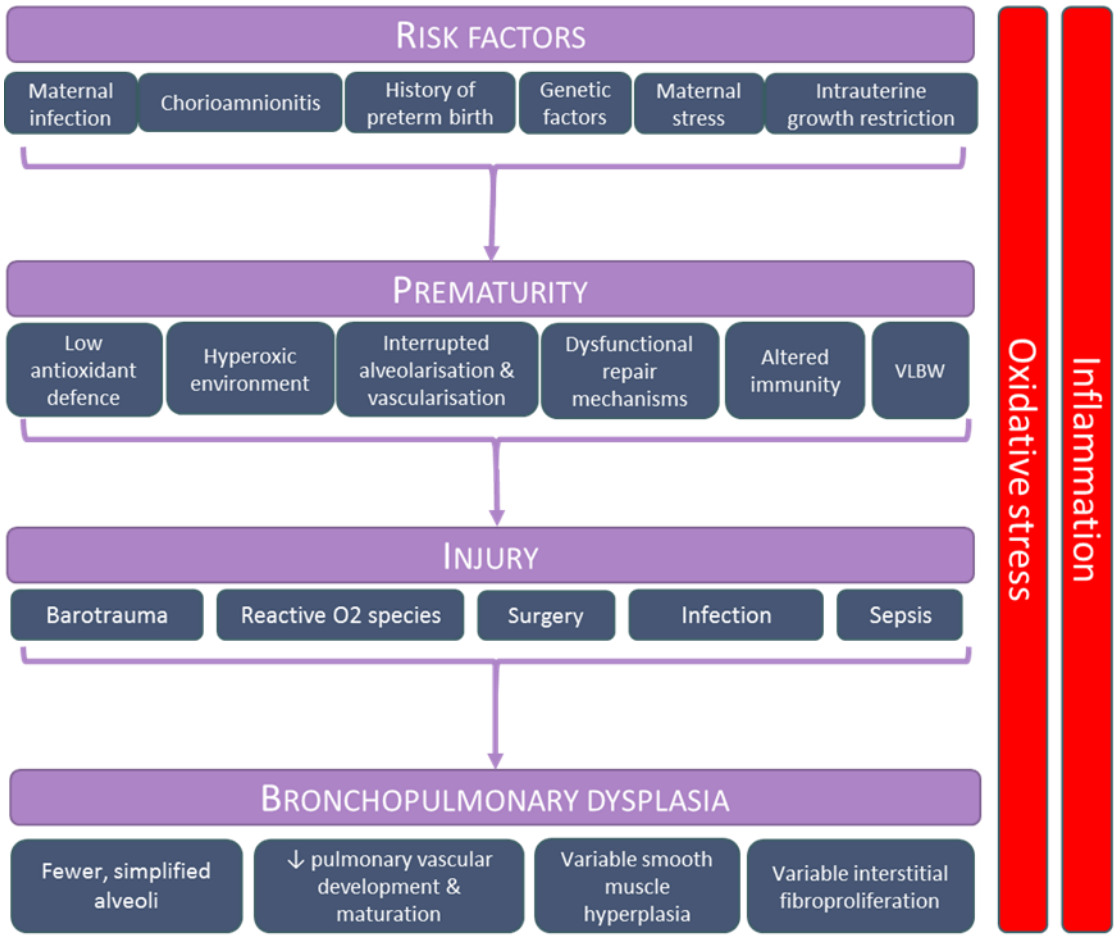

Figure 2. Pathophysiology and risk factors for bronchopulmonary dysplasia (BPD). Development of BPD is complex and multifactorial. Inflammation and oxidative stress are associated with many of the risk factors and have been implicated in the pathophysiology of BPD, although the precise mechanisms are not entirely understood. BPD is characterised by altered lung development, with simplified alveoli, potential airway remodelling and a decrease in the growth and maturation of the pulmonary vasculature.

BEYOND THE NICU

Structural abnormalities

Most of the evidence underpinning our current understanding of the pulmonary histopathology of BPD arise from studies of a few fatal cases in infants; with only one case described beyond three years of age (35). These studies describe simplified alveoli, decreased vasculature and variable smooth muscle hyperplasia in BPD, and all suggest a delay in alveolar development in survivors of prematurity. Animal models of 'new' BPD describe decreased alveolar septation and hyperplasia (36), enlarged and simplified alveoli and increased pulmonary fibrosis, which worsen over time (37). These are associated with abnormal lung mechanics (38) and shortened life-span (39). These findings suggest that arrested development and insults to the lung in the neonatal period can result in long-term function-limiting structural alterations.

Computed tomography (CT) scans from preterm infants and children born in the surfactant era show high rates of structural abnormalities with the presence of bronchial wall thickening (suggesting inflammation or post-inflammatory changes), linear and triangular sub-pleural opacities (likely scarring) and decreased pulmonary attenuation with limited amounts of emphysema detected (3 %) (40, 41). Similar findings of sub-pleural opacities were found in several cross-sectional studies at school age (42-44), with emphysema, bronchial wall thickening and fibrosis also noted (42, 45). More severe structural lung disease is associated with increased BPD severity and poorer lung function outcomes (40, 41, 43, 44). Mixed patterns of reduced lung attenuation, bronchial wall thickening and inverse bronchopulmonary artery diameter ratios have also been observed in preterm-born adults (46), with one study of young adult survivors of severe "old" BPD reporting an 84 % incidence of emphysema, which correlated with lower forced expiratory volume in 1 second (FEV_1) values (47). A more recent report of three cases of lung transplantation for 'new' bronchopulmonary dysplasia in adults reported similar radiologic findings including hyperexpanded lungs, air trapping and mosaic attenuation, with pathologic examination revealing large, simplified alveoli, fibrosis, muscular hypertrophy and fibromuscular hyperplasia (48). As no longitudinal CT imaging studies have been performed, it remains unknown whether the decreased pulmonary attenuation seen during mid-childhood resolves, persists or progresses to the emphysema observed in young adults.

In contrast to the results noted by chest CT, the helium-3 magnetic resonance (^{3}HeMR) imaging technique, which assesses alveolar dimensions and uniformity, has shown normalisation after preterm birth (49). Despite lower FEV_1 values in preterm-born children, alveolar dimensions were not different to term-born children; possibly suggesting catch-up alveolarisation in preterm-born children (49). Even with this apparent recovery in alveolarisation after preterm birth, there is no evidence of improved functional outcomes (50). As such, it is important to further investigate the contribution of structural alterations of the lung, or alternate pathological pathways, to this persistent functional deficit.

Ongoing respiratory morbidity

Beyond the neonatal period, survivors of preterm birth experience persistent respiratory symptoms. In the first years of life, preterm-born infants experience more wheeze, use inhaled medications and are re-hospitalised more frequently than their term-born counterparts (51). Approximately 50 % of those with BPD are re-hospitalised in the first year (52). At school age, preterm-born children are up to 5 times more at risk of wheezing disorders than their term born counterparts (53), and commonly report respiratory symptoms independent of a neonatal diagnosis of BPD (54). Additionally, preterm-born children are more likely to have exercise-induced respiratory symptoms, be diagnosed with asthma and are twice as likely to be prescribed inhaler medications, including inhaled corticosteroids (ICS), than term born children (55, 56).

The burden of respiratory disease likely persists beyond childhood with adolescents from a large Swedish cohort (born in the late-preterm period) reporting more wheeze than their term counterparts (57). Conversely, young adults born preterm from a small study by Landry *et al.* (58) reported no differences in respiratory symptoms compared to those born at term. However, adult survivors of 'old' BPD report increased symptoms, with much higher rates of wheeze and asthma medication use than full-term controls (59). Additionally, a microsimulation study on the lifetime burden of BPD found that the expected quality-adjusted life years of the average individual diagnosed with BPD is only 42, with an annual quality of life score of less than 0.58, with 1 indicating perfect health and 0 indicating death (60).

Although cross-sectional studies generally show increased rates of respiratory symptoms in those born preterm compared to those born at term, longitudinal data describing how these symptoms change over time is lacking. In extremely preterm children, prevalence of respiratory symptoms, hospitalisation and medication use is reported to significantly decrease between 2 and 6 years of age, although high rates of chest deformities at 6 years suggest ongoing respiratory morbidity (61). Simpson *et al.* (62) showed that in children born at less than 32 weeks gestation, rates of wheeze, cough and asthma medication use remained consistent between early and mid-childhood. As the oldest survivors of prematurity in the post-surfactant era are only now reaching early adulthood, more adequately powered studies are needed to establish the prevalence and characteristics of respiratory symptoms, and the consequences of 'new' BPD throughout life.

Impaired lung function

Respiratory symptoms are often reported in the presence of lung function abnormalities in preterm children and lung function is further decreased in children with BPD (34). Cross-sectional studies report obstructive lung disease throughout childhood and into adulthood with abnormal spirometry parameters in preterm children – namely lower forced expiratory volumes in 1 second (FEV1), lower forced mid-expiratory flow (FEF_{25-75}) with normal forced vital capacities (42, 45, 54-58, 63-67). Airway obstruction is partially reversible with bronchodilators in about one third of preterm-born infants (68), with studies reporting between 25-60 % of those with BPD responding to bronchodilators at school age (42, 55, 64, 68). However, it remains unknown whether regular, long-term use of bronchodilators is associated with improved lung function outcomes in this population.

Some studies report airway obstruction in the presence of modest restriction (69, 70) with lower lung volumes in preterm infants with and without BPD (71, 72) and reduced residual volume to total lung capacity ratio (RV/TLC) in preterm-born children at school-age (56) compared to those born at term. Preterm born children also exhibit altered respiratory mechanics in comparison to term born controls, which is more pronounced in those with BPD, with increased respiratory system resistance and altered

elastic properties of the respiratory system (reactance); indicating peripheral lung disease from infancy to at least school-age (42, 45, 55, 73-75).

There is conflicting evidence of ventilation inhomogeneity in preterm born children using multiple breath washout techniques, with one study reporting elevated inhomogeneity (76) in preterm infants compared to controls, with another detecting no difference (77). In the EPICure cohort of extremely preterm-born participants in mid-childhood, slightly elevated ventilation inhomogeneity (lung clearance index) was reported in preterm children compared to those born at term (69) while no differences were reported in a West Australian cohort of similar age (54), although this cohort included children up to 32 weeks gestational age. Another recent study found that although lung clearance index was not different, the alveolar phase III of washout revealed elevated ventilation inhomogeneity in the conducting airways (Scond) but not the acinar airways (Sacin) of extremely preterm-born children at school age, suggesting a functionally normal alveolar compartment with impaired function of the proximal airways in comparison to controls (78). From these studies it may be likely that ventilation inhomogeneity, if it exists in this population, is mild and may only be seen in those born extremely preterm.

Assessments of gas exchange (DLCO) also provide conflicting results, with some studies suggesting decreased alveolar-capillary membrane function in preterm-born children throughout childhood and adolescence (55, 56, 58, 79), while others fail to detect a difference (45, 80). Those who reported impaired gas exchange assessed cohorts that were born more premature than those who reported no difference, which may account for the inconsistencies between studies. Taken together, it therefore remains unclear whether the alveolar compartment and pulmonary vasculature of preterm-born children develops normally and whether any altered alveolar or pulmonary vascular development is functionally significant in mid-childhood and beyond.

Longitudinal lung function

Few studies have examined longitudinal lung function beyond infancy in survivors of preterm birth, and often in small numbers of participants, of the most extreme neonatal course, prior to routine use of exogenous surfactant and in the absence of all-ages reference equations. Consequently, findings are conflicting.

Across childhood, Filippone *et al.* (81) showed poor lung function in 17 BPD survivors, which tracked (but did not change trajectory compared to controls) through 2, 9 and 15 years of age. Similarly, no 'catch-up' in lung function from mid-childhood to adolescence was observed in moderate-late preterm-born children with poor lung function (57). Some studies have documented improvements in FEV_1 z-score with individuals born at 33-34 week gestation improving from 8 to 16 years of age (63). Other studies of preterm children of lower gestational age report significant declines in FEV_1 z-scores from childhood to adolescence or young adulthood (82, 83) and more decline in those with BPD or current smoking status (65). Studies into adulthood report both persistently low (66, 67) and declining (84, 85) lung function in those born preterm in the pre-surfactant era, with one study reporting lung function declines of at least 0.1 z-scores per year during childhood in those with BPD (Figure 3.) (62).

Consequently, it remains to be established whether lung function deficits in those born preterm track through life or deteriorate over time, especially those born in the era of "new" BPD. Regardless, low 'peak' lung function (FEV_1) and unknown age-related rate of FEV_1 decline during childhood may put ex-preterm children at risk of early onset chronic lung disease in adulthood and early mortality (86). Indeed, a recent meta-analysis demonstrated that individuals born very preterm did not reach the expected peak airway capacity in adolescence and young-adulthood (87). A recent systematic review found that individuals born extremely prematurely (less than 27 weeks gestation) had a 2- to 4-fold relative risk of all-cause mortality (88), largely attributed to cardiorespiratory causes (89). Additionally, the preterm population are highly susceptible to known predictors of lung function decline, such as increased risk of respiratory infection during early childhood, increased asthma diagnoses and increased airway hyper-responsiveness (86). Those born preterm may also be subject to other mechanisms of persistent or progressive lung disease that are not influenced by exposures, such as altered growth, genetics, immunity and persistent inflammation and oxidative stress, which are described below.

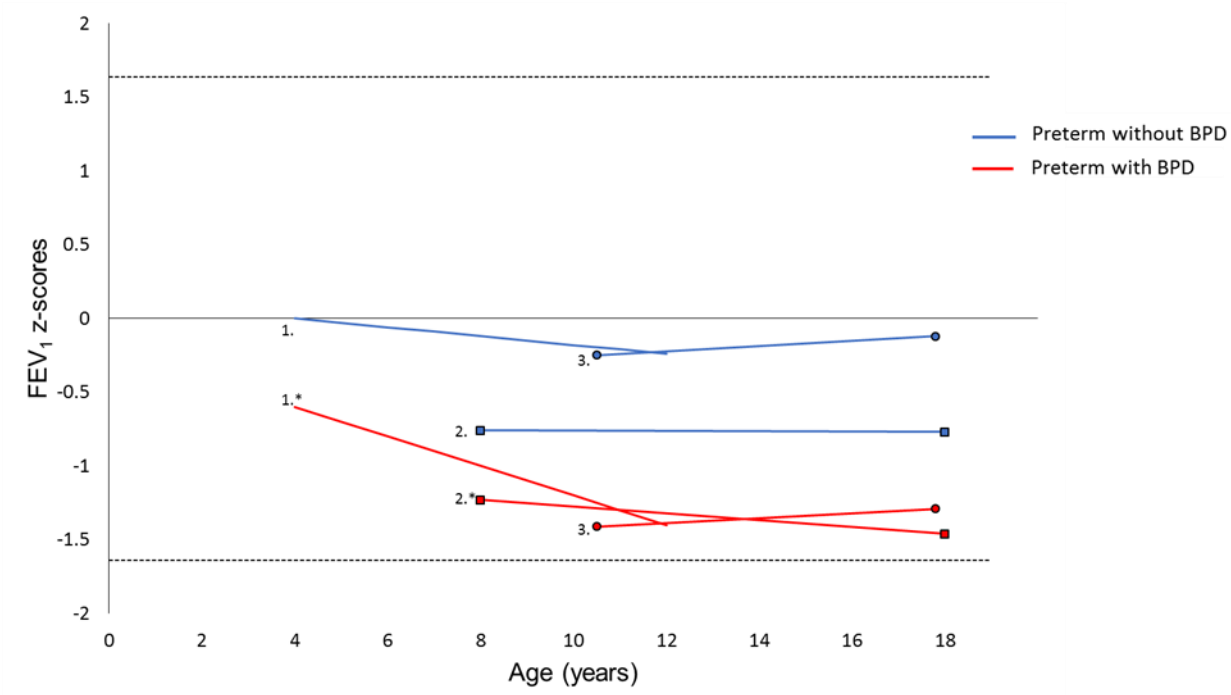

Figure 3. Longitudinal change in forced expiratory volume in 1 s (FEV_1) over time in children born preterm in the post surfactant era with and without bronchopulmonary dysplasia (BPD) (2). 1. Simpson *et al.* report declining lung function trajectories in children born preterm, modelled from 347 lung function visits of children aged 4 to 12 years. FEV_1 declined by a rate of at least 0.1 z-scores per year in children with BPD over the course of the study (62). 2. Doyle *et al.* show increased impairments in airflow from 8 to 18 years of age in survivors of extreme premature birth, which was more pronounced in those with BPD and those smoking at 18 years (65). 3. Vollsaeter *et al.* reported low FEV_1 in extremely preterm children which tracked between 10 and 18 years of age for those with and without BPD (66). Upper and lower limits of normal are represented by dotted lines.
*Indicates significant lung function decline over time relative to a representative population of term-born controls. These studies have shown lung function tracks at z-scores of zero for healthy term-born children. Note: Confidence intervals are not displayed due to inconsistent reporting of results between studies.

POTENTIAL MECHANISMS:

Genetics

Although genetics are known to play a role in the susceptibility of developing BPD (21), it is unknown whether these genetic variances influence the persistence of respiratory morbidity beyond the neonatal period. Because of the multi-factorial nature of the disease, no specific genetic predictors of BPD have been identified, although associations have been made between BPD and altered innate and adaptive immune responses, surfactant metabolism and potential reductions in growth factors like vascular endothelial growth factor (VEGF) (90). Siezen and colleagues reported increased genetic susceptibility to respiratory syncytial virus (RSV) in preterm-born compared to term-born children, with these differences likely manifested in airway remodelling and innate immunity such as altered interferon and transforming growth factor-beta (TGF-β) function and altered response to inflammation (91). This current evidence suggests that genetics may predispose preterm-born individuals to altered immunity, lung repair and lung growth, which can have long-term implications on respiratory health (Figure 2).

Immunity

Normal 'programming' of the immune system occurs *in utero* and throughout the first year of life (92). Birth prior to term results in incomplete maternal transfer of antibodies (which occurs during the 3rd trimester) to the preterm infant, which may play a role in the increased susceptibility to infection during the first year of life (92). Prenatal exposures like antenatal glucocorticoids, prenatal infections and inflammation contribute to altered immunity in preterm infants, who have deficiencies in antimicrobial peptides, altered cellular responses to infection (92) and may have a different and unstable microbial colonisation (93, 94). These early life influences may result in altered immune programming and long-term immune deficiencies (92). Indeed, young adults who were born extremely premature exhibit significant dysbiosis in their airway microbiome (95). Long-term alterations in immunity may put survivors of preterm birth at risk of increased incidence and severity of respiratory infection, a known risk factor for accelerated lung function decline in adulthood (86).

Inflammation and OS

Beyond the neonatal period, data to identify and characterise ongoing inflammation and oxidative stress in survivors of preterm birth is limited. Increased levels of chemokines, growth factors, T-helper cytokines (Th-1, Th-2 and Th-17 cytokines) and immunomodulatory mediators have been detected in nasopharyngeal aspirates from preterm infants at 1 year of age (96). One small study reported that inflammatory markers in exhaled breath condensate did not differ between school-aged healthy and preterm-born children, although as only a few subjects were studied this finding may lack statistical power (97). Other small studies report increased neutrophilic inflammation, such as a 16-fold increase in sputum neutrophils and a 3-fold increase in sputum IL-8 in 16 children with "old" BPD (98) and increased levels of urinary leukotriene E_4 in children born preterm regardless of BPD diagnosis (99). It is clear that inflammation is a common underlying process that affects and is affected by predictors of respiratory morbidity like environmental exposures, immune responses, growth factors and genetics. As such, it is likely that inflammation persists in the preterm lung throughout childhood, and must be considered in light of persisting and possibly declining lung function throughout life.

Markers of oxidative stress are abundant in other chronic lung disorders and are linked to ongoing airway inflammation and remodelling (100), however there are limited data in survivors of preterm birth beyond infancy. Filippone *et al.* (101) reported that adolescents born preterm with and without a history of BPD, had "unexpected" ongoing oxidative stress with increased levels of 8-isoprostane in exhaled breath condensates in the presence of lower lung function when compared to term-born adolescents. The underlying mechanisms of this continuing oxidative stress remain unclear, but the implication that oxidative stress persists through to young adulthood suggests that oxidative damage may play a role in ongoing respiratory morbidity.

Given the integral role of inflammatory and oxidative stress pathways in the pathogenesis of BPD – and their key role in similar respiratory diseases like chronic obstructive pulmonary disease (COPD) – it is an important area for future investigation.

TOOLS TO HELP CHARACTERISE LUNG DISEASE:

Measuring biomarkers of lung disease

Identifying biomarkers of lung disease in premature infants would not only provide a clearer understanding of the underlying pathology but also unlock potential avenues for early intervention. Biomarkers can be identified in different biological fluids such as blood, urine and saliva. Samples that are specific to the lung are often collected invasively, such as with bronchoalveolar lavage fluids and tracheal aspirates, or can be challenging to collect especially from children, like induced sputum. The appeal of exhaled breath condensate is the relative ease and non-invasive nature of sample collection. The utility of exhaled breath condensate as a lung-specific sample has been explored in small studies, showing evidence of oxidative stress in the airways of preterm-born children compared to those born at term (101, 102). However, despite a few targeted markers of lung inflammation and oxidative stress being identified, it is unlikely that a single marker of disease will be reliable. Rather, it will be useful to identify a panel of biomarkers that characterise ongoing lung disease of prematurity (103).

Risk factors for long-term respiratory morbidity are multifactorial and influenced by prenatal, postnatal and childhood events. To examine the complex, multifactorial mechanisms of lung function decline in this vulnerable group more comprehensive, systems biology approaches are needed. ‘Omics methods – which include genomics, epigenomics, microbiomics, transcriptomics, proteomics and metabolomics – have been able to distinguish between neonates who go on to develop BPD and those who do not (104).

Metabolomics identifies the end-products of metabolism and as such may also identify the most modifiable pathways of disease. In small metabolomics studies, analysis of tracheal aspirates and urine from neonates could discriminate between those who would and would not go on to develop BPD. (105-107). These studies show metabolomic patterns associated with oxidative stress and altered lipid and amino acid metabolism as potentially predictive of BPD development (103).

Beyond infancy, however, only one metabolomics investigation has been reported in survivors of preterm birth - a metabolomics study which clearly delineated between the metabolomic profile of exhaled breath condensate in healthy term adolescents and those with BPD (108). This study suggests differing surfactant lipid profiles, despite the small number of adolescents (all with BPD) exhibiting heterogeneous clinical symptoms and inhaled corticosteroid usage (108).

Metabolomics studies have also distinguished between health and disease in other respiratory conditions such as acute respiratory distress syndrome (109), COPD (110) and asthma (111, 112). To date there are several human metabolomic studies using EBC, urine, plasma and serum which have discriminated between healthy and asthmatic individuals (112). These studies often report that pathways associated with oxidative stress, inflammation, immunity and lipid metabolism differ between groups (112). A 2011 study of 41 school-aged atopic asthmatic children and 12 age-matched controls showed that urine metabolomics could distinguish between groups, and the asthmatic group had a metabolite signature correlated with immune modulation (113). It is unknown whether similar pathways are altered in children born preterm with underlying lung disease.

Although more adequately powered studies are needed, 'omics tools provide a promising approach to better understanding the mechanisms underlying ongoing lung disease in survivors of preterm birth. Importantly, these methods may help to identify potential therapeutic targets and capture the metabolic response to pharmaceutical interventions.

Conclusion

Survivors of preterm birth face a lifetime of respiratory morbidity, with impaired lung structure and function which may worsen over time. It is becoming increasingly clear that not only do prenatal and neonatal events increase the risk of impaired pulmonary function in preterm-born individuals, but exposures through childhood – such as poor growth, infection, inflammation and oxidative stress - may play a larger role than previously thought. Adequately powered systems biology approaches are needed to discern predictors and targets for preventing chronic lung disease after prematurity, due to its complex and multi-factorial nature.

REFERENCES

1. Phases of lung development.: University of Fribourg; 2005 [Available from: http://www.embryology.ch/dutch/rrespiratory/phasen01.html.
2. Urs R, Kotecha S, Hall GL, Simpson SJ. Persistent and progressive long-term lung disease in survivors of preterm birth. Paediatric respiratory reviews. 2018.
3. Smith LJ, McKay KO, van Asperen PP, Selvadurai H, Fitzgerald DA. Normal development of the lung and premature birth. Paediatric respiratory reviews. 2010;11(3):135-42.
4. Kotecha S. Lung growth: implications for the newborn infant. Archives of Disease in Childhood-Fetal and Neonatal Edition. 2000;82(1):F69-F74.
5. Burri PH. Fetal and postnatal development of the lung. Annual review of physiology. 1984;46(1):617-28.
6. Burri PH. Structural aspects of postnatal lung development–alveolar formation and growth. Neonatology. 2006;89(4):313-22.
7. Otto-Verberne CJ, Ten Have-Opbroek AA, Balkema JJ, Franken C. Detection of the type II cell or its precursor before week 20 of human gestation, using antibodies against surfactant-associated proteins. Anatomy and embryology. 1988;178(1):29-39.
8. Butler JP, Loring SH, Patz S, Tsuda A, Yablonskiy DA, Mentzer SJ. Evidence for adult lung growth in humans. New England Journal of Medicine. 2012;367(3):244-7.
9. Zeltner TB, Burri PH. The postnatal development and growth of the human lung. II. Morphology. Respiration physiology. 1987;67(3):269-82.
10. Northway WH, Jr., Rosan RC, Porter DY. Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. N Engl J Med. 1967;276(7):357-68.
11. Kamath BD, Macguire ER, McClure EM, Goldenberg RL, Jobe AH. Neonatal mortality from respiratory distress syndrome: lessons for low-resource countries. Pediatrics. 2011;127(6):1139-46.
12. Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller AB, Narwal R, et al. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. Lancet. 2012;379(9832):2162-72.
13. Australian, Network NZN, Chow SS, Creighton P, Kander V, Haslam R, et al. Report of the Australian and New Zealand Neonatal Network, 20162018.
14. Abman SH. Bronchopulmonary Dysplasia: "A Vascular Hypothesis". American journal of respiratory and critical care medicine. 2001;164(10):1755-6.
15. Jobe AH. The new bronchopulmonary dysplasia. Current opinion in pediatrics. 2011;23(2):167.
16. Speer CP. Inflammation and bronchopulmonary dysplasia: a continuing story. Semin Fetal Neonatal Med. 2006;11(5):354-62.
17. Lal MK, Manktelow BN, Draper ES, Field DJ. Chronic lung disease of prematurity and intrauterine growth retardation: a population-based study. Pediatrics. 2003;111(3):483-7.
18. Hoo A-F, Henschen M, Dezateux C, Costeloe K, Stocks J. Respiratory function among preterm infants whose mothers smoked during pregnancy. American journal of respiratory and critical care medicine. 1998;158(3):700-5.
19. Costeloe KL, Hennessy EM, Haider S, Stacey F, Marlow N, Draper ES. Short term outcomes after extreme preterm birth in England: comparison of two birth cohorts in 1995 and 2006 (the EPICure studies). Bmj. 2012;345:e7976.
20. Avery ME, Tooley WH, Keller JB, Hurd SS, Bryan MH, Cotton RB, et al. Is chronic lung disease in low birth weight infants preventable? A survey of eight centers. Pediatrics. 1987;79(1):26-30.
21. Somaschini M, Castiglioni E, Volonteri C, Cursi M, Ferrari M, Carrera P. Genetic predisposing factors to bronchopulmonary dysplasia: preliminary data from a multicentre study. The Journal of Maternal-Fetal & Neonatal Medicine. 2012;25(sup4):119-22.
22. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. Am J Respir Crit Care Med. 2001;163(7):1723-9.
23. Saugstad OD, editor Bronchopulmonary dysplasia—oxidative stress and antioxidants. Seminars in Neonatology; 2003: Elsevier.

24. Schmalisch G, Wilitzki S, Roehr CC, Proquitté H, Bührer C. Differential effects of immaturity and neonatal lung disease on the lung function of very low birth weight infants at 48–52 postconceptional weeks. Pediatric pulmonology. 2013;48(12):1214-23.
25. Hislop AA, Wigglesworth JS, Desai R, Aber V. The effects of preterm delivery and mechanical ventilation on human lung growth. Early Human Development. 1987;15(3):147-64.
26. Schmalisch G, Wilitzki S, Fischer HS, Bührer C. Effect of intubation and mechanical ventilation on exhaled nitric oxide in preterm infants with and without bronchopulmonary dysplasia measured at a median postmenstrual age of 49 weeks. BMC research notes. 2014;7(1):389.
27. Dennery PA. Role of redox in fetal development and neonatal diseases. Antioxidants and Redox Signaling. 2004;6(1):147-53.
28. Gerber C, Bruchelt G, Stegmann H, Schweinsberg F, Speer CP. Presence of bleomycin-detectable free iron in the alveolar system of preterm infants. Biochemical and biophysical research communications. 1999;257(1):218-22.
29. Schock BC, Sweet DG, Halliday HL, Young IS, Ennis M. Oxidative stress in lavage fluid of preterm infants at risk of chronic lung disease. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2001;281(6):L1386-L91.
30. Clerch LB, Wright AE, Coalson JJ. Lung manganese superoxide dismutase protein expression increases in the baboon model of bronchopulmonary dysplasia and is regulated at a posttranscriptional level. Pediatric research. 1996;39(2):253-8.
31. Speer C. Pulmonary inflammation and bronchopulmonary dysplasia. Journal of Perinatology. 2006;26(S1):S57.
32. Perrone S, Tataranno ML, Buonocore G. Oxidative stress and bronchopulmonary dysplasia. Journal of clinical neonatology. 2012;1(3):109.
33. Speer CP, Groneck P, editors. Oxygen radicals, cytokines, adhesion molecules and lung injury in neonates. Seminars in Neonatology; 1998: Elsevier.
34. Kotecha SJ, Edwards MO, Watkins WJ, Henderson AJ, Paranjothy S, Dunstan FD, et al. Effect of preterm birth on later FEV1: a systematic review and meta-analysis.(forced expiratory volume)(Report). Thorax. 2013;68(8):760.
35. Husain AN, Siddiqui NH, Stocker JT. Pathology of arrested acinar development in postsurfactant bronchopulmonary dysplasia. Human Pathology. 1998;29(7):710-7.
36. Coalson JJ, Winter VT, Siler-Khodr T, Yoder BA. Neonatal chronic lung disease in extremely immature baboons. American journal of respiratory and critical care medicine. 1999;160(4):1333-46.
37. Warner BB, Stuart LA, Papes RA, Wispé JR. Functional and pathological effects of prolonged hyperoxia in neonatal mice. American Journal of Physiology-Lung Cellular and Molecular Physiology. 1998;275(1):L110-L7.
38. Yee M, Chess PR, McGrath-Morrow SA, Wang Z, Gelein R, Zhou R, et al. Neonatal oxygen adversely affects lung function in adult mice without altering surfactant composition or activity. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2009;297(4):L641-L9.
39. Yee M, White RJ, Awad HA, Bates WA, McGrath-Morrow SA, O'Reilly MA. Neonatal hyperoxia causes pulmonary vascular disease and shortens life span in aging mice. The American journal of pathology. 2011;178(6):2601-10.
40. Tonson La Tour A, Spadola L, Sayegh Y, Combescure C, Pfister R, Barazzone Argiroffo C, et al. Chest CT in bronchopulmonary dysplasia: Clinical and radiological correlations. Pediatric Pulmonology. 2013;48(7):693-8.
41. Mahut B, De Blic J, Emond S, Benoist M-R, Jarreau P-H, Lacaze-Masmonteil T, et al. Chest computed tomography findings in bronchopulmonary dysplasia and correlation with lung function. Archives of Disease in Childhood - Fetal and Neonatal Edition. 2007;92(6):F459.
42. Broström EB, Thunqvist P, Adenfelt G, Borling E, Katz-Salamon M. Obstructive lung disease in children with mild to severe BPD. Respir Med. 2010;104(3):362-70.
43. Aukland SM, Halvorsen T, Fosse KR, Daltveit AK, Rosendahl K. High-resolution CT of the chest in children and young adults who were born prematurely: findings in a population-based study. AJR American journal of roentgenology. 2006;187(4):1012.
44. Aukland SM, Rosendahl K, Owens CM, Fosse KR, Eide GE, Halvorsen T. Neonatal bronchopulmonary dysplasia predicts abnormal pulmonary HRCT scans in long-term survivors of extreme preterm birth. Thorax. 2009;64(5):405.

45. Simpson SJ, Logie KM, O'Dea CA, Banton GL, Murray C, Wilson AC, et al. Altered lung structure and function in mid-childhood survivors of very preterm birth. Thorax. 2017.
46. Howling SJ, Northway WH, Hansell DM, Moss RB, Ward S, Müller NL. Pulmonary sequelae of bronchopulmonary dysplasia survivors: high-resolution CT findings. AJR American journal of roentgenology. 2000;174(5):1323.
47. Wong PM, Lees AN, Louw J, Lee FY, French N, Gain K, et al. Emphysema in young adult survivors of moderate-to-severe bronchopulmonary dysplasia. The European respiratory journal. 2008;32(2):321.
48. Liu N, Cummings OW, Lagstein A, Hage CA, Chan KM, Zhang C. Lung transplantation for bronchopulmonary dysplasia in adults: a clinical and pathologic study of 3 cases. The American journal of surgical pathology. 2020;44(4):509-15.
49. Narayanan M, Beardsmore CS, Owers-Bradley J, Dogaru CM, Mada M, Ball I, et al. Catch-up alveolarization in ex-preterm children. evidence from 3He magnetic resonance. American journal of respiratory and critical care medicine. 2013;187(10):1104-9.
50. Jobe AH, Bancalari E. An All-Inclusive Perspective on Bronchopulmonary Dysplasia. The Journal of Pediatrics. 2021.
51. Pramana IA, Latzin P, Schlapbach LJ, Hafen G, Kuehni CE, Nelle M, et al. Respiratory symptoms in preterm infants: burden of disease in the first year of life.(Clinical report). 2011;16(197).
52. Greenough A, Alexander J, Burgess S, Chetcuti P, Cox S, Lenney W, et al. Home oxygen status and rehospitalisation and primary care requirements of infants with chronic lung disease. Archives of disease in childhood. 2002;86(1):40-3.
53. Been JV, Lugtenberg MJ, Smets E, van Schayck CP, Kramer BW, Mommers M, et al. Preterm birth and childhood wheezing disorders: a systematic review and meta-analysis. PLoS Med. 2014;11(1):e1001596.
54. Simpson SJ, Hall GL, Wilson AC. Lung function following very preterm birth in the era of 'new' bronchopulmonary dysplasia. Respirology. 2015;20(4):535-40.
55. Fawke J, Lum S, Kirkby J, Hennessy E, Marlow N, Rowell V, et al. Lung function and respiratory symptoms at 11 years in children born extremely preterm: the EPICure study. American journal of respiratory and critical care medicine. 2010;182(2):237-45.
56. Korhonen P, Laitinen J, Hyoudynmaa E, Tammela O. Respiratory outcome in school-aged, very-low-birth-weight children in the surfactant era. Acta Pædiatrica. 2004;93(3):316-21.
57. Thunqvist P, Gustafsson PM, Schultz ES, Bellander T, Berggren-Broström E, Norman M, et al. Lung Function at 8 and 16 Years After Moderate-to-Late Preterm Birth: A Prospective Cohort Study. Pediatrics. 2016;137(4).
58. Landry J, Tremblay G, Li P, Wong C, Benedetti A, Taivassalo T. Lung Function and Bronchial Hyperresponsiveness in Adults Born Prematurely A Cohort Study. Annals of the American Thoracic Society. 2016;13(1):17-24.
59. Gough A, Linden M, Spence D, Patterson CC, Halliday HL, McGarvey LPA. Impaired lung function and health status in adult survivors of bronchopulmonary dysplasia. The European respiratory journal. 2014;43(3):808.
60. van Katwyk S, Augustine S, Thébaud B, Thavorn K. Lifetime patient outcomes and healthcare utilization for Bronchopulmonary dysplasia (BPD) and extreme preterm infants: a microsimulation study. BMC pediatrics. 2020;20(1):1-10.
61. Hennessy EM, Bracewell M, Wood N, Wolke D, Costeloe K, Gibson A, et al. Respiratory health in pre-school and school age children following extremely preterm birth. Archives of disease in childhood. 2008;93(12):1037-43.
62. Simpson SJ, Turkovic L, Wilson A, Verheggen M, Logie KM, Pillow JJ, et al. Lung function trajectories throughout childhood in survivors of very preterm birth: A longitudinal cohort study. The Lancet Child and Adolescent Health. 2018.
63. Kotecha SJ, Watkins WJ, Paranjothy S, Dunstan FD, Henderson AJ, Kotecha S. Effect of late preterm birth on longitudinal lung spirometry in school age children and adolescents. Thorax. 2012;67(1):54.
64. Filippone M, Sartor M, Zacchello F, Baraldi E. Flow limitation in infants with bronchopulmonary dysplasia and respiratory function at school age. The Lancet. 2003;361(9359):753-4.

65. Doyle LW, Adams A-M, Robertson C, Ranganathan S, Davis NM, Lee KJ, et al. Increasing airway obstruction from 8 to 18 years in extremely preterm/low-birthweight survivors born in the surfactant era. Thorax. 2017;72(8):712.
66. Vollsaeter M, Roksund OD, Eide GE, Markestad T, Halvorsen T. Lung function after preterm birth: development from mid-childhood to adulthood.(Report). Thorax. 2013;68(8):767.
67. Vollsæter M, Clemm H, Satrell E, Eide G, Røksund O, Markestad T, et al. Adult Respiratory Outcomes of Extreme Preterm Birth: A Regional Cohort Study. Annals of the American Thoracic Society. 2015;12(3):313-22.
68. Fakhoury KF, Sellers C, Smith EOB, Rama JA, Fan LL. Serial measurements of lung function in a cohort of young children with bronchopulmonary dysplasia. Pediatrics. 2010;125(6):e1441.
69. Lum S, Kirkby J, Welsh L, Marlow N, Hennessy E, Stocks J. Nature and severity of lung function abnormalities in extremely pre-term children at 11 years of age. European Respiratory Journal. 2011;37(5):1199-207.
70. Filbrun AG, Popova AP, Linn MJ, McIntosh NA, Hershenson MB. Longitudinal measures of lung function in infants with bronchopulmonary dysplasia. Pediatric pulmonology. 2011;46(4):369.
71. Greenough A, Dimitriou G, Bhat RY, Broughton S, Hannam S, Rafferty GF, et al. Lung volumes in infants who had mild to moderate bronchopulmonary dysplasia. European journal of pediatrics. 2005;164(9):583-6.
72. Hjalmarson O, Sandberg KL. Lung function at term reflects severity of bronchopulmonary dysplasia. The Journal of pediatrics. 2005;146(1):86-90.
73. Vrijlandt EJLE, Boezen HM, Gerritsen J, Stremmelaar EF, Duiverman EJ. Respiratory Health in Prematurely Born Preschool Children with and without Bronchopulmonary Dysplasia. The Journal of Pediatrics. 2007;150(3):256-61.
74. Thunqvist P, Gustafsson P, Norman M, Wickman M, Hallberg J. Lung function at 6 and 18 months after preterm birth in relation to severity of bronchopulmonary dysplasia. Pediatric pulmonology. 2015;50(10):978-86.
75. Udomittipong K, Sly PD, Patterson HJ, Gangell CL, Stick SM, Hall GL. Forced oscillations in the clinical setting in young children with neonatal lung disease. The European respiratory journal. 2008;31(6):1292.
76. Hülskamp G, Pillow JJ, Dinger J, Stocks J. Lung function tests in neonates and infants with chronic lung disease of infancy: functional residual capacity. Pediatric pulmonology. 2006;41(1):1-22.
77. Latzin P, Roth S, Thamrin C, Hutten GJ, Pramana I, Kuehni CE, et al. Lung volume, breathing pattern and ventilation inhomogeneity in preterm and term infants. PloS one. 2009;4(2):e4635.
78. Yammine S, Schmidt A, Sutter O, Fouzas S, Singer F, Frey U, et al. Functional evidence for continued alveolarisation in former preterms at school age? The European respiratory journal. 2016;47(1):147.
79. Satrell E, Røksund O, Thorsen E, Halvorsen T. Pulmonary gas transfer in children and adolescents born extremely preterm. The European respiratory journal. 2013;42(6):1536.
80. Vom Hove M, Prenzel F, Uhlig HH, Robel-Tillig E. Pulmonary Outcome in Former Preterm, Very Low Birth Weight Children with Bronchopulmonary Dysplasia: A Case-Control Follow-Up at School Age. The Journal of Pediatrics. 2014;164(1):40-5.e4.
81. Filippone M, Bonetto G, Cherubin E, Carraro S, Baraldi E. Childhood course of lung function in survivors of bronchopulmonary dysplasia. Jama. 2009;302(13):1418-20.
82. Narang I, Rosenthal M, Cremonesini D, Silverman M, Bush A. Longitudinal Evaluation of Airway Function 21 Years after Preterm Birth. American Journal of Respiratory and Critical Care Medicine. 2008;178(1):74-80.
83. Chambers DC. Lung Function in Ex-preterm Adults. American journal of respiratory and critical care medicine. 2009;179(6):517-.
84. Gibson AM, Reddington C, McBride L, Callanan C, Robertson C, Doyle LW. Lung function in adult survivors of very low birth weight, with and without bronchopulmonary dysplasia. Pediatric Pulmonology. 2015;50(10):987-94.
85. Saarenpaa H-K, Tikanmaki M, Sipola-Leppanen M, Hovi P, Wehkalampi K, Siltanen M, et al. Lung function in very low birth weight adults.(Report). 2015;136(4).
86. Martinez FD. Early-life origins of chronic obstructive pulmonary disease. New England Journal of Medicine. 2016;375(9):871-8.

87. Doyle LW, Andersson S, Bush A, Cheong JL, Clemm H, Evensen KAI, et al. Expiratory airflow in late adolescence and early adulthood in individuals born very preterm or with very low birthweight compared with controls born at term or with normal birthweight: a meta-analysis of individual participant data. The Lancet respiratory medicine. 2019;7(8):677-86.
88. Crump C. Preterm birth and mortality in adulthood: a systematic review. Journal of Perinatology. 2020;40(6):833-43.
89. Crump C, Sundquist J, Winkleby MA, Sundquist K. Gestational age at birth and mortality from infancy into mid-adulthood: a national cohort study. The Lancet Child & Adolescent Health. 2019;3(6):408-17.
90. Lavoie PM, Dubé M-P. Genetics of bronchopulmonary dysplasia in the age of genomics. Current opinion in pediatrics. 2010;22(2):134.
91. Siezen CL, Bont L, Hodemaekers HM, Ermers MJ, Doornbos G, Van't Slot R, et al. Genetic susceptibility to respiratory syncytial virus bronchiolitis in preterm children is associated with airway remodeling genes and innate immune genes. The Pediatric infectious disease journal. 2009;28(4):333-5.
92. Melville JM, Moss TJ. The immune consequences of preterm birth. Frontiers in neuroscience. 2013;7.
93. Collado MC, Cernada M, Neu J, Pérez-Martínez G, Gormaz M, Vento M. Factors influencing gastrointestinal tract and microbiota immune interaction in preterm infants. Pediatric research. 2015;77(6):726-31.
94. Gentle SJ, Lal CV. Predicting BPD: lessons learned from the airway microbiome of preterm infants. Frontiers in pediatrics. 2020;7:564.
95. Rofael SA, McHugh TD, Troughton R, Beckmann J, Spratt D, Marlow N, et al. Airway microbiome in adult survivors of extremely preterm birth: the EPICure study. European Respiratory Journal. 2019;53(1).
96. Matías V, San Feliciano L, Fernández JE, Lapeña S, Garrido E, Ardura J, et al. Host and environmental factors influencing respiratory secretion of pro-wheezing biomarkers in preterm children. Pediatric Allergy and Immunology. 2012;23(5):441-7.
97. Korhonen PH, Suursalmi PH, Kopeli T, Nieminen R, Lehtimaki L, Luukkaala T, et al. Inflammatory activity at school age in very low birth weight bronchopulmonary dysplasia survivors. Pediatr Pulmonol. 2015;50(7):683-90.
98. Teig N, Allali M, Rieger C, Hamelmann E. Inflammatory markers in induced sputum of school children born before 32 completed weeks of gestation. J Pediatr. 2012;161(6):1085-90.
99. Halvorsen T, Skadberg BT, Eide GE, Roksund O, Aksnes L, Oymar K. Characteristics of asthma and airway hyper-responsiveness after premature birth. Pediatr Allergy Immunol. 2005;16(6):487-94.
100. MacNee W, Tuder RM. New paradigms in the pathogenesis of chronic obstructive pulmonary disease I. Proceedings of the American Thoracic Society. 2009;6(6):527-31.
101. Filippone M, Bonetto G, Corradi M, Frigo AC, Baraldi E. Evidence of unexpected oxidative stress in airways of adolescents born very pre-term. The European respiratory journal. 2012;40(5):1253-9.
102. Carraro S, Giordano G, Stocchero M, Baraldi E. Exhaled breath condensate: metabolomics. Eur Respir Mon. 2010;49:231-6.
103. Piersigilli F, Bhandari V. Metabolomics of bronchopulmonary dysplasia. Clinica Chimica Acta. 2020;500:109-14.
104. Piersigilli F, Bhandari V. Biomarkers in neonatology: the new "omics" of bronchopulmonary dysplasia. The Journal of Maternal-Fetal & Neonatal Medicine. 2016;29(11):1758-64.
105. Piersigilli F, Lam TT, Vernocchi P, Quagliariello A, Putignani L, Aghai ZH, et al. Identification of new biomarkers of bronchopulmonary dysplasia using metabolomics. Metabolomics. 2019;15(2):20.
106. Pintus MC, Lussu M, Dessì A, Pintus R, Noto A, Masile V, et al. Urinary 1H-NMR metabolomics in the first week of life can anticipate bpd diagnosis. Oxidative medicine and cellular longevity. 2018;2018.
107. Fanos V, Cristina Pintus M, Lussu M, Atzori L, Noto A, Stronati M, et al. Urinary metabolomics of bronchopulmonary dysplasia (BPD): preliminary data at birth suggest it is a congenital disease. The Journal of Maternal-Fetal & Neonatal Medicine. 2014;27(sup2):39-45.

108. Carraro S, Giordano G, Pirillo P, Maretti M, Reniero F, Cogo PE, et al. Airway metabolic anomalies in adolescents with bronchopulmonary dysplasia: new insights from the metabolomic approach. J Pediatr. 2015;166(2):234-9 e1.
109. Evans CR, Karnovsky A, Kovach MA, Standiford TJ, Burant CF, Stringer KA. Untargeted LC–MS metabolomics of bronchoalveolar lavage fluid differentiates acute respiratory distress syndrome from health. Journal of proteome research. 2014;13(2):640-9.
110. Adamko DJ, Nair P, Mayers I, Tsuyuki RT, Regush S, Rowe BH. Metabolomic profiling of asthma and chronic obstructive pulmonary disease: a pilot study differentiating diseases. Journal of Allergy and Clinical Immunology. 2015;136(3):571-80. e3.
111. Reinke SN, Gallart-Ayala H, Gómez C, Checa A, Fauland A, Naz S, et al. Metabolomics analysis identifies different metabotypes of asthma severity. European Respiratory Journal. 2017;49(3).
112. Kelly RS, Dahlin A, McGeachie MJ, Qiu W, Sordillo J, Wan ES, et al. Asthma metabolomics and the potential for integrative omics in research and the clinic. Chest. 2017;151(2):262-77.
113. Mattarucchi E, Baraldi E, Guillou C. Metabolomics applied to urine samples in childhood asthma; differentiation between asthma phenotypes and identification of relevant metabolites. Biomedical Chromatography. 2012;26(1):89-94.

Chapter 2: Aims and Hypotheses

The gaps:

Although it is becoming clear that lung function outcomes are impaired in survivors of preterm birth, additional physiological information can be gained when traditional techniques such as spirometry are accompanied by other techniques such as respiratory oscillometry and exhaled nitric oxide testing, as well as utilising new techniques such as within-breath oscillometry. Establishing a clinical phenotype in this population will improve understanding of the functional implications of preterm birth and is useful for identifying potential underlying mechanisms of impaired function.

The functional outcomes become even more useful when considered in the context of biomarkers of disease. However, there are very limited studies into inflammatory biomarkers beyond the neonatal period in survivors of preterm birth, despite inflammatory and oxidative stress processes playing a key role in the development of chronic lung disease of prematurity.

There is very little research investigating the mechanisms of long-term respiratory morbidity in survivors of preterm birth. As such, systems biology approaches, including metabolomics, have the ability to identify pathways of disease that are implicated in persistent respiratory morbidity and may serve as targets for identification of individuals at-risk of ongoing poor lung health and potential interventions.

This research project was designed to investigate the underlying pathophysiology of impaired lung function and respiratory morbidity in survivors of very preterm birth. Multiple Perth-based cohorts of preterm-born and term-born children participated in studies as neonates, infants, school-aged children and young adults which enabled this research to cross-sectionally examine risk factors and potential biomarkers contributing to the long-term respiratory morbidity of preterm-born children. The main aims and hypothesis of the research project are outlined below:

<u>AIM 1:</u>

Characterise the respiratory functional phenotype in survivors of preterm birth at school-age and young adulthood in comparison to term-born controls using exhaled nitric oxide, respiratory flows and volumes, and respiratory system mechanics; including the novel application of within-breath oscillometry. Additionally, we aimed to assess if lung function parameters were different between preterm-born individuals with and without a neonatal diagnosis of bronchopulmonary dysplasia. We also aimed to determine the impacts of neonatal treatments on lung function in later childhood and adolescence.

Hypothesis 1:

Children born preterm will have reduced lung function, and this will be more pronounced in those with a neonatal diagnosis of bronchopulmonary dysplasia and who required more intensive neonatal interventions.

AIM 2:

Identify markers of inflammation in exhaled breath condensate and urine from infants and children born preterm, and determine which inflammatory markers are associated with poorer lung health during childhood in survivors of preterm birth.

Hypothesis 2:

Inflammatory markers will be increased in preterm-born infants and children compared to those born at term, and further increased in those with reduced lung function.

AIM 3:

Identify a panel of metabolites associated with the diagnosis of bronchopulmonary dysplasia in the neonatal stage of life.

Hypothesis 3:

Neonates who develop bronchopulmonary dysplasia will have a significantly altered metabolome to those who do not.

AIM 4:

Determine if the metabolome differs between survivors of very preterm birth and those born at term, and identify any markers associated with poorer respiratory outcomes in later childhood.

Hypothesis 4:

The metabolome will differ between individuals born preterm and those born at term. Pathways that are altered between groups will help elucidate the pathophysiology of lung function deficits in survivors of preterm birth in later childhood.

SIGNIFICANT CONTRIBUTIONS

Novelty

The research presented in this thesis:

- Is the first to describe intra-breath respiratory mechanics in preterm-born children in childhood and young-adulthood.
- Comprehensively assesses the inflammatory biomarkers in non-invasively collected biofluids in a large cohort for the first time, in order to determine the inflammatory profile of survivors of preterm birth
- Relates the inflammatory profile to lung function in survivors of preterm birth
- Is one of very few metabolomics studies in preterm born neonates with and without chronic lung disease, and the first to look at metabolomics past the first few weeks of life.
- Is the largest metabolomics study beyond infancy in survivors of preterm birth, accompanying only one other small study in this population, which distinguishes between term and preterm-born individuals with and without bronchopulmonary dysplasia. This study extends novelty by relating metabolomic profiles to clinical outcomes, such as lung function.

Significance

This research will help identify early risk factors of long-term respiratory morbidity in survivors of preterm birth, which in turn will enable easier identification of the most high-risk infants who will benefit from closer respiratory follow-up. Additionally, the identification of biomarkers of disease will help elucidate the underlying pathophysiology contributing to persistent and progressive lung function decline in survivors of preterm birth. The identification of a panel of biomarkers which predict the development of chronic lung disease as a neonate and into later life will help guide clinical management both in the neonatal intensive care unit and into childhood. Identifying potentially modifiable pathways of disease using metabolomics is an important first step for more targeted treatment to improve respiratory trajectories and prevent decline in survivors of preterm birth.

Chapter 3: Methodology

This chapter includes an overall description of the participant cohorts involved and methods used in the studies within this thesis. The sections below outline details of the study population, ethics, general lung function principles and sample collection and analyses which are referenced in the subsequent results chapters.

To understand and predict poor respiratory outcomes for survivors of preterm birth, a "life-course" approach was taken. Respiratory outcomes including lung function and biomarker analysis were assessed in preterm-born participants across infancy, childhood and young adulthood. Details of the involvement of participants across all these age groups is described below:

INFANT COHORT

Study population:

Preterm-born infants both with and without a diagnosis of BPD were enrolled into the Preterm Infant Functional and Clinical Outcome (PIFCO) study. Participants were born very preterm (GA<32 weeks) between September 2013 and February 2017 and admitted to King Edward Memorial Hospital (KEMH). Infants were excluded from the study if they had major congenital abnormalities influencing cardiorespiratory function. The overall objectives of the PIFCO study were to better understand the interplay between lung, diaphragm and heart function in very preterm infants and determine the impact of clinical treatment on physiological outcomes. Participants underwent assessment at 36 weeks postmenstrual age and were followed up between 12- and 16-months corrected age to see if heart and lung function improved over the first year of life. Lung function and biological sample collection occurred at both time points. Infant lung function tests were performed in the following order: tidal breathing, multiple breath washout, forced oscillation technique (FOT), oxygen reduction test and exhaled breath condensate collection. Infant lung function testing at 12-16 months was performed under sedation with 80 mg/kg oral chloral hydrate. A validated respiratory questionnaire was completed by the infant's parents. Medical history was obtained from hospital records.

Healthy, term-born controls were recruited by sending information to local day-cares and playgroups, and via word of mouth and social media advertising. Healthy participants were infants aged between 9-18 months, born at or after 37 weeks gestation with no history of wheeze and/or recurrent cough, doctor diagnosis of respiratory disease or any neonatal respiratory disease.

Ethics:

The neonatal component of the PIFCO study was approved by the Women and Newborn Health Service (WNHS) Human Research Ethics Committee (HREC) [Approval #2013091EW]; Follow-up approved by the Child and Adolescent Health Service (CAHS) HREC [Approval #2014083EP]. Sample analysis

was approved by Curtin University (HRE2020-0097). Approval for the recruitment and sample collection from healthy term-born infants was obtained from Curtin University (HRE2018-0407). Written informed consent was received from the parents/guardians of the participants.

Protocol:

Of the infant lung function tests performed, only tidal breathing and oxygen reduction test measures are reported in this thesis. Biofluids were collected for downstream analysis including EBC (at both 36 weeks postmenstrual age and 12-16 months corrected) and urine (only at 36 weeks postmenstrual age).

Urine collection:

Urine samples were collected from neonates at 36 weeks postmenstrual age by extracting urine from cotton wool placed in the infant's nappy. Samples were aliquoted into 1 mL tubes and stored at -80 degrees Celsius.

EBC Collection:

Exhaled breath condensate collection from neonates occurred at bedside in the intensive care nursery in neonates receiving no respiratory support at the time, using a novel method developed as part of this thesis (114). For preterm-born participants at 12-16 months of age, EBC was collected while the infant was sedated for lung function. EBC was collected from healthy, term-born participants during home visits while the infant was in natural sleep. EBC was collected using an R-Tube collection device (Respiratory Research Inc, Charlottesville, VA) adapted for collection in neonates and infants by reducing dead-space, as described in Chapter 4*, "Collecting exhaled breath condensate from non-ventilated preterm-born infants: A modified method*" (114). EBC was collected with the infants in supine position during tidal breathing. Infants breathed into the device through an infant face-mask (size 1, Laerdal Medical AS, Stavanger, Norway) placed over the infant's nose and mouth to create a leak-free seal. Collection took place over 10-15 minutes of tidal breathing through the R-Tube (114). After collection was complete, the condensate was aliquoted into Eppendorf tubes and stored at -80 degrees Celsius (114).

Tidal breathing:

Baseline tidal breathing and minute ventilation was measured in the infants prior to EBC collection using an infant facemask attached to an ultrasonic flow meter (Ecomedics AG, Duernten Switzerland) and calculated using analysis software (Wbreath v3.2.0, Ndd Medizintechnik AG, Zurich, Switzerland) as previously described (115).

Oxygen reduction test:

In neonates, the oxygen reduction test was performed with a headbox placed over the infant's head during natural sleep. In infants at 12-16 months, the test was performed using an infant facemask attached to an ultrasonic flow meter. Nitrogen was introduced into either the headbox or facemask to

adjust the partial pressure of inspired oxygen (P_IO_2) in a stepwise manner. Peripheral oxyhaemoglobin saturation (SpO_2) was measured with a pulse oximeter on the right hand after P_IO_2 had stabilised at each level. The paired measurements of SpO_2 at each P_IO_2 were plotted, and the rightward shift of the SpO_2 / P_IO_2 curve from the oxyhaemoglobin dissociation curve was determined (116).

CHILDHOOD COHORT

Study population:

School-aged children between 6 and 12 years were recruited as part of the Preterm Paediatric Inhaled CorticoSteroid Intervention (PICSI) study. Preterm-born children who were born at or below 32 weeks gestation between 2006 and 2014 and admitted to KEMH were enrolled in the study. Preterm-born participants included those with and without a neonatal diagnosis of BPD. Age-matched term-born controls with no history of respiratory symptoms were also enrolled in the study. The primary objective of this study is to determine how lung inflammation contributes to ongoing respiratory morbidity in survivors of preterm birth and how treatment with inhaled corticosteroids impacts lung inflammation and function in children born preterm.

Ethics:

This study was approved by the CAHS HREC (Approval: RGS367) and reciprocal ethics was obtained from Curtin University (HRE2018-0385). Written informed consent was received from the parents/guardians of the participants.

Protocol:

Participants attended Perth Children's Hospital Research Outpatient clinic for the baseline visit of the trial. At this visit, participants had a general medical check-up, and height, weight, temperature, blood pressure and oxygen saturations were measured. Lung function testing was then performed, including fractional exhaled nitric oxide (FeNO), FOT and spirometry with bronchodilator response. Skin prick testing for 10 common aeroallergens was also performed. Biological samples including EBC, urine, blood and nasal brushings were also collected.

Methods used for childhood and young adult protocols were identical and are detailed below.

YOUNG ADULT COHORT

Study population:

Young adults between 16 and 22 years old were enrolled into the West Australian Lung Health In Prematurity (WALHIP) follow-up study. The WALHIP study is a longitudinal lung-health follow-up of preterm- and term-born participants (54). Preterm participants were born at or below 32 weeks

gestation and admitted to KEMH. Healthy, term-born participants had no history of respiratory symptoms.

Ethics:

This study was approved by the CAHS HREC (Approval: RGS815) and reciprocal ethics was obtained from Curtin University (HRE2019-0723). Written informed consent was received from the participants.

Protocol:

Participants attended Perth Children's Hospital Research Outpatient clinic for lung function testing, including FeNO, FOT and spirometry with bronchodilator response. Participants also had a general medical check-up, and height, weight, temperature, blood pressure and oxygen saturations were measured. Biological samples including EBC, urine, blood and nasal brushings were also collected.

Lung function tests and biological sample collection took place in the following order: history and symptoms, exhaled breath condensate collection, fractional exhaled nitric oxide, spectral and intrabreath oscillometry, spirometry, bronchodilator administration then post-bronchodilator oscillometry and spirometry. Urine sample collection took place when most convenient throughout testing.

History and respiratory symptoms:

Neonatal information was collected via the King Edward Memorial Neonatal Database for preterm infants and includes (but is not limited to) details of exogenous surfactant, postnatal corticosteroids, duration and type of respiratory and oxygen support. Current and past respiratory symptoms at rest and with exercise as well as previous and current treatment use was obtained using modified version of the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire (117). Consent to consult hospital health records to allow verification of participants health information was obtained.

Fractional exhaled nitric oxide (FeNO):

Nitric oxide is normally found in human exhaled breath in varying levels. Increased levels of exhaled nitric oxide are associated with airway eosinophilic inflammation and levels are often increased in asthmatics and those with atopy (118). Fractional exhaled nitric oxide (FeNO) can be measured by exhaling into an analyser. Participants exhaled at a rate of approximately 50 mL/second into a mouthpiece connected to the Medisoft Hypair FeNO device (Medisoft, Sorinnes, Belgium) according to American Thoracic Society/ European Respiratory Society (ATS/ERS) guidelines (119). The mean of 2 or more FeNO values from acceptable manoeuvres that agreed within 10 % was reported.

Spectral and intra-breath oscillometry:

The forced oscillation technique measures respiratory mechanics by superimposing oscillations (or sound waves) at a range of frequencies over a participant's tidal breathing. The response of the

respiratory system to these oscillations is measured with the main outcomes being respiratory resistance (Rrs) and reactance (Xrs) (120). Resistance is the measure of how much pressure is required to drive a certain airflow through the airways, with an increased resistance associated with airway obstruction. Reactance is a measure of lung stiffness, with large negative values associated with a stiffer lung.

For this study the forced oscillation technique was performed using a commercially available tremoFlo C-100 system (Thorasys Inc. Montreal, QC). Oscillometry measurements were performed to the ERS task force guidelines (120) across frequencies ranging from 5 to 37 Hz. During each measurement, the participant wore a nose clip and breathed tidally for 30 seconds through a filtered mouthpiece while seated upright. The participant's cheeks and chin were supported to minimize shunting of the oscillatory signal. Measurements were deemed unacceptable if there was any swallow, mouth movement, air leak, vocalisation or irregular breathing. At least 3 acceptable measurements were obtained where the coefficient of variation for resistance and reactance measured within 10 %. The average values of resistance at 5 Hz (R_5), reactance at 5 Hz (X_5), resonant frequency (Fres) and area under the reactance curve (AX) were reported and converted to z-scores using the Calogero *et al.* reference values (121) for children aged 6-13 years and Oostveen *et al.* reference values for those aged 18-23 years (122). A positive bronchodilator response was calculated according to the 2020 ERS technical guidelines for respiratory oscillometry, namely a −40 % change in R_5, +50 % change in X_5 and −80 % change in AX (120).

Intra-breath oscillometry has been proposed as a more sensitive measure of respiratory mechanics, particularly airway obstruction, compared to conventional spectral oscillometry in children. Within-breath changes are tracked using a single 10 Hz signal to measure resistance and reactance throughout the breathing cycle. The difference between end-inspiration and end-expiration resistance (R_{in-ex}) and reactance (X_{in-ex}) reflect their volume dependence during tidal breathing. These values are independent of age, sex, height and weight in preschool children (123) and may be more sensitive at discriminating between preschool children with and without wheeze (123). Intrabreath measures were collected using the tremoFlo system as above, except with a 10 Hz signal superimposed over the participant's tidal breathing for 60 seconds.

Both spectral and intra-breath oscillometry were performed before and 15 minutes after administration with 400 mcg of salbutamol.

Spirometry:

Spirometry testing to assess airway function and lung volume is the basic screening tool for respiratory abnormality. Forced spirometry involves the measurement of flows and volumes during a maximal forced expiration from total lung capacity (TLC) to residual volume (RV). The largest volume of air that can be expired from the lungs from a position of full inspiration is the Forced Vital Capacity (FVC).

The amount of air that can be expired from the lungs in the first second of a forced expiration is the Forced Expiratory Volume in One Second (FEV_1) (124).

Measuring flow at different lung volumes as in spirometry can indicate restricted or obstructed airways. Lung stiffness or loss of elastic recoil (as in fibrosis or emphysema) results in increased compression of the airways and reduced flows at all lung volumes. Airways obstructed by inflammation or airway smooth muscle (as in asthma or bronchitis) directly limits flow.

Spirometry was performed on the commercially available HypAir system (Medisoft, Sorinnes, Belgium). Spirometry was performed according to ATS/ERS standardisation of spirometry guidelines (124). Participants sat upright and wore nose clips while breathing into a filtered mouthpiece connected to the system. Participants were instructed to take a maximal breath in and a forced exhale until RV. The best value for FEV_1 and FVC were reported of 3 acceptable and repeatable results as per ATS/ERS spirometry guidelines (124). The FEV_1, FVC, FEF_{25-75} and FEV_1/FVC were reported and converted to z-scores using the Global Lung Initiative reference values (125).

Spirometry was performed before and after administration with 400 mcg of salbutamol. A significant bronchodilator response was defined according to ATS/ERS guidelines as an increase in FEV_1 and/or FVC of $\geq$12 % from baseline (126).

Exhaled Breath Condensate (EBC):

Exhaled breath condensate (EBC) was collected using the R-Tube collection device (Respiratory Research Inc, Charlottesville, VA). Participants breathed tidally through the device mouthpiece with a one-way valve connected to a collection tube cooled to approximately -20 degrees Celsius (°C) for 15 minutes. The exhaled air cooled and condensed in the collection tube, with droplets of EBC aliquoted into 1 mL vials and stored at -80 °C until analysed.

Urine:

Participants provided a urine sample into a collection jar. Samples were aliquoted into 1 mL tubes and frozen at -80 °C.

SAMPLE ANALYSIS

This section describes the methods used to analyse samples for markers of inflammation and oxidative stress. EBC samples from neonates and infants were only analysed for leukotriene B4 and 8-isoprostane due to limited sample volume. Otherwise, all samples from all study cohorts were stored and analysed identically using the methods outlined below. Targeted analyses for the known markers for oxidative stress (8-isoprostane), neutrophilic inflammation and immunity (leukotriene B4 and interleukin-8), eosinophilic inflammation (cysteinyl leukotrienes) and airway remodelling (matrix metalloproteinase 9) were undertaken via enzyme-linked immunosorbent-assay (ELISA). Untargeted analyses were

undertaken via the metabolomics methods of liquid chromatography and mass spectrometry, with the aim of discovering potential metabolic pathways that were altered between groups.

Enzyme-linked immune-sorbent assays (ELISAs)

Enzyme-linked immunosorbent assay is a plate-based assay technique commonly used for measuring the presence and concentration of soluble substances such as peptides, antibodies, proteins and antigens in biological samples.

Sample preparation

EBC samples were analysed neat for all ELISAs.

Urine samples were diluted 8-fold for 8-isoprostane analysis and 16-fold for cysteinyl leukotriene analysis, and were undiluted for all other assays, based on manufacturer recommendation and following assay optimisation.

ELISA protocols

Cysteinyl leukotriene, leukotriene B4 and 8-isoprostane assays:

ELISA plates are pre-coated with mouse anti-rabbit IgG antibody by the assay manufacturer (Cayman Chemical, USA). For each assay, every plate contained wells for two blank samples, two non-specific binding wells, two maximum binding wells and an eight-point standard curve run in duplicates, as well as samples run in duplicate.

ELISA buffer, ELISA standards, samples, acetylcholinesterase (AChE) tracers and ELISA antiserums for cysteinyl leukotrienes, leukotriene B4 and 8-isoprostane were added to the assay plates according to the protocol outlined by the Cayman Chemical Cysteinyl leukotrienes EIA Kit (Item No. 500390), Leukotriene B4 EIA Kit (Item No. 520111) and 8-Isoprostane EIA Kit (Item No. 516351).

Following an incubation period of 18 hours, the plates were washed to remove any unbound reagents, and then acetylcholinesterase (AChE) substrate was added to the wells. The plates were then placed on an orbital shaker and allowed to develop in the dark at room temperature for 90-120 minutes. Once colour change occurred (clear to yellow), absorbance was read at a wavelength between 405-420 nm.

Analysis

A standard curve was plotted using the absorbance readings of the maximum binding, non-specific binding and standard wells. The standard curve was then used to determine the concentrations of cysteinyl leukotrienes, leukotriene B4 and 8-isoprostane in the EBC samples.

The cysteinyl leukotriene ELISA typically displays a half maximal inhibitory concentration (IC50) (50 % B/B0) of approximately 97 pg/mL and a detection limit (80 % B/B0) of approximately 40 pg/mL. The leukotriene B4 ELISA typically displays an IC50 of approximately 50 pg/mL and a detection limit (80 % B/B0) of approximately 13 pg/mL. The 8-isoprostane ELISA typically displays an IC50 (50 %

B/B0) of approximately 10 pg/mL and a detection limit (80 % B/B0) of approximately 2.7 pg/mL. For samples with %B/B0 values less than 20 %, data were recorded as zero as they fell out of the linear range of the standard curve.

Interleukin-8 (IL-8) assay:

Interleukin-8 levels were analysed using the BD OptEIA Human Interleukin-8 set (BD Biosciences, USA). Plates were coated plates overnight with the coating buffer provided in the BD OptEIA Reagent Set B (BD Biosciences, USA) and blocked with assay diluent. Samples and standards were then added and incubated for 2 hours at room temperature. After incubation, absorbance was read at 450 nm with a wavelength correction at 570 nm.

Analysis

A standard curve was generated by plotting a linear best fit curve for the log of the standard absorbance readings against the log of known standards concentrations. This standard curve was then used to determine the IL-8 concentrations in samples. For samples below the lowest detection limit of 3.1 pg/mL, data were expressed as zero.

Matrix Metalloproteinase 9 (MMP-9) assay:

MMP-9 levels were analysed using the R&D Systems Human MMP-9 Quantikine kit (R&D Systems, USA). This kit employed solid phase sandwich ELISA techniques. Wells were pre-coated with a monoclonal antibody specific for human MMP-9. Reagents, standards, samples and controls were added according to the protocol provided by R&D systems. The sensitivity of the assay was 0.156 ng/mL with a detection limit of 0.3 ng/mL.

Analysis

A standard curve was created using a 4-parameter logistic regression against readings for known standard absorbances. This standard curve was then used to calculate the concentrations of MMP-9 within each sample. For samples below the lowest detection limit, data were expressed as zero.

Table 1. Summary of targeted enzyme-linked immune-assays performed. The targeted assays were chosen for those compounds which have been shown to play a potential role in lung disease.

Assay target:	**Marker for:**
8-isoprostane	Oxidative stress
Leukotriene B4	Neutrophilic inflammation
Cysteinyl leukotrienes	Eosinophilic inflammation
Interleukin 8 (IL-8)	Inflammation/immunity
Matrix metalloproteinase 9 (MMP-9)	Airway remodelling

METABOLOMICS

Metabolomics is the analysis of the end-products of metabolism in biological systems. Through chromatography and mass spectrometry (LC/MS) methods, metabolomics can identify compounds that differ in health and disease including proteins, carbohydrates, fatty acids, lipids, hormones, vitamins, drugs, and other small molecules that are present in certain biofluids. Metabolomics methods are well established for some biofluids including plasma and urine. However, methods for EBC metabolomics analysis have not been established.

EBC metabolomics method development

EBC metabolomics analysis was attempted using LC/MS methods. EBC samples were freeze-dried and reconstituted using UltraPure water. Samples were then inserted into the ThermoFisher Triple quadrupole LC/MS system autosampler for analysis. However, metabolites remained unquantifiable as the noise to signal ratio was too large. Despite multiple attempts at optimisation, EBC metabolomics remained out of the scope of this current work.

Urine

Randomisation

Samples were prepared to be run in 1 mode of LC/MS analysis across 2 batches of 112 and 128 samples respectively, as the instrument could only allow for batches of around 130 samples at a time. In addition to samples, each batch contained 25 quality-control aliquots which consisted of all samples pooled in equal quantities. 8 quality-control aliquots were injected at the start and end of each batch to condition the instrument, as well as a single quality-control (QC) sample after every block of 8 participant samples. Samples were block randomised in blocks of 8 based on participant sex, age at testing, gestational age at birth and neonatal diagnosis of BPD to ensure random distribution across and within batches and within blocks.

Sample preparation

Urine samples were defrosted in preparation for metabolomic analysis. Sample preparation was performed in batches to ensure that the amount of time outside of the freezer was consistent across samples (~2 hours per batch). Sample preparation included measurement of specific gravity via refractometer, sub-aliquoting and quality-control pooling.

Samples were thawed on ice for about 30 minutes, then vortexed to homogenise the sample. Samples were then centrifuged for 5 minutes at 14,000 rotations per minute (rpm) at 4 °C.

The specific gravity of each sample was measured by placing 100 µL of the sample on a refractometer (ATAGO-UGα, Atago, Tokyo, Japan).

An aliquot of 120 µL of each sample was then transferred into an aliquot tube, labelled with the batch number and injection order number and placed on ice. Another 120 µL of sample was transferred to a 15 mL Falcon tube to create the pooled quality-control. This process was repeated for each sample before samples were re-frozen at -80 °C.

Data Analysis

On the day of analysis, urine sample concentration was normalised by dilution with water, based on the sample's specific gravity measurement in order to achieve uniform dilution across all samples.

Samples were crashed with 150 µL of MeOH, vortexed and centrifuged for 10 minutes at 14,000 rpm at 4 °C and inserted into the autosampler for analysis, through which 50 µL was ran in a reversed-phase chromatography method through a Thermo-Q-Exactive (Orbitrap) liquid-chromatography mass spectrometry system (Thermo Fisher Scientific, San Jose, CA, USA).

Data processing

The data were cleaned based on the following criteria:

1. Quality Control-Relative Standard Deviation (QC-RSD) – a metric of analytical precision for each metabolite that is measured by calculating the relative standard deviation of metabolite measurements in biological samples compared to the standard deviation of pooled QC samples. Metabolites with a QC-RSD of >30 % were excluded from analysis, as per guidelines for untargeted clinical metabolomic studies (127).

2. Dispersion Ratio (D-Ratio) – a metric which describes the measurement precision of a detected metabolite by considering the statistical dispersion of the pooled QC samples to the dispersion of biological test samples i.e. a measure of the ratio of analytical variance to overall biological variance. Metabolites with a D-Ratio above 35 % were excluded from analysis as per guidelines for untargeted clinical metabolomic studies (127).

3. Metabolite identification – metabolites were identified if the chromatography peak matched to at least one of the following: (a) the ECU in-house MS/MS spectral library, (b) the ECU in-house retention time and accurate mass library, or (c) the Thermo-specific online MS/MS spectral database (mzCloud; https://www.mzcloud.org/, HighChem LLC, Slovakia). Metabolites that passed this inclusion criterion started with a higher level of identification confidence. For peaks that did not pass this criterion, the name was based on mass or molecular formula only. Metabolites were then manually reviewed and excluded if they were exogenous and/or had no clear biological relevance.

A principal component analysis (PCA) was performed in order to visualise the distribution of individual metabolomic profiles and this method identified no sample outliers for exclusion.

These criteria conform to the guidelines for the use of systems suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies (127).

BIOINFORMATIC AND STATISTICAL ANALYSES

To address Aim 1, the difference in lung function between healthy term-born individuals and those born preterm with and without BPD was assessed by one-way analysis of variance with post-hoc comparisons, independent samples t-test or Mann-Whitney U test depending on the normality of the data.

The impacts of neonatal treatments on lung function in later childhood and adolescence was assessed by regression analyses adjusted for multicollinearities. This was performed by using the un-standardised residuals of independent regressions of the confounding variables. For example, the residual from a regression of gestational age (independent) and days of mechanical ventilation (dependent) was used to test for the independent association of days of mechanical ventilation on respiratory outcomes.

In order to address Aim 2, differences in inflammatory markers between healthy and preterm groups (those with and without BPD) were analysed by one-way analysis of variance with post-hoc comparisons, independent samples t-test or Mann-Whitney U test depending on normality of the data. Bivariate correlation was used to assess associations between biomarker levels and lung function parameters.

The metabolomic studies to address Aims 3 and 4 were analysed as below:

Neonatal samples were analysed separately as they were distinctly different from samples from older children and young adults after preliminary PCA analysis. Analysis was also split between childhood and young-adulthood groups to minimise the influence of age on metabolite differences between preterm and term-born groups.

The relative abundance of metabolites was not normally distributed, and therefore univariate comparison between groups was performed using Mann-Whitney U tests or Kruskal Wallis rank tests. Correlations between metabolite abundances and neonatal factors were tested using pairwise Spearman's rank correlation coefficients (r).

Principal component-cononical variate analysis (PC-CVA) was used for multivariate analyses of metabolite differences between groups. Circle plots were created to display metabolites with similar pathways and spring plots display metabolite associations with neonatal variables.

Analysis was performed using Python in Jupyter notebooks (128).

A p-value of <0.05 was considered significant for all analyses.

REFERENCES

54. Simpson SJ, Hall GL, Wilson AC. Lung function following very preterm birth in the era of 'new' bronchopulmonary dysplasia. Respirology. 2015;20(4):535-40.
114. Urs R, Stoecklin B, Pillow JJ, Hartmann B, Hall GL, Simpson SJ. Collecting exhaled breath condensate from non-ventilated preterm-born infants: a modified method. Pediatric Research. 2021:1-3.
115. Singer F, Kieninger E, Abbas C, Yammine S, Fuchs O, Proietti E, et al. Practicability of nitrogen multiple-breath washout measurements in a pediatric cystic fibrosis outpatient setting. Pediatric pulmonology. 2013;48(8):739-46.
116. Quine D, Wong CM, Boyle EM, Jones JG, Stenson BJ. Non-invasive measurement of reduced ventilation: perfusion ratio and shunt in infants with bronchopulmonary dysplasia: a physiological definition of the disease. Archives of Disease in Childhood-Fetal and Neonatal Edition. 2006;91(6):F409-F14.
117. Asher M, Keil U, Anderson H, Beasley R, Crane J, Martinez F, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. European respiratory journal. 1995;8(3):483-91.
118. Menzies-Gow A, Mansur AH, Brightling CE. Clinical utility of fractional exhaled nitric oxide in severe asthma management. European Respiratory Journal. 2020;55(3).
119. Dweik RA, Boggs PB, Erzurum SC, Irvin CG, Leigh MW, Lundberg JO, et al. An official ATS clinical practice guideline: interpretation of exhaled nitric oxide levels (FENO) for clinical applications. American journal of respiratory and critical care medicine. 2011;184(5):602-15.
120. King GG, Bates J, Berger KI, Calverley P, de Melo PL, Dellacà RL, et al. Technical standards for respiratory oscillometry. European Respiratory Journal. 2020;55(2).
121. Calogero C, Simpson SJ, Lombardi E, Parri N, Cuomo B, Palumbo M, et al. Respiratory impedance and bronchodilator responsiveness in healthy children aged 2–13 years. Pediatric pulmonology. 2013;48(7):707-15.
122. Oostveen E, Boda K, van der Grinten CP, James AL, Young S, Nieland H, et al. Respiratory impedance in healthy subjects: baseline values and bronchodilator response. European Respiratory Journal. 2013;42(6):1513-23.
123. Czövek D, Shackleton C, Hantos Z, Taylor K, Kumar A, Chacko A, et al. Tidal changes in respiratory resistance are sensitive indicators of airway obstruction in children. Thorax. 2016;71(10):907-15.
124. Graham BL, Steenbruggen I, Miller MR, Barjaktarevic IZ, Cooper BG, Hall GL, et al. Standardization of spirometry 2019 update. An official American thoracic Society and European respiratory Society technical statement. American journal of respiratory and critical care medicine. 2019;200(8):e70-e88.
125. Stanojevic S, Cole T, Baur X, Hall G, Culver B, Enright P. Multi-ethnic reference values for spirometry for the 3-95 year age range: The global lung function 2012 equations. The European respiratory journal. 2012;40(6):1324-43.
126. Pellegrino R, Viegi G, Brusasco V, Crapo R, Burgos F, Casaburi R, et al. Interpretative strategies for lung function tests. European respiratory journal. 2005;26(5):948-68.
127. Broadhurst D, Goodacre R, Reinke SN, Kuligowski J, Wilson ID, Lewis MR, et al. Guidelines and considerations for the use of system suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies. Metabolomics. 2018;14(6):1-17.
128. Mendez KM, Pritchard L, Reinke SN, Broadhurst DI. Toward collaborative open data science in metabolomics using Jupyter Notebooks and cloud computing. Metabolomics. 2019;15(10):1-16.

Chapter 4: Collecting exhaled breath condensate from non-ventilated preterm-born infants: a modified method

This is the peer-reviewed but unedited manuscript version of the following article: Urs, R., Stoecklin, B., Pillow, J.J. et al. Collecting exhaled breath condensate from non-ventilated preterm-born infants: a modified method. Pediatr Res (2021). (DOI: 10.1038/s41390-021-01474-x). The final, published version is available at https://doi.org/10.1038/s41390-021-01474-x.(114)

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AUTHOR CONTRIBUTIONS

R.U. collected and analysed the data, interpreted the data, drafted the initial manuscript, performed literature search, drafted the figures, and approved the final manuscript as submitted. B.S. assisted with collection and analysis of the data, reviewed and revised the manuscript, and approved the final manuscript as submitted. B.H. co-developed the design for the adapted R-Tube and 3D-printed the custom connector, reviewed and revised the manuscript; and approved the final manuscript as submitted. J.J.P. was the principal investigator of the PIFCO study, critically reviewed and revised the manuscript, and approved the final manuscript as submitted. G.H. contributed to the overall study design and reviewed and approved the final manuscript as submitted. S.S. contributed to the overall study design, critically reviewed and revised the manuscript, and approved the final manuscript as submitted.

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Disclosure Statement

The authors have no conflicts of interest to declare.

Category of Study: Correspondence

Consent Statement

This study took place as part of the Preterm Infant Clinical and Functional Outcome (PIFCO) study (ACTRN126130010627181) and was approved by the West Australian Women and Newborn Health Service Human Research Ethics Committee (20130193EW), the Child and Adolescent Health Service Human Research Ethics Committee (2014083EP) and the University of Western Australia (RA/3/1/5942). Informed parental written consent was obtained for all participants.

Impact

- Our low-dead space adaptor improves feasibility of acquiring exhaled breath condensate from preterm neonates and infants

- We adapted a commercially available system to collect exhaled breath condensate in preterm-born neonates and infants during natural sleep by minimising dead space as recommended by the European Respiratory Society (ERS)

- Non-invasive measurement of airway biomarkers via analysis of exhaled breath condensate from preterm-born neonates and infants may allow early identification and intervention in chronic lung disease

ABSTRACT

Background: Non-invasive measurement of airway biomarkers via analysis of exhaled breath condensate (EBC) may allow early identification and intervention in preterm lung disease. However, no commercially available EBC collection system is suitable for non-ventilated infants.

Objectives: We aimed to adapt a commercially available system to collect EBC in non-ventilated preterm-born neonates and infants.

Method: An infant nasal- or face-mask was attached to the R-Tube collection device (Respiratory Research Inc, Charlottesville, VA) using a custom 3D-printed connector. EBC was collected from preterm-born neonates aged 35-40 weeks postmenstrual age and infants aged 12-16 months corrected

Results: The dead space of the device was reduced from 30 mL to 4 mL with our customised adaptor. EBC samples greater than 100 µL were collected successfully in 14/19 attempts in neonates and in all 32 attempts in infants aged 12-16 months.

Conclusion: Our low-dead space adaptor improves feasibility of acquiring EBC from preterm neonates and infants.

INTRODUCTION

Exhaled breath condensate (EBC) collection is a non-invasive, safe method for measurement of biomarkers in patients with lung disease. Other methods of obtaining samples from the lungs, such as bronchoalveolar lavage, are invasive and require anaesthesia/sedation in neonates and infants. EBC is particularly appealing for assessing biomarkers in preterm-born infants, a population at risk of ongoing lung disease (2). Previous small studies of EBC from ventilated, preterm-born neonates identified potential biomarkers of respiratory disease (129, 130). A method for EBC collection in non-ventilated infants may allow for longitudinal monitoring of lung disease biomarkers beyond the intensive care unit and potentially identify infants most at risk of ongoing disease. However, EBC collection is more challenging in infants compared to older children and adults. The 2017 European Respiratory Society (ERS) technical standard for exhaled biomarkers in lung disease highlighted the importance of standardising EBC collection methods and recommended the development of EBC collection systems suitable for infants and preschool children (131). To date, EBC collection in infants has used custom-made devices (132, 133). However, we are unaware of successful EBC collection in non-ventilated, preterm-born neonates and infants using a commercially available system. We aimed to adapt a commercially available system to collect EBC in non-ventilated preterm-born neonates and infants.

METHODS

Modification of a commercial system: EBC was collected using an R-Tube collection device (Respiratory Research Inc, Charlottesville, VA) adapted by reduction of equipment dead-space. The mouthpiece and 'Tee' section of the R-Tube was removed and replaced with a 3D-printed connector (Polylactic acid plastic), with three connection points (Fig. 1). The polypropylene condensation tube of the R-Tube device was attached to one connection point. The second point connected to a blue duckbill silicone rubber valve from another R-Tube device, which served as a one-way inspiratory valve. The third connection point was attached to a neonatal nasal mask (size 11, EME Ltd., Brighton, UK) or infant mask (size 0/1, 12 mL effective dead space, Laerdal Medical AS, Stavanger, Norway). All connections were sealed tightly to prevent air leak. Room air was inspired through the duckbill valve connection and exhaled into the condensation tube through the existing one-way valve. Both duckbill valves were checked prior to and during collection to ensure they were not stuck and were opening during the breathing cycle. An insulating cover and aluminium sleeve (-20 ^{o}C) was placed around the condensation tube during collection. Care was taken to maintain the temperature throughout the collection period in neonates, with replacement of the aluminium sleeve with another cooled to -20 ^{o}C halfway through collection. New polypropylene condensation tubes were used for each collection, with connectors and duckbill valves sterilised between each use with laboratory-grade disinfectant.

Validation in preterm infants: Measurements were performed as a substudy of the Preterm Infant Clinical and Functional Outcome (PIFCO) study (ACTRN126130010627181) and approved by the Women and Newborn Health Service Human Research Ethics Committee (20130193EW), the Child and Adolescent Health Service Human Research Ethics Committee (2014083EP) and the University of Western Australia (RA/3/1/5942). Infants were eligible for the substudy if they were < 32 weeks' gestation and informed parental written consent had been obtained. EBC was obtained with the infant in supine position. The device was hand-held upwards manually at a 90-degree angle to the infant's face. Collections of EBC in preterm neonates were obtained at the infant's bedside in the neonatal intensive care unit, at 35 – 40 weeks' postmenstrual age, over 20–30 minutes during natural sleep.

Collections from preterm-born infants at 12 – 16 months corrected postnatal age took 15 minutes, and were obtained after lung function testing under sedation with chloral hydrate (80–100 mg/kg). All collections took place when infants were clinically stable and oxygen saturations and heart rate were monitored throughout collection. Baseline minute ventilation was measured in the infants prior to EBC collection using an ultrasonic flow meter (Ecomedics AG, Duernten Switzerland) and calculated using analysis software (Wbreath v3.2.0, Ndd Medizintechnik AG, Zurich, Switzerland). Condensate was collected using the R-Tube plunger device and stored at -80 °C. Successful collection was defined as volumes greater than 100 µL based on immuno-assay sample requirements.

RESULTS

Our modified device reduced the dead space of the system from 30 mL to 4 mL. EBC was successfully collected in the neonatal period via nasal mask in 14/19 attempts at mean (SD) age of 35.8 (1.7) weeks' postmenstrual age. Three of these neonates met the criteria for mild chronic lung disease defined as requiring supplemental oxygen for at least 28 days after birth but not at 36 weeks postmenstrual age. None of the infants were receiving respiratory support or oxygen supplementation at the time of EBC collection. Insufficient EBC volume was collected in five neonates. Collection was successful in two neonates who had nasogastric feeding tubes *in situ* while breathing through nasal masks. EBC was collected successfully in all 32 infants at 12 – 16 months' age, 9 of whom had a neonatal diagnosis of mild chronic lung disease. All infants remained clinically stable throughout EBC collection.

Median (IQR) successful EBC collection volumes are shown in Table 1. There was a weak positive correlation between baseline minute ventilation and EBC volume collected (Spearman correlation; r = 0.387, p = .029). The volume of EBC collected was independent of weight or age in either subgroup.

Table 1: Participant details and EBC volumes collected from neonates and infants.

Demographics	Neonates (n=14)	Infants (n=32)
Male (n, %)	9 (64 %)	25 (78 %)
Gestation at birth (w)	28.2 ± 2.4	27.8 ± 1.3
Age at collection (w PMA/m cPNA)	35.8 ± 1.7	14.4 (13.7-15)
Weight at collection (kg)	2.5 ± 0.4	10.0 ± 1.4
EBC volume collected (µL)	100 (100-163)	425 (300-500)
Tidal volume (mL)	14.4 ± 4.7	78.4 ± 9.5
Minute ventilation (mL/min)	1030 ± 254	2395 ± 295

Parametric data are reported as mean ± SD, non-parametric data as median (IQR). PMA, postmenstrual age of neonates; cPNA, corrected postnatal age of infants.

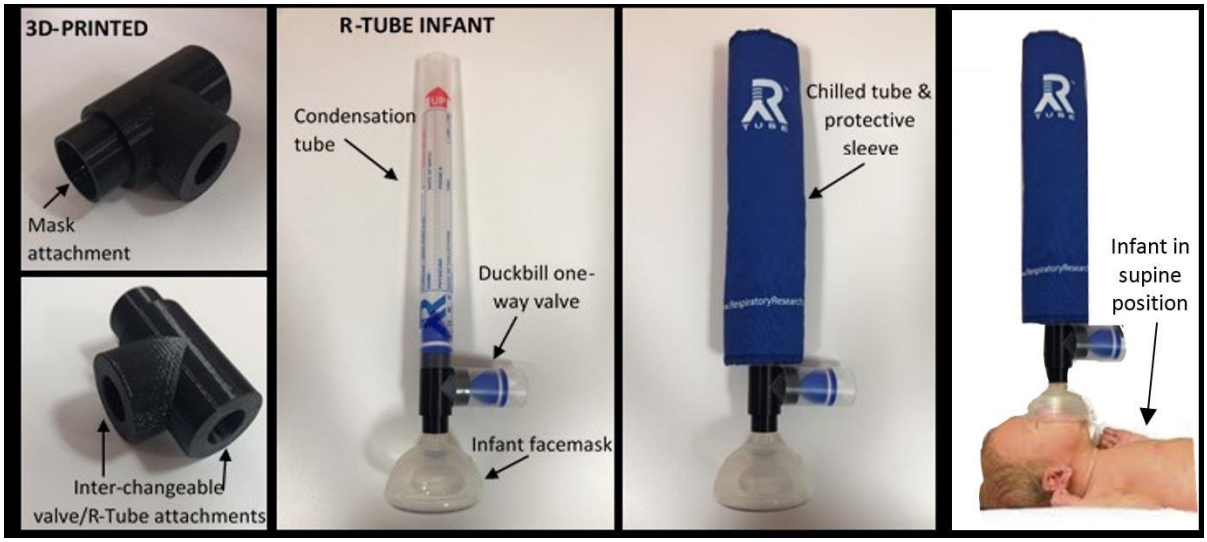


Fig. 1. Configuration of the 3D-printed connector and setup of the infant facemask, 3D-printed connector and R-Tube attachment for the collection of EBC in preterm-born infants. The 3D-printed connector model can be found on the NIH 3D Print Exchange website: https://3dprint.nih.gov/discover/3dpx-013974.

The infant nasal or face mask fits into the mask attachment on the 3D-printed connector. For EBC collection in infants in the supine position, the duckbill one-way valve is placed on the perpendicular connection point on the 3D-printed connector with the duckbill pointed inwards towards the 3D-printed connector. The condensation tube is then placed on the remaining connection point, with the red 'Up' arrow pointing away from the 3D-printed connector. Both duckbill valves are prepared for use by gently squeezing the surrounding tubing to ensure they are not stuck. The chilled aluminium tube and protective sleeve can then be placed over the condensation tube ready for EBC collection. The device is then handheld and the nasal- or face-mask placed over the infant's nose and mouth taking care to ensure no air-leak for the duration of EBC collection. The duckbill valves are checked again to ensure they open during the breathing cycle of the infant.

DISCUSSION

We adapted a commercially available system to collect EBC in preterm-born neonates and infants during natural sleep by minimising dead space as recommended by the European Respiratory Society (ERS) (134). Collection was well-tolerated in all infants, yielding sufficient sample for most targeted assays in 89 % of studies.

The ERS/American Thoracic Society (ATS) Workforce specifications for equipment used in infant pulmonary function testing recommend equipment dead space <2 mL/kg. According to these guidelines, the 30 mL dead space in the original commercial EBC collection system is unsuitable for infants below 15 kg. With a dead space of 4 mL, and the use of a nasal mask which fits snugly over the nose (no effective dead space), our adapted collection system enables EBC collection in infants and neonates as small as 2 kg, in accordance with the ERS/ATS guidelines. While the use of a nasal mask allowed collection during quiet sleep and removed any potential salivary contamination (135), some studies suggest variability of certain biomarkers between nasal and mouth breathing, which may confound longitudinal measurement or comparisons to older populations (131).

Our adapted device paired with the use of a face mask with 12 mL effective dead space (size 1, Laerdal Medical AS, Stavanger, Norway) brings the total effective dead space to 16 mL, making this combination suitable for EBC collection in infants as small as 8 kg. This setup allowed successful EBC collection in all attempts made in infants aged 12-16 months.

The small tidal volume of neonates resulted in collection times of approximately 20 minutes to produce sufficient EBC volumes for downstream analyses, such as with targeted enzyme-linked immune-assays and metabolomics previously used in EBC analysis, which typically require a minimum sample volume of 50-100 µL (108, 136). Further analyses should investigate if longer collection times increase risk of metabolite oxidation or alterations in sample pH (131). Collection time in the 12-16 month old infants is similar to that in adults. Although infants generate smaller EBC volumes, the sample yield is still sufficient for multiple assays which previous studies have conducted (129, 137-139). Minute ventilation correlates with condensate volume in term infants and older subjects (133). We found no correlation between baseline minute ventilation and sample volume. Despite the absence of this correlation, the EBC volumes collected using our device are comparable to those reported in studies of infant EBC collection using homemade devices (133). Cooling and maintaining the collection device at lower temperatures comparable to other studies (-40 °C – -80 °C) which report greater EBC volumes from ventilated neonates (129, 130, 139) may be an important consideration to increase EBC volume in future studies (140). The feasibility of EBC collection in non-sedated infants beyond the neonatal stage remains unknown. However, other lung function tests such as forced oscillation technique and multiple breath washout are achievable via facemask in sleeping infants beyond the neonatal period (135). It is therefore likely collection of EBC is also probable during quiet sleep.

In conclusion, we demonstrate that EBC collection is feasible and can be collected safely using an adapted commercial device in non-ventilated preterm-born neonates and infants. For a population particularly susceptible to chronic lung disease, a non-invasive approach of measuring biomarkers in the airway may allow early identification of and intervention in lung disease.

REFERENCES

2. Urs R, Kotecha S, Hall GL, Simpson SJ. Persistent and progressive long-term lung disease in survivors of preterm birth. Paediatric respiratory reviews. 2018.
108. Carraro S, Giordano G, Pirillo P, Maretti M, Reniero F, Cogo PE, et al. Airway metabolic anomalies in adolescents with bronchopulmonary dysplasia: new insights from the metabolomic approach. J Pediatr. 2015;166(2):234-9 e1.
114. Urs R, Stoecklin B, Pillow JJ, Hartmann B, Hall GL, Simpson SJ. Collecting exhaled breath condensate from non-ventilated preterm-born infants: a modified method. Pediatric Research. 2021:1-3.
129. Kononikhin A, Starodubtseva N, Chagovets V, Ryndin A, Burov A, Popov I, et al. Exhaled breath condensate analysis from intubated newborns by nano-HPLC coupled to high resolution MS. Journal of Chromatography B. 2017;1047:97-105.
130. Rosso MI, Roark S, Taylor E, Ping X, Ward JM, Roche K, et al. Exhaled breath condensate in intubated neonates-a window into the lung's glutathione status. Respiratory research. 2014;15(1):1-9.
131. Horváth I, Barnes PJ, Loukides S, Sterk PJ, Högman M, Olin A-C, et al. A European Respiratory Society technical standard: exhaled biomarkers in lung disease. European Respiratory Journal. 2017;49(4):1600965.
132. Muller W, Morini F, Eaton S, Peters M, Jaffe A. Safety and feasibility of exhaled breath condensate collection in ventilated infants and children. European Respiratory Journal. 2006;28(3):479-85.
133. Moeller A, Franklin P, Hall GL, Horak Jr F, Wildhaber JH, Stick SM. Measuring exhaled breath condensates in infants. Pediatric pulmonology. 2006;41(2):184-7.
134. Frey U, Stocks J, Coates A, Sly PD, Bates J. Specifications for equipment used for infant pulmonary function testing. The European respiratory journal. 2000;16:731-40.
135. Schulzke S, Deeptha K, Sinhal S, Baldwin D, Pillow J. Nasal versus face mask for multiple-breath washout technique in preterm infants. Pediatric pulmonology. 2008;43(9):858-65.
136. Horváth I, Hunt J, Barnes PJ. Exhaled breath condensate: methodological recommendations and unresolved questions. European Respiratory Journal. 2005;26(3):523-48.
137. Carpagnano GE, Barnes PJ, Geddes DM, Hodson ME, Kharitonov SA. Increased leukotriene B4 and interleukin-6 in exhaled breath condensate in cystic fibrosis. American journal of respiratory and critical care medicine. 2003;167(8):1109-12.
138. Patel K, Davis SD, Johnson R, Esther Jr CR. Exhaled breath condensate purines correlate with lung function in infants and preschoolers. Pediatric pulmonology. 2013;48(2):182-7.
139. Yen E, Weinberger B, Laumbach R, Ohman-Strickland P, Vetrano A, Gow A, et al. Exhaled breath condensate nitrite in premature infants with bronchopulmonary dysplasia. Journal of neonatal-perinatal medicine. 2018;11(4):399-407.
140. Goldoni M, Caglieri A, Andreoli R, Poli D, Manini P, Vettori MV, et al. Influence of condensation temperature on selected exhaled breath parameters. BMC Pulmonary Medicine. 2005;5(1):1-9.

Chapter 5: Impaired respiratory function in survivors of preterm birth using spirometry and oscillometry measures

INTRODUCTION

Survivors of very preterm birth, particularly those with a neonatal diagnosis of bronchopulmonary dysplasia (BPD), exhibit significant respiratory morbidity into the first years of life and later childhood (34). In early childhood, preterm-born children report increased respiratory symptoms, experiencing more wheeze, inhaled medication use and re-hospitalization than their term-born counterparts (51). At school-age, preterm-born children are more likely to be diagnosed with asthma and be prescribed inhaler medications than those born at term (53).

Fractional exhaled nitric oxide (FeNO), a marker of airway eosinophilic inflammation, is elevated in children with typical asthma (141). However, preterm-born children have been shown to have similar FeNO levels to term-born children, suggesting that there are alternate underlying mechanisms contributing to the increased symptoms and lung function deficits observed in children born preterm (142). Several cross-sectional studies report airway obstruction in children born preterm as indicated by lower forced expiratory volumes in 1 second (FEV_1) and forced mid-flows (FEF_{25-75}) with normal forced vital capacity (FVC) (42, 45, 54-58, 63, 64). Airway obstruction is partially reversible in approximately one third of preterm born children, with studies reporting between 25-60 % of those with BPD with bronchodilator responsiveness at school-age (42, 55, 64, 68). Preterm-born children also exhibit altered respiratory mechanics with increased airway resistance and worse reactance outcomes as assessed with spectral oscillometry measures, indicating peripheral lung disease from infancy through to school-age (42, 45, 55, 73-75). Intra-breath oscillometry has been proposed as a more sensitive measure of airway obstruction and respiratory mechanics compared to conventional spectral oscillometry outcomes in children with recurrent wheeze (123). However, intra-breath measures have not yet been assessed in preterm-born children.

Since changes to neonatal practice in the 1990s with the introduction of surfactant use, there have been more survivors of extreme prematurity who are just now reaching adolescence and young adulthood (12). Two recent longitudinal studies of preterm children born in the post-surfactant era report significant declines in FEV_1 z-scores from childhood to young adulthood (62, 65). This is concerning considering that low 'peak' lung function (FEV_1) and an unknown rate of FEV_1 decline over time may put survivors of preterm birth at risk of early-onset chronic lung disease in adulthood and even early mortality (86, 143). It is therefore crucial to better characterise lung function deficits in survivors of

preterm birth in the post-surfactant era and identify risk factors for worse respiratory and potential avenues for intervention.

AIMS & HYPOTHESIS

This chapter aimed to characterise respiratory function in children and young-adults born very prematurely in comparison to those born at term, including assessing the novel intra-breath oscillometry along with respiratory flows, mechanics & nitric oxide. Additionally, we aimed to assess if lung function parameters were different between preterm-born individuals with and without a neonatal diagnosis of bronchopulmonary dysplasia. We also aimed to determine the impacts of neonatal treatments on lung function in later childhood and adolescence.

We hypothesised that preterm born children will have reduced lung function and this will be more pronounced in those with a neonatal diagnosis of BPD and who required more intensive neonatal interventions.

METHODS

Participants

Preterm children and young adults, with and without a diagnosis of BPD, and healthy term-born controls, were assessed for lung function between the ages of 6 and 23 years. Preterm-born participants were born at 32 weeks gestation or less at King Edward Memorial Hospital (KEMH) in Perth, Western Australia. Participants born preterm were classified as having bronchopulmonary dysplasia if they received 28 days of oxygen supplementation or more, as assessed at 36 weeks postmenstrual age. Healthy term born participants were born at 37 weeks gestation or more and had no history of recurrent respiratory symptoms or lung disease. Written informed consent was obtained from participants over 18 and from parents or guardians for participants under 18. Ethical approval was obtained from the Child and Adolescent Health Service Human Research Ethics Committee (RGS367, RGS815).

History and Symptoms

Neonatal and maternal health data was obtained from medical records and the KEMH neonatal database. Current and past respiratory symptoms at rest and with exercise as well as previous and current treatment use was obtained using validated general and respiratory questionnaires adapted from the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaires (117). Recent respiratory symptoms such as wheeze, shortness of breath, cough and rattly chest in the 3 months prior to the participant's study visit was reported by parents. Anthropometric data collected at the participant's study visit included height, weight, oxygen saturation and blood pressure.

Lung function assessment

Participants attended Perth Children's Hospital Outpatients for lung function assessment. Fractional exhaled nitric oxide was measured using the Medisoft Hypair FeNO device (Medisoft, Sorinnes, Belgium). Respiratory mechanics was assessed using the tremoFlo FOT Device (Thorasys Inc. Montreal, QC). Spectral oscillometry was performed across 5-37 Hz and intrabreath oscillometry was performed using a single 10 Hz frequency. Spirometry was performed according to American Thoracic Society/European Respiratory Society standards. Oscillometry and spirometry was performed before and after administration of 400 µg salbutamol. Spectral oscillometry and spirometry outcomes were expressed as z-scores to adjust for anthropometric factors. Intra-breath oscillometry is not dependent on anthropometrics (144).

Statistics

Differences between groups were analysed by one-way analysis of variance with post-hoc comparisons, independent samples t-test or Mann-Whitney U test depending on normality of the data. Normally distributed data are presented as means and standard deviations. Non-normally distributed data are presented as medians and interquartile ranges. The chi-squared test was used to compare proportions between groups. Neonatal risk factors such as gestational age and days of respiratory support were adjusted for multicollinearities by using the un-standardised residuals of independent regressions of the confounding variables. These residuals were then used in regression analyses against lung function parameters to assess the independent association of these neonatal risk factors on lung function outcomes.

RESULTS

Study participants at visit

Term-born children (n=45) and preterm-born children (n=119), 55 of whom had a neonatal diagnosis of BPD, participated in the study. Age, height, weight and BMI were not significantly different between preterm and term-born participants (Table 1). Preterm participants were born at a median (IQR) gestation of 28 (26 – 30) weeks (Table 2). Participants with BPD were born at a lower median gestation than those without BPD, and had a lower birth weight ($p < 0.01$) and lower birth weight z-score ($p < 0.05$). As expected, due to the definition of BPD, those with BPD required significantly longer duration of oxygen supplementation, mechanical ventilation and continuous positive airway pressure (CPAP) ($p< 0.01$). The proportion of participants with BPD who had surfactant administered was also greater compared to those without BPD ($p < 0.01$).

Table 1. Participant demographics and recent symptoms at time of study visit

	Term Controls	Preterm	Preterm, no BPD	Preterm with BPD
Participants, n	45	119	64	55
Age (years)	15.7 (± 5.0)	15.7 (± 4.7)	14.8 (± 4.3)	16.8 (± 5.0)
Male, n (%)	23 (51)	66 (55)	35 (55)	31 (56)
Height (cm)	160.9 (± 21.9)	157.3 (± 17.3)	156.6 (± 17.1)	158.0 (± 17.7)
Weight (kg)	56.3 (± 21.6)	53.3(± 20.3)	52.8 (± 20.4)	53.9 (± 20.3)
Body Mass Index (BMI)	20.7 (± 4.0)	20.7 (± 4.7)	20.7 (± 4.6)	20.7 (± 4.9)

Data presented as mean (± SD) or number (% of population).
*Represents significant difference from term controls ($p<0.05$)
ǂ Represents significant difference between the preterm groups with and without BPD
3m, within 3 months prior to study visit. BPD, bronchopulmonary dysplasia.

Table 2. Neonatal information about preterm-born participants

	Preterm	Preterm, no BPD	Preterm with BPD
Gestation (weeks)	28.0 (26.0-30.0)	30.0 (28.1-31.0)	26.0 (24.7-28.0)*
Birth weight (grams)	1125.3 (± 391.0)	1375.2 (± 326.8)	834.6 (± 222.3)**
Birth weight (z-score)	-0.06 (± 0.83)	0.11 (± 0.73)	-0.26 (±0.90)*
O_2 supplementation (days)	9.50 (0-73.0)	0.04 (0-2)	77.0 (49.9-104.1)**
Mechanical ventilation (days)	1 (0-12)	0.06 (0-0.78)	15.0 (0-32.7)**
Continuous positive airway pressure (CPAP) (days)	5.63 (0-25.71)	1.27 (0-6.75)	21.0 (3.23–38.8)**
Surfactant administered, n (%)	82 (68.9)	34 (53.1)	48 (87.3)**
Antenatal steroids, n (%)	78 (65.5)	41 (64.1)	37 (67.3)

Data presented as mean (± standard deviation), median (interquartile range) or number (% of population).
*Represents significant difference between preterm groups with and without bronchopulmonary dysplasia (BPD) ($p<0.05$)
**Represents significant difference between preterm groups with and without bronchopulmonary dysplasia (BPD) ($p<0.01$)

Over 60% of preterm-born participants reported recent symptoms either during the daytime or on exertion (Table 3). The proportion of participants with BPD who reported recent respiratory symptoms on exertion was greater than those without a BPD diagnosis (X^2 (1, N=119) = 5.54, p=.018) (Table 3). There was a greater proportion of those with BPD who reported recent daytime wheeze (X^2 (1, N=119) = 4.41, p=0.036), wheeze on exertion (X^2 (1, N=119) = 4.77, p=0.029), cough on exertion (X^2 (1, N=119) = 9.85, p=0.002) and shortness of breath during the day (X^2 (1, N=119) = 5.49, p=0.019) compared to those without BPD (Table 3).

Table 3. Recent symptoms (within 3 months prior to study visit) for preterm-born participants

Symptoms, n (%)	Preterm	Preterm, no BPD	Preterm with BPD
Asthma medication use	14 (11.7)	6 (9.4)	8 (14.5)
Wheeze during the day	13 (10.9)	3 (4.7)	10 (18.2)*
Cough during the day	44 (37.0)	20 (31.3)	24 (43.6)
Rattly chest during the day	11 (9.2)	3 (4.7)	8 (14.6)
Shortness of breath during the day	28 (23.5)	10 (15.6)	18 (32.7)*
Any daytime symptoms	56 (47.1)	27 (42.2)	29 (52.7)
Wheeze on exertion	19 (16.0)	6 (9.4)	13 (23.6)*
Cough on exertion	35 (29.4)	11 (17.2)	24 (43.6)**
Rattly chest on exertion	12 (10.1)	4 (6.3)	8 (14.6)
Shortness of breath on exertion	50 (42.0)	22 (34.4)	28 (50.9)
Any symptoms on exertion	55 (46.2)	23 (35.9)	32 (58.2)*
Any symptoms (daytime or on exertion)	73 (61.3)	35 (54.7)	38 (69.1)

Data presented as number (% of population).
*Represents significant difference between preterm groups with and without BPD ($p<0.05$)
**Represents significant difference between preterm groups with and without BPD ($p<0.01$)

Lung function

FeNO: There was no difference in FeNO levels between term and preterm-born participants, or between those with and without BPD (Table 4).

Spirometry: Preterm participants had significantly lower z-scores for spirometry parameters FEV_1, FVC, FEV_1/FVC and FEF_{25-75}, with FEV_1/FVC and FEF_{25-75} further reduced in those with BPD (Table 4). There was no difference in FEV_1/FVC z-scores between preterm participants without BPD and term-born participants (Figure 1). The proportion of participants with airway obstruction, defined as

FEV_1/FVC<-1.64 z-scores, was significantly higher in preterm participants with BPD group compared to those without BPD (X^2 (1, N = 113) = 4.43, p = .04) and those born at term (X^2 (1, N = 98) = 11.9, p = .001) (Table 4). Of the 33 participants with airway obstruction, 22 had a significant response to bronchodilator, only 9 of whom reached normal spirometry limits after bronchodilator. The proportion of participants with a significant response to bronchodilator on spirometry was not different between term- and preterm-born participants. However, the proportion of those with BPD with a significant bronchodilator response was greater compared to those born at term (X^2 (1, N = 100) = 7.06, p = .008) and those without BPD (X^2 (1, N = 113) = 4.1, p = .044) (Table 4). Lower FEV_1, FEV_1/FVC and FEF_{25-75} was associated with decreased GA and more days of mechanical ventilation and oxygen supplementation in the NICU (Table 6). Age was not significantly correlated with either FEV_1 or FVC z-scores for term- and preterm-born participants (Figure 2).

FEV_1/FVC was lower in participants whose mother received antenatal steroids compared to those who did not (no steroids -0.75 ± 0.97, steroids -0.89 ± 1.33; p=0.029). There were no differences in later lung function between those who did and did not receive surfactant in the NICU.

Table 4. FeNO and spirometry results in term- and preterm-born participants

	Term (n=45)	Preterm (n=113)	Preterm, no BPD (n=60)	Preterm with BPD (n=53)
FeNO (ppb)	16 (9.5–22.5)	14 (7 – 21)	14 (5 – 23)	13.5 (8 – 19)
FEV_1 (z-score)	0.17 ± 1.16	-0.75 ± 1.19**	-0.57 ± 1.16 **	-0.96 ± 1.20 **
FVC (z-score)	0.28 ± 1.10	-0.16 ± 1.08 *	-0.21 ± 1.13 *	-0.11 ± 1.02
FEV_1/FVC (z-score)	-0.21 ± 0.98	-0.85 ± 1.21**	-0.59 ± 1.11	-1.17 ± 1.26 **ǂ
FEF_{25-75} (z-score)	-0.06 ± 1.07	-0.84 ± 1.24 **	-0.52 ± 1.06 *	-1.22 ± 1.34 **ǂ
Airway obstruction, n (%)	3 (6.7)	30 (26.5)**	11 (18.3)	19 (35.9)**ǂ
BDR, n (%)	5 (11.6)	29 (24.8)	11 (18.3)	18 (34.6)*ǂ

Data presented as mean ± standard deviation, median (interquartile range) or number (% of population)

*significantly different to term-born controls p<0.05

**significantly different to term-born controls p<0.01

ǂ significant difference to preterm participants with no bronchopulmonary dysplasia (BPD)

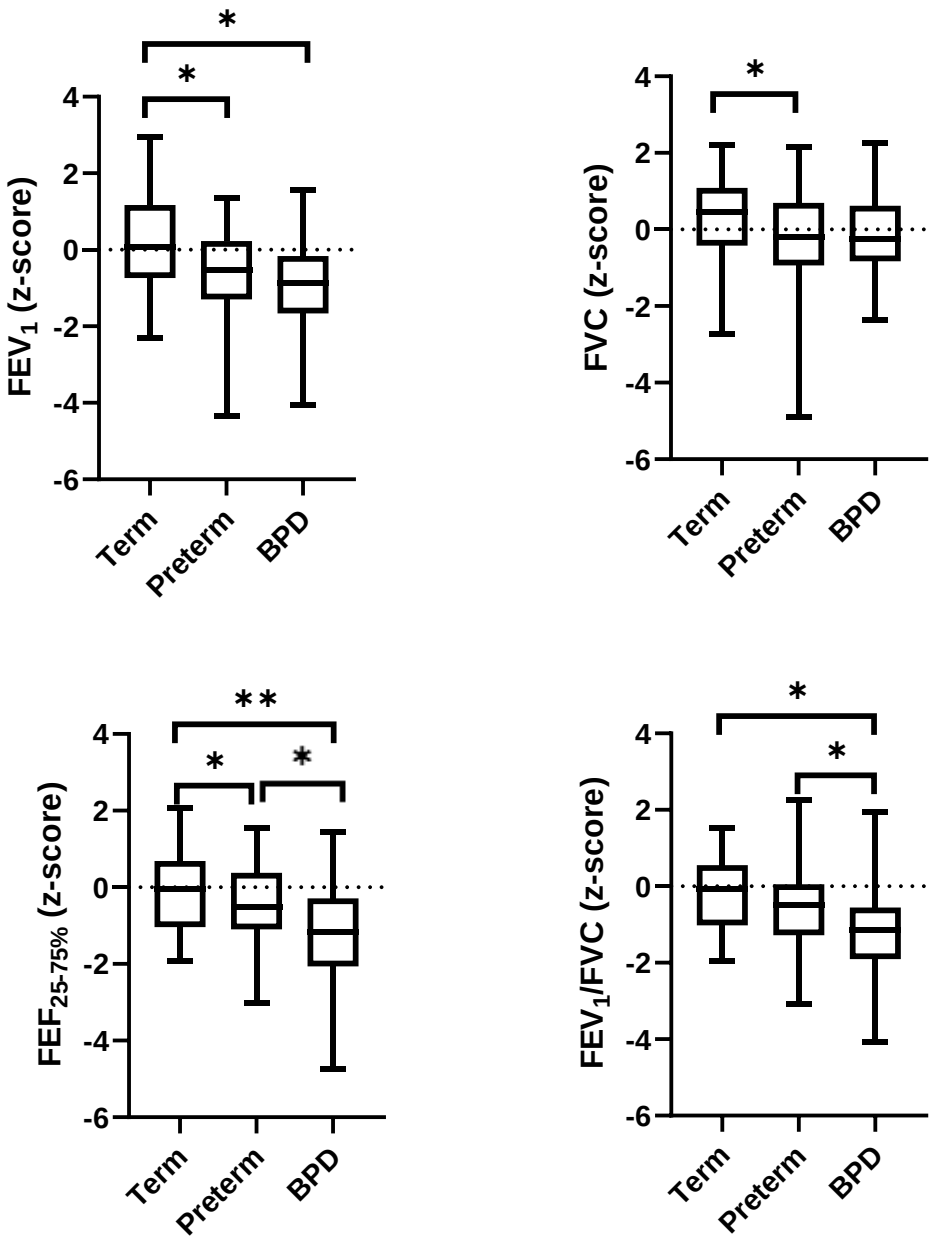
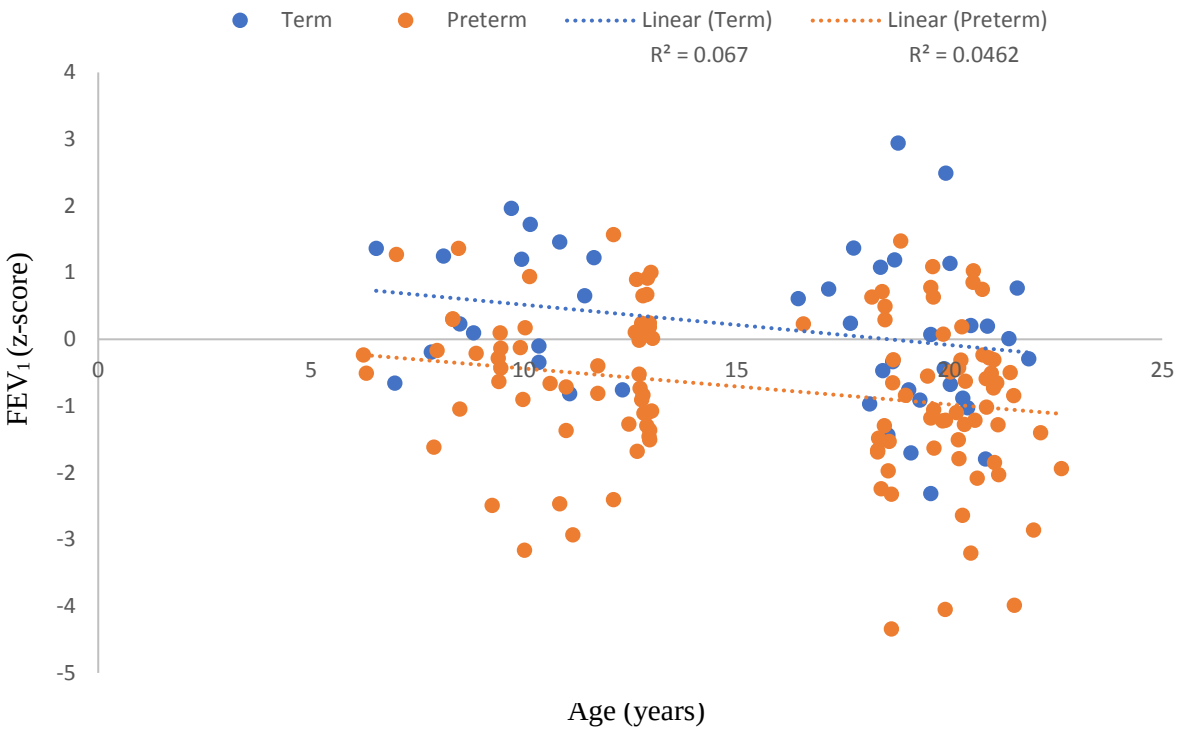


Figure 1. Differences in median spirometry outcomes between term-born participants "Term", preterm participants without BPD "Preterm" and preterm participants with BPD "BPD". Spirometry outcomes are expressed in z-scores as derived from the Global Lung Initiative reference equations (145). *Significant difference ($p<0.05$); **Significant difference ($p<0.01$)



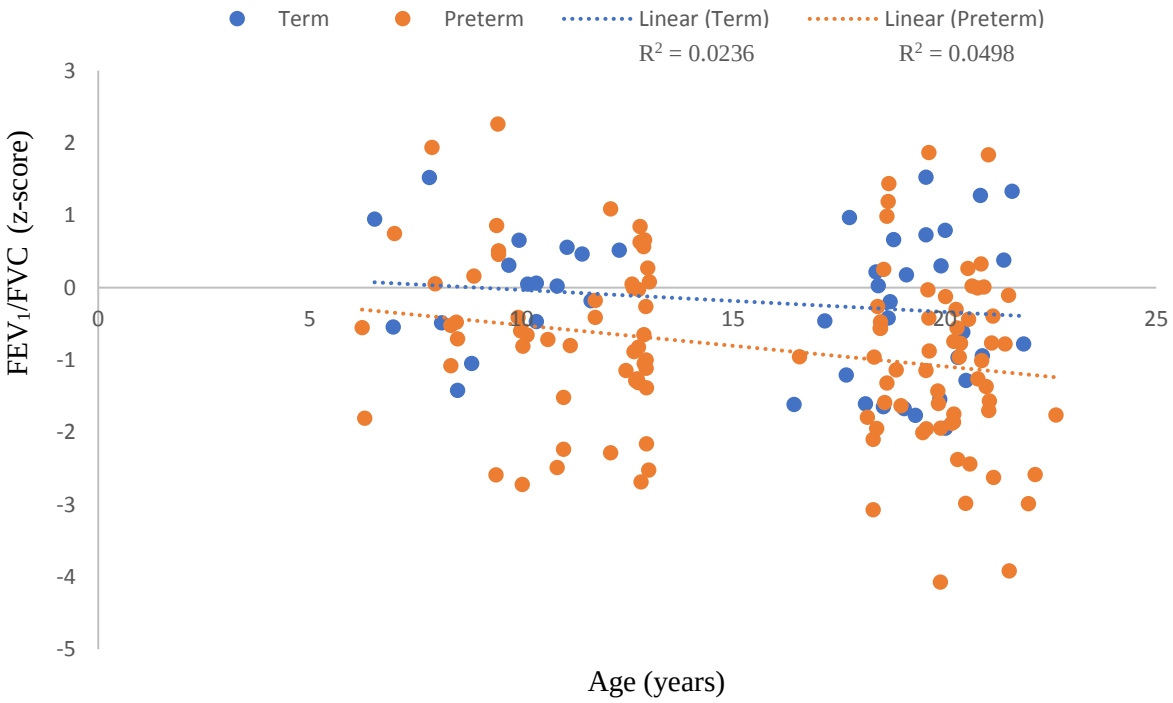


Figure 2. Relationship between FEV_1 and FEV_1/FVC z-scores and age (years). Age was not significantly correlated with either FEV_1 or FVC z-scores for term- and preterm-born participants.

Spectral oscillometry: R_5 and X_5 z-scores were not different between term and preterm-born participants. Preterm-born children had increased resonant frequency (Fres) and area under the reactance curve (AX) z-scores compared to term-born children ($p<0.01$) (Table 5). When comparing those with and without BPD to term-born controls, R_5 z-scores were not different across all groups. Those without BPD had decreased X_5 z-scores compared to those with BPD and those born at term ($p<0.01$). AX z-scores were increased compared to term-born participants in both those with and without BPD. Those with BPD had increased Fres z-scores compared to term participants and those without BPD (Figure 3). The proportion of those with a significant bronchodilator response according to the 2020 ERS technical guidelines for respiratory oscillometry was significantly different between those born at term and those with BPD (X^2 (1, N = 100) = 5.30, p = .02), but not different between the other groups. Of the 19 participants with a significant bronchodilator response according to oscillometry guidelines, only 9 also had a significant bronchodilator response with spirometry. Decreased GA and increased days of oxygen supplementation was associated with increased X_5 and Fres z-scores (Table 7). Increased days of mechanical ventilation was associated with increased X_5, AX and Fres z-scores (Table 7, Figure 4).

Intrabreath oscillometry: Intrabreath measures $R_{10in-ex}$ and $X_{10in-ex}$ are independent of body size and are reported in absolute units (Hz). $R_{10in-ex}$ and $X_{10in-ex}$ were not different between term and preterm groups. Those without BPD had a higher median $X_{10in-ex}$ than those with BPD (Table 5). Age and height was not associated with either $R_{10in-ex}$ or $X_{10in-ex}$. Increased days of oxygen supplementation was associated with increased $R_{10in-ex}$ and decreased $X_{10in-ex}$.

Table 5. Spectral and intra-breath oscillometry outcomes for term- and preterm-born participants.

	Term	Preterm	Preterm no BPD	Preterm with BPD
R_5 (z-score)	0.53 ± 1.25	0.68 ± 1.31	0.59 ± 1.40	0.79 ± 1.21
X_5 (z-score)	0.39 (-0.78–1.17)	0.07 (-0.76–0.9)	-0.28 (-0.98-0.42)**	0.65 (-0.26-1.56)ǂ
AX (z-score)	0.88 ± 1.02	1.55 ± 1.33**	1.43 ± 1.38*	1.69 ± 1.28**
Fres (z-score)	0.64 (0.06–1.23)	1.42 (0.65–2.20)**	1.09 (0.38-1.81)	1.64 (0.74–2.54)** ǂ
BDR spectral n (%)	2 (4.4)	17 (14.3)	6 (9.4)	11 (20.0)*
$R_{10in-ex}$ (Hz)	-0.2 (-0.4–0)	-0.17 (-0.42–0.08)	-0.2 (-0.7–0)	-0.1 (-0.4–0.4)
$X_{10in-ex}$ (Hz)	0.1 (0–0.375)	0.18 (-0.18–0.54)	0.4 (0-1.05)	0.1 (-0.1–0.6) ǂ

Data presented as mean ± SD or median (IQR).
*significantly different to term-born controls $p<0.05$
**significantly different to term-born controls $p<0.01$
ǂ significant difference to preterm participants with no BPD

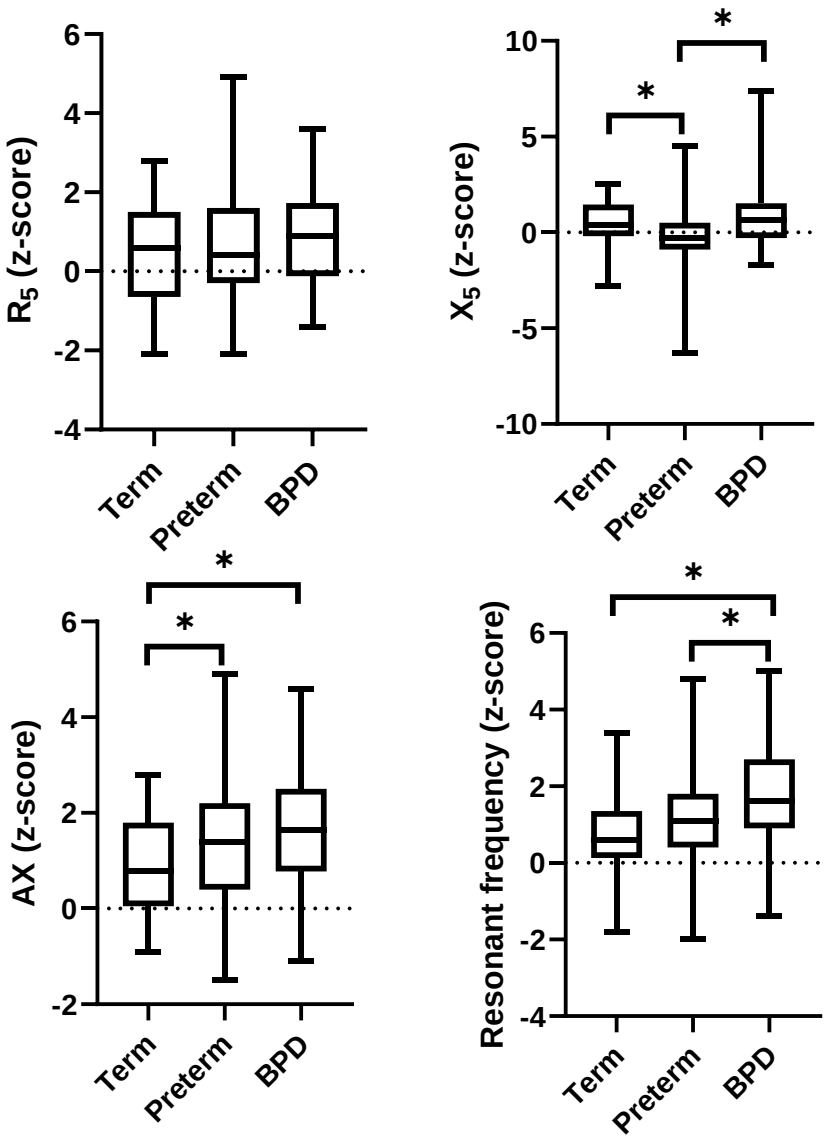


Figure 3. Differences in median spectral oscillomtery outcomes – resistance at 5 Hz (R_5), reactance at 5 Hz (X_5), area under the reactance curve (AX) and resonant frequency (Fres) – between term-born participants "Term", preterm participants without BPD "Preterm" and preterm participants with BPD "BPD". Oscillometry outcomes are expressed in z-scores.
*Significant difference ($p<0.05$)

Table 6. Neonatal factors influencing spirometry parameters. Neonatal factors were adjusted for multicollinearities, such as gestational age with duration of respiratory support, by using the un-standardised residuals of independent regressions of the confounding variables. These residuals were then used in regression analyses against lung function parameters to assess the independent association of each of these neonatal factors on lung function outcomes.

	B	p-value	95 % CI Lower	95 % CI Upper
FEV_1 z-score				
GA	0.215	0.020*	0.016	0.188
BW z-score	0.234	0.011*	0.081	0.612
IMV residual	-0.263	0.004*	-0.524	-0.100
O_2 residual	-0.233	0.011*	-0.491	-0.064
CPAP residual	0.188	0.042*	0.008	0.444
FEV_1/FVC z-score				
GA	0.217	0.019*	0.018	0.195
BW z-score	0.106	0.256	-0.117	-0.436
IMV residual	-0.244	0.008*	-0.514	-0.078
O_2 residual	-0.245	0.008*	-0.520	-0.080
CPAP residual	0.186	0.045*	0.005	0.449
$FEF_{25-75\%}$ z-score				
GA	0.331	p<0.001*	0.078	0.251
BW z-score	0.181	0.051	-0.001	0.560
IMV residual	-0.312	0.001*	-0.604	-0.169
O_2 residual	-0.349	p<0.001*	-0.647	-0.217
CPAP residual	0.310	0.001*	0.169	0.609

*significant association (p<0.05)

Table 7. Neonatal factors influencing spectral and intra-breath oscillometry parameters

	B	p-value	95 % CI Lower	95 % CI Upper
R_5				
GA	-0.124	0.182	-0.161	0.031
BW z-score	-0.041	0.659	-0.357	0.227
IMV residual	0.172	0.063	-0.013	0.472
O_2 residual	0.132	0.155	-0.067	0.414
CPAP residual	-0.437	p<0.001*	-0.790	-0.355
X_5				
GA	-0.295	0.001*	-0.334	-0.084
BW z-score	-0.052	0.581	-0.505	-.284
IMV residual	0.275	0.003*	0.176	0.816
O_2 residual	0.326	p<0.001*	0.269	0.890
CPAP residual	-0.433	p<0.001*	-1.062	-0.471
AX				
GA	-0.126	0.177	-0.164	0.031
BW z-score	-0.177	0.056	-0.576	0.007
IMV residual	0.259	0.005*	0.109	0.592
O_2 residual	0.164	0.078	-0.025	0.461
CPAP residual	-0.339	p<0.001*	-0.682	-0.220
Fres				
GA	-0.283	0.003*	-0.246	-0.052
BW z-score	-0.181	0.060	-0.589	0.013
IMV residual	0.268	0.005*	0.113	0.614
O_2 residual	0.365	p<0.001*	0.0249	0.727
CPAP residual	-0.335	p<0.001*	-0.675	-0.202
$R_{10in-ex}$				
GA	-0.173	0.067	-0.106	0.004
BW z-score	0.011	0.907	-0.161	0.181
IMV residual	-0.010	0.916	-0.151	0.135
O_2 residual	0.195	0.039*	0.008	0.279
CPAP residual	-0.089	0.346	-0.203	0.072
$X_{10in-ex}$				
GA	0.185	0.050	0.000	0.133
BW z-score	-0.057	0.546	-0.273	0.145
IMV residual	0.038	0.691	-0.140	0.210
O_2 residual	-0.244	0.009*	-0.384	-0.056
CPAP residual	0.166	0.079	-0.017	0.315

*significant association (p<0.05)

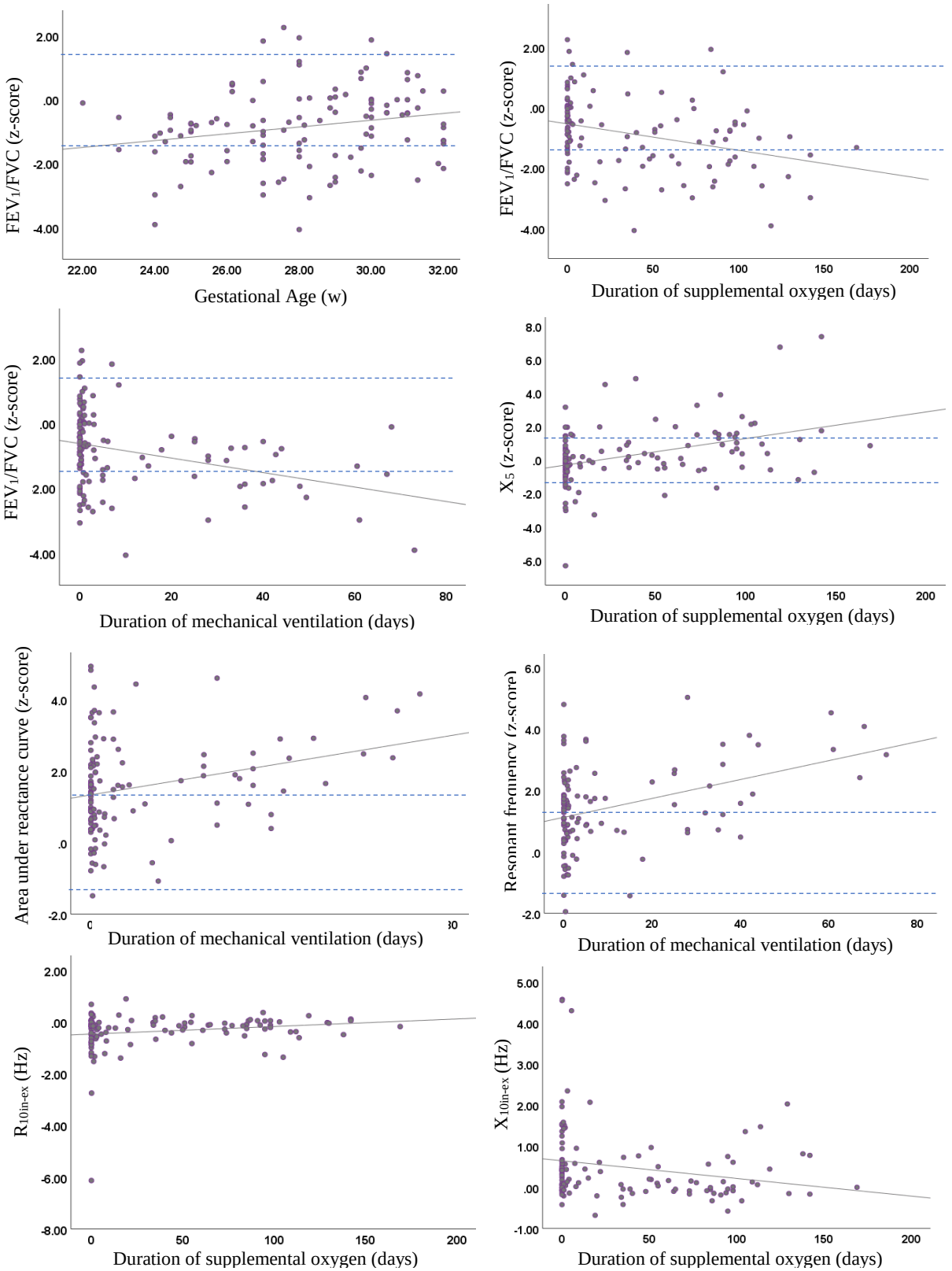


Figure 4. Neonatal factors influencing lung function in preterm-born children in later-childhood. Lung function parameters are expressed as z-scores, except for $R_{10in-ex}$ and $X_{10in-ex}$ which are expressed in Hz. Dashed lines indicate upper and lower limits of normal, at ±1.64 z-scores.

Symptoms and lung function

Those with recent respiratory symptoms (n=70) such as cough, wheeze or shortness of breath had lower FEV_1, FEV_1/FVC and $FEF_{25-75\%}$ than those without symptoms (n=47) (Figure 5, Table 8). Symptomatic participants also had increased resistance (R_5), increased area under the curve (AX) and increased resonant frequency (Fres) (Figure 5, Table 8). Over 40 % of preterm-born participants reported shortness of breath on exertion (Table 3) and midflows ($FEF_{25-75\%}$) were reduced in this group compared to those who did not report shortness of breath on exertion (mean difference ± SEM; -0.54 ± 0.23, p=0.02).

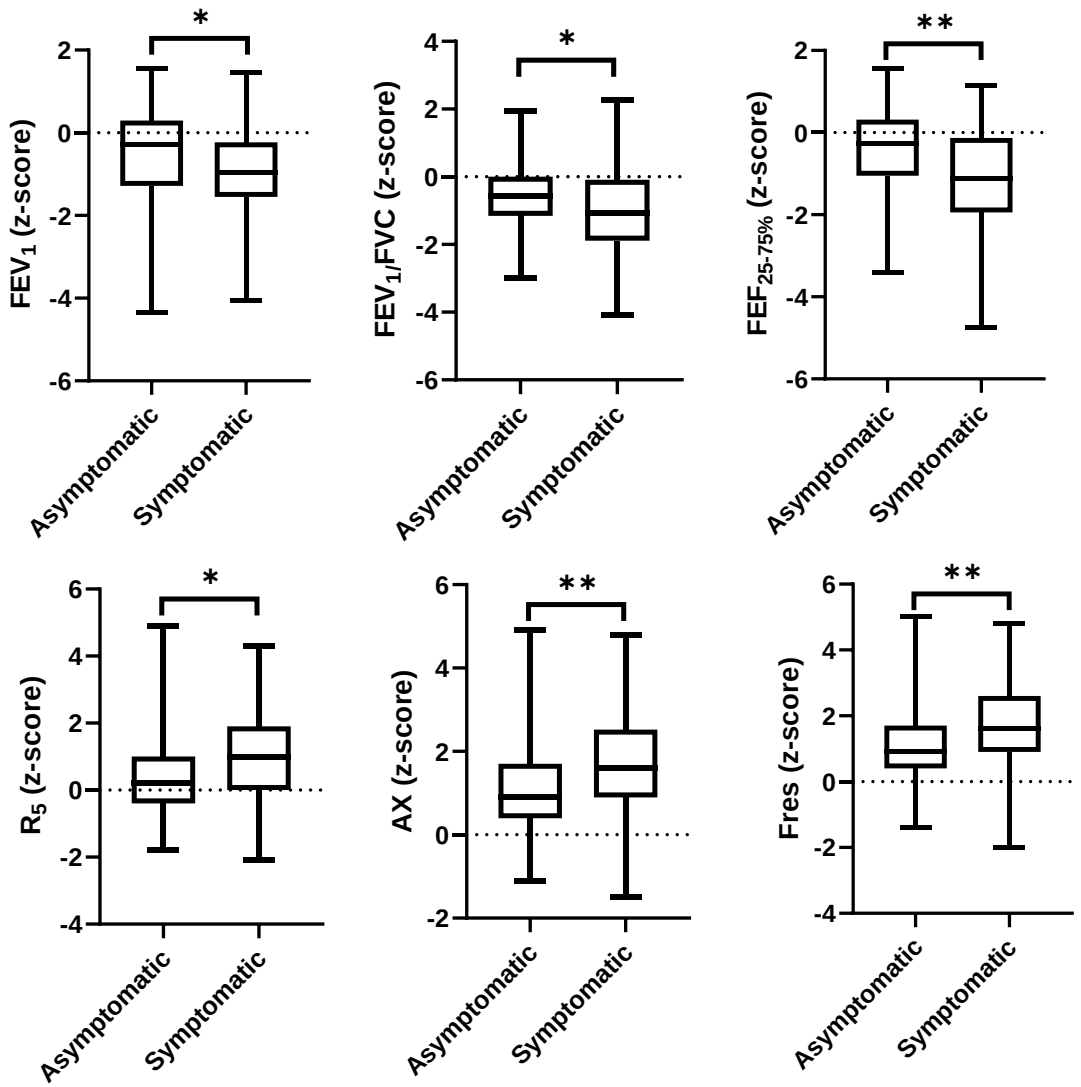


Figure 5. Differences in median spirometry and oscillometry outcomes between preterm participants who reported no recent respiratory symptoms within 3 months prior to lung function testing (Asymptomatic) and those who had any recent symptoms (Symptomatic). Lung function outcomes are expressed in z-scores.
*Significant difference ($p<0.05$); **Significant difference ($p<0.01$)

Table 8. Lung function in preterm participants with and without recent respiratory symptoms

	No symptoms (n=47)	Any symptoms (n=70)
FeNO (ppb)	16 (7-25)	13 (7-19)
FEV_1 (z-score)	-0.29 (1.58)	-0.96 (1.32)*
FVC (z-score)	0.28 (1.78)	-0.35 (1.27)
FEV_1/FVC (z-score)	-0.57 ± 1.09	-1.03 ± 1.26*
FEF_{25-75} (z-score)	-0.45 ± 1.03	-1.11 ± 1.31**
R_5 (z-score)	0.22 (1.39)	0.96 (1.86)*
X_5 (z-score)	-0.28 (1.02)	0.37 (1.97)
AX (z-score)	0.93 (1.36)	1.62 (1.31)**
Fres (z-score)	1.02 ± 1.19	1.71 ± 1.37 **
$R_{10in-ex}$	-0.20 (0.47)	-0.14 (0.54)
$X_{10in-ex}$	0.48 (1.02)	0.11 (0.54)

Data expressed as mean ± SD or median (IQR).
*significantly different between symptomatic and asymptomatic groups ($p<0.05$)
**significantly different between symptomatic and asymptomatic groups ($p<0.01$)

DISCUSSION

This study shows that preterm-born children have reduced lung function and altered respiratory mechanics compared to those born at term, with poorer outcomes associated with lower gestational age, the requirement of more mechanical ventilation and oxygen supplementation at birth, and the neonatal diagnosis of BPD.

Approximately half of preterm-born participants reported recent respiratory symptoms, documented as either cough, wheeze, shortness of breath or a rattly chest in the 3 months prior to their study visit. Those with a BPD diagnosis reported higher rates of recent daytime wheeze and shortness of breath, along with more wheeze and cough on exertion, with over 40 % of all preterm participants reporting shortness of breath on exertion. Preterm participants who reported any recent symptoms also had poorer spirometry and oscillometry outcomes compared to those who did not. These high rates of respiratory symptoms are consistent with previous studies and demonstrate the high burden of respiratory disease in preterm individuals well beyond early childhood (54-58, 146). Despite high rates of symptoms, the rates of asthma medication use were relatively low. Closer respiratory follow-up for those reporting symptoms may be beneficial, considering the increasing evidence of ongoing and perhaps progressive respiratory morbidity in preterm-born individuals.

Those who reported shortness of breath on exertion had lower mid-flow outcomes on spirometry. The relatively high incidence of shortness of breath on exertion in preterm participants may reflect the sequalae of new BPD, where infants born extremely prematurely exhibit less severe acute lung injury but have arrested alveolar and vascular growth resulting in long-term respiratory symptoms (14). Alternatively, others have shown that the upper airway may play a role in persistent symptoms such as wheeze and exercise induced symptoms (147). Upper airway damage and resulting dysfunction are differentials of exercise-induced asthma (148), and laryngeal pathology is reported in preterm-born children at school-age (149) likely from damage following invasive ventilation strategies in the neonatal period. Underlying mechanisms of exercise-induced symptoms in children born preterm may extend beyond chronic disease and include altered pulmonary vascularisation and long-term upper airway pathology. Additionally, shortness of breath in those born preterm may have implications for their exercise capacity. However, a recent study reported that despite almost half of the preterm-born participants having parentally reported respiratory symptoms on exertion, there were no differences in aerobic exercise capacity between term and preterm-born children (150). Additionally, a study in preterm-born adults found no difference in the degree of dyspnoea when compared to those born at term during exercise testing (151). Evidence of reduced exercise capacity in preterm-born children with and without BPD is conflicting, with some studies reporting reduced exercise capacity in those born preterm (152-154), while others report no differences to those born at term (150, 155, 156). Although not examined in the current study, the relationship, if any, of exertional dyspnoea to exercise capacity in children born preterm remains to be understood.

Consistent with other studies, we showed an obstructive pattern of disease on spirometry in preterm-born participants, reporting reduced FEV_1, FEV_1/FVC ratio and midflows with similar FVC values to term-born controls (45, 54, 55, 57, 65). Those with BPD had significantly lower FEV_1/FVC ratio and midflows than those without BPD, with poorer spirometry outcomes associated with lower GA and the requirement of respiratory support in the NICU. This indicates that the lung function observed in our cohort is typical of the preterm-born population in the post-surfactant era, with other studies noting the association of reduced GA (157) and the requirement of more respiratory intervention (45) with worse lung function outcomes in childhood. This is not unexpected considering that more immature lungs not only require more support but are also likely more susceptible to subsequent injury from invasive ventilation and inflammatory events such as sepsis in the NICU. These early events may result in chronic airway inflammation and airway remodelling – as suggested by abnormal chest CT findings in mid-childhood by Simpson *et al.* (45) – leading to the observed obstructed lung function in later childhood. Interestingly, we saw lower FEV_1/FVC ratio in those born preterm who received antenatal steroids compared to those who did not. This is similar to findings by Simpson et al who also report

decreased ratio in mid-childhood survivors of preterm birth who received antenatal steroids (62). However, other studies have reported either no long-term effect on lung function (158, 159) or improved lung function (160, 161) with antenatal steroid treatment. Antenatal steroid treatment increases survival but has little impact on the rates of BPD (158), and the lower lung function observed in those treated with antenatal steroids in our study may reflect mothers with recognised antenatal risk factors for preterm birth which may also have long-term impacts on lung health regardless of BPD diagnosis. Alternatively, antenatal steroids may lead to alterations in lung morphogenesis despite promoting lung cellular differentiation and surfactant production as suggested by primate studies (162). Together with the risk of antenatal steroid administration restricting foetal growth (163), the long-term effects of antenatal steroids may be associated structural lung changes resulting in lower lung function into childhood. As the risks, benefits and optimal dosing of antenatal steroid administration continues to be reviewed (164), further investigation into their effect on long-term lung structure and function is warranted. Together our ur results add to the growing evidence of airway obstruction across childhood and young-adulthood in individuals born very prematurely and the case for routine lung function testing in this population.

Spirometry is the most common measure of lung function reported in survivors of preterm birth. However, other lung function tests, particularly those providing information on the peripheral lung such as oscillometry, are likely to yield additional information about disease pathophysiology. These tests are under-reported in survivors of preterm birth. Some studies which measured osillometry outcomes report both increased resistance and decreased reactance in children born preterm (165, 166), where others reported normal resistance values in childhood but more negative reactance and increased AX and Fres (45, 157). The latter is more in line with our oscillometry outcomes, where we observed no difference in resistance (R_5) between term and preterm-born children with and without BPD. Considering R_5 is reflective of both large and small airways (120), this suggests that total airway resistance is not different between preterm and term-born children. The measure of R_{5-20} may have been a more informative measure considering it is more sensitive to small airways disease (120), however there is a lack of robust reference data for this measure which is required to further explore these outcomes. Reactance was only decreased in preterm children without BPD but was not different overall between preterm and term-born participants. We observed large variability of X_5 outcomes across our preterm group in comparison to those born at term which may contribute to no difference being observed between groups. We also observed that AX was increased in preterm children and further increased in those with BPD. Fres was also increased in those born preterm, mostly driven by the increased Fres in those with BPD. Together these outcomes suggest no difference in large airway mechanics but altered peripheral lung mechanics and reduced elasticity of the lungs in those born preterm (120). Considering

increased mechanical ventilation was associated with increased AX and Fres, and decreased GA and increased oxygen supplementation also associated with increased Fres, it is implied that lung injury from preterm birth or subsequent treatment may result in alterations in small airway calibre and reduction in lung elastance in later life. The large variability in our oscillometry measures is likely due to the use of two different reference equations, namely the Calogero reference equations for children up to 13 years, and Oostveen reference equations for those above this age. This variability highlights the need for normative, global reference values for oscillometry as outlined in the Technical Standard for Respiratory Oscillometry (120).

Recent studies have suggested that intra-breath oscillometry measures may be more useful in detecting wheeze (123) and predicting lower respiratory tract infections (LRTI) (167) in infants and young children than spectral oscillometry. By measuring the change in impedance during the inspiratory and expiratory phases of the breathing cycle separately, these studies were able to detect disease changes likely attributable to factors least affected by breathing pattern and the upper airway, such as inhomogeneity of the peripheral airways and an increased baseline constrictor tone of the airways (123, 167). Ours is the first study to report within-breath changes with single-frequency oscillometry with $R_{10in-ex}$ and $X_{10in-ex}$ measures in preterm-born children and suggests these measures may be less useful in this population. Our study showed no difference in $R_{10in-ex}$ and $X_{10in-ex}$ measures between preterm and term-born groups. In adults with COPD, the within-breath changes in resistance outcomes were not different to healthy controls, likely because the mechanics of upper airway flow within breaths is similar regardless of disease status (168). This may also explain the similarity in within-breath resistance measures between our healthy and preterm participants. The studies of within breath measures in infants with LRTI and adults with COPD did however see altered within-breath reactance values, which they suggest is likely due to inhomogenous peripheral airway obstruction (167, 168). We did not observe differences in $X_{10in-ex}$ measures between preterm and healthy participants or any associations of within-breath measure with spirometry outcomes, which may mean that airway inhomogeneity is likely not the primary driver of airway obstruction in preterm-born individuals. Indeed, small studies measuring ventilation inhomogeneity using multiple breath washout report no differences between preterm and term-born infants (77, 169, 170). Airway obstruction in preterm-born individuals may instead be more attributable to reduced compliance as suggested by our spectral oscillometry outcomes. The observed association between increased days of oxygen supplementation and increased $R_{10in-ex}$ and decreased $X_{10in-ex}$, may suggest some influence of neonatal factors on within-breath mechanics and warrants further investigation. As the literature around within-breath oscillometry is limited, there are no references for 'normal' measures and the physiology behind within-breath outcomes remains somewhat

speculative. Further work is needed to explore the physiology of within-breath changes and its implications in individuals born preterm.

Despite exhibiting more airway obstruction and altered respiratory mechanics, there was no difference in FeNO levels between term and preterm-born children both with and without BPD. This is consistent with other literature (142) which suggests the observed reduction in lung function in preterm-born children is not due to eosinophilic inflammation but perhaps other underlying pathways of inflammation or airway structural abnormalities.

Our study reported that a greater proportion of those with BPD, but not those born preterm without BPD, responded to bronchodilator compared to those born at term. With 34.6 % of our BPD group exhibiting a significant bronchodilator response by spirometry, our findings are consistent with the limited existing evidence of bronchodilator responsiveness in preterm-born children, with studies reporting between 25-60 % of those with BPD with significant FEV_1 improvement post-bronchodilator (171). Thunqvist *et al*. reported a 28 % rate of bronchodilator response in 6-year-old children who were born very prematurely, while the EpiCure study found 27 % of 11-year-old children born <25weeks had significant bronchodilator response (172). Our results also found that 22 of 33 participants with airway obstruction on spirometry had a significant bronchodilator response, with 11 who did not. With reversibility of airway obstruction only observed in a subset of those born preterm, the distinction of the lung disease observed in the group from typical asthma is further reinforced. This also suggests that there is a proportion of preterm-born children with fixed airway obstruction. Together with oscillometry data suggesting increased respiratory system stiffness and reports of structural lung disease on CT in this population (40, 41, 43, 45-47), it may be that structural lung damage as a result of early lung injury persists, leading to irreversible functional deficits. However, bronchodilator use may still be useful in a subset of those born preterm. Evidently, there is some heterogeneity in respiratory phenotype in those born preterm with persistent respiratory deficits, in a similar way to different phenotypes in asthmatics. Further work is needed to understand underlying mechanisms of disease for those with seemingly fixed airway obstruction and possibly reduced pulmonary vasculature to identify potential interventions for this group. Better characterisation of the respiratory trajectory of those born preterm combining biological markers, radiology, symptoms, clinical features and more comprehensive functional testing will assist in clarifying these mechanisms.

Assessing bronchodilator response using oscillometry may provide additional insight into the utility of bronchodilators in those born preterm. In the present study, a significant bronchodilator response was observed in 19 participants according to oscillometry guidelines with 34 participants exhibiting a significant bronchodilator response according to spirometry guidelines; of these only 9 had a

bronchodilator response according to both techniques. It is possible that oscillometry identifies additional individuals for whom respiratory mechanics are improved with bronchodilator use. However, the lack of overlap between participants identified as having a bronchodilator response according to spirometry and oscillometry classifications may also be explained by the different criteria used in each technique to determine a significant response. A significant bronchodilator response using oscillometry is based on the presence of 95th percentile measures for each outcome, allowing determination of a threshold value (120). Oscillometry bronchodilator response assessment is also based on absolute values which are dependent on baseline values (124). Oscillometry z-score bronchodilator response calculations have been proposed to adjust for this limitation, however the healthy reference data needed for these calculations is not yet available (120). Once z-score bronchodilator response calculations are available for oscillometry, it will be worthwhile assessing oscillometry outcomes after bronchodilator in those born preterm, which may provide more information on potentially modifiable areas of the respiratory system to improve function in this population.

Longitudinal studies suggest that lung function (spirometry) is declining through life in preterm-born individuals, however in these 2 cross-sectional studies spanning 6 to 22 years, we saw no association between lung function and age. Considering that lung function peaks in early adulthood (173), it is likely that many preterm-born individuals exhibit lung function within normal limits as they approach this age. As such, a distinct age-related decline may not be observable when analysing lung function at a single time-point, particularly considering the heterogeneity of lung disease in those born preterm. Certainly, cross-sectional analysis does not track trajectories of lung function as in longitudinal studies. Previous longitudinal studies which have tracked trajectories suggest declining lung function in children and young-adults born preterm (62, 65). However, many of these individuals may still have lung function within normal ranges during these ages. Despite this, if these trajectories of decline continue into adulthood, this poses a risk for early-onset respiratory morbidity for those born preterm perhaps in their thirties and beyond (174). Additionally, the heterogeneity of lung disease in those born preterm may mean that while some individuals exhibit declining lung function trajectories, some may track throughout childhood or even improve throughout life (174). Furthermore, lower spirometry outcomes in childhood (as observed in those born preterm) predict later morbidity (86). As such it is important to identify those individuals at risk of persistent and progressive lung disease in order to appropriately intervene to prevent early-onset respiratory morbidity.

We show that preterm-born children both with and without BPD exhibit obstructed lung function on spirometry and altered peripheral airway mechanics suggesting reduced lung elastance compared to those born at term. These functional deficits occur in the absence of elevated eosinophilic inflammation

with only about a third of children with BPD having a short-term response to bronchodilator. Further investigation of within-breath oscillometry measures may provide further insight into the mechanics of the observed airway obstruction in individuals born preterm, In light of recent evidence suggesting lung function may trend further away from normal throughout childhood, it is crucial that we better understand the underlying processes contributing to functional abnormalities after premature birth to enable us to explore interventions to prevent further decline.

REFERENCES

12. Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller AB, Narwal R, et al. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. Lancet. 2012;379(9832):2162-72.
14. Abman SH. Bronchopulmonary Dysplasia: "A Vascular Hypothesis". American journal of respiratory and critical care medicine. 2001;164(10):1755-6.
34. Kotecha SJ, Edwards MO, Watkins WJ, Henderson AJ, Paranjothy S, Dunstan FD, et al. Effect of preterm birth on later FEV1: a systematic review and meta-analysis.(forced expiratory volume)(Report). Thorax. 2013;68(8):760.
40. Tonson La Tour A, Spadola L, Sayegh Y, Combescure C, Pfister R, Barazzone Argiroffo C, et al. Chest CT in bronchopulmonary dysplasia: Clinical and radiological correlations. Pediatric Pulmonology. 2013;48(7):693-8.
41. Mahut B, De Blic J, Emond S, Benoist M-R, Jarreau P-H, Lacaze-Masmonteil T, et al. Chest computed tomography findings in bronchopulmonary dysplasia and correlation with lung function. Archives of Disease in Childhood - Fetal and Neonatal Edition. 2007;92(6):F459.
42. Broström EB, Thunqvist P, Adenfelt G, Borling E, Katz-Salamon M. Obstructive lung disease in children with mild to severe BPD. Respir Med. 2010;104(3):362-70.
43. Aukland SM, Halvorsen T, Fosse KR, Daltveit AK, Rosendahl K. High-resolution CT of the chest in children and young adults who were born prematurely: findings in a population-based study. AJR American journal of roentgenology. 2006;187(4):1012.
45. Simpson SJ, Logie KM, O'Dea CA, Banton GL, Murray C, Wilson AC, et al. Altered lung structure and function in mid-childhood survivors of very preterm birth. Thorax. 2017.
46. Howling SJ, Northway WH, Hansell DM, Moss RB, Ward S, Müller NL. Pulmonary sequelae of bronchopulmonary dysplasia survivors: high-resolution CT findings. AJR American journal of roentgenology. 2000;174(5):1323.
47. Wong PM, Lees AN, Louw J, Lee FY, French N, Gain K, et al. Emphysema in young adult survivors of moderate-to-severe bronchopulmonary dysplasia. The European respiratory journal. 2008;32(2):321.
51. Pramana IA, Latzin P, Schlapbach LJ, Hafen G, Kuehni CE, Nelle M, et al. Respiratory symptoms in preterm infants: burden of disease in the first year of life.(Clinical report). 2011;16(197).
53. Been JV, Lugtenberg MJ, Smets E, van Schayck CP, Kramer BW, Mommers M, et al. Preterm birth and childhood wheezing disorders: a systematic review and meta-analysis. PLoS Med. 2014;11(1):e1001596.
54. Simpson SJ, Hall GL, Wilson AC. Lung function following very preterm birth in the era of 'new' bronchopulmonary dysplasia. Respirology. 2015;20(4):535-40.
55. Fawke J, Lum S, Kirkby J, Hennessy E, Marlow N, Rowell V, et al. Lung function and respiratory symptoms at 11 years in children born extremely preterm: the EPICure study. American journal of respiratory and critical care medicine. 2010;182(2):237-45.
56. Korhonen P, Laitinen J, Hyoudynmaa E, Tammela O. Respiratory outcome in school-aged, very-low-birth-weight children in the surfactant era. Acta Pædiatrica. 2004;93(3):316-21.
57. Thunqvist P, Gustafsson PM, Schultz ES, Bellander T, Berggren-Broström E, Norman M, et al. Lung Function at 8 and 16 Years After Moderate-to-Late Preterm Birth: A Prospective Cohort Study. Pediatrics. 2016;137(4).
58. Landry J, Tremblay G, Li P, Wong C, Benedetti A, Taivassalo T. Lung Function and Bronchial Hyperresponsiveness in Adults Born Prematurely A Cohort Study. Annals of the American Thoracic Society. 2016;13(1):17-24.
62. Simpson SJ, Turkovic L, Wilson A, Verheggen M, Logie KM, Pillow JJ, et al. Lung function trajectories throughout childhood in survivors of very preterm birth: A longitudinal cohort study. The Lancet Child and Adolescent Health. 2018.

63. Kotecha SJ, Watkins WJ, Paranjothy S, Dunstan FD, Henderson AJ, Kotecha S. Effect of late preterm birth on longitudinal lung spirometry in school age children and adolescents. Thorax. 2012;67(1):54.
64. Filippone M, Sartor M, Zacchello F, Baraldi E. Flow limitation in infants with bronchopulmonary dysplasia and respiratory function at school age. The Lancet. 2003;361(9359):753-4.
65. Doyle LW, Adams A-M, Robertson C, Ranganathan S, Davis NM, Lee KJ, et al. Increasing airway obstruction from 8 to 18 years in extremely preterm/low-birthweight survivors born in the surfactant era. Thorax. 2017;72(8):712.
68. Fakhoury KF, Sellers C, Smith EOB, Rama JA, Fan LL. Serial measurements of lung function in a cohort of young children with bronchopulmonary dysplasia. Pediatrics. 2010;125(6):e1441.
73. Vrijlandt EJLE, Boezen HM, Gerritsen J, Stremmelaar EF, Duiverman EJ. Respiratory Health in Prematurely Born Preschool Children with and without Bronchopulmonary Dysplasia. The Journal of Pediatrics. 2007;150(3):256-61.
74. Thunqvist P, Gustafsson P, Norman M, Wickman M, Hallberg J. Lung function at 6 and 18 months after preterm birth in relation to severity of bronchopulmonary dysplasia. Pediatric pulmonology. 2015;50(10):978-86.
75. Udomittipong K, Sly PD, Patterson HJ, Gangell CL, Stick SM, Hall GL. Forced oscillations in the clinical setting in young children with neonatal lung disease. The European respiratory journal. 2008;31(6):1292.
77. Latzin P, Roth S, Thamrin C, Hutten GJ, Pramana I, Kuehni CE, et al. Lung volume, breathing pattern and ventilation inhomogeneity in preterm and term infants. PloS one. 2009;4(2):e4635.
86. Martinez FD. Early-life origins of chronic obstructive pulmonary disease. New England Journal of Medicine. 2016;375(9):871-8.
117. Asher M, Keil U, Anderson H, Beasley R, Crane J, Martinez F, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. European respiratory journal. 1995;8(3):483-91.
120. King GG, Bates J, Berger KI, Calverley P, de Melo PL, Dellacà RL, et al. Technical standards for respiratory oscillometry. European Respiratory Journal. 2020;55(2).
123. Czövek D, Shackleton C, Hantos Z, Taylor K, Kumar A, Chacko A, et al. Tidal changes in respiratory resistance are sensitive indicators of airway obstruction in children. Thorax. 2016;71(10):907-15.
124. Graham BL, Steenbruggen I, Miller MR, Barjaktarevic IZ, Cooper BG, Hall GL, et al. Standardization of spirometry 2019 update. An official American thoracic Society and European respiratory Society technical statement. American journal of respiratory and critical care medicine. 2019;200(8):e70-e88.
141. Ferraro V, Carraro S, Bozzetto S, Zanconato S, Baraldi E. Exhaled biomarkers in childhood asthma: old and new approaches. Asthma research and practice. 2018;4(1):1-7.
142. Course CW, Kotecha S, Kotecha SJ. Fractional exhaled nitric oxide in preterm-born subjects: A systematic review and meta-analysis. Pediatric pulmonology. 2019;54(5):595-601.
143. Magnussen C, Ojeda FM, Rzayeva N, Zeller T, Sinning CR, Pfeiffer N, et al. FEV1 and FVC predict all-cause mortality independent of cardiac function—Results from the population-based Gutenberg Health Study. International journal of cardiology. 2017;234:64-8.
144. Shackleton C. Improving the clinical utility of the forced oscillation technique in preschool-aged children. 2018.
145. Quanjer PH, Stanojevic S, Cole TJ, Baur X, Hall GL, Culver BH, et al. Multi-ethnic reference values for spirometry for the 3-95-yr age range: the global lung function 2012 equations. The European respiratory journal. 2012;40(6):1324-43.
146. Gough A, Spence D, Linden M, Halliday HL, McGarvey LPA. General and Respiratory Health Outcomes in Adult Survivors of Bronchopulmonary Dysplasia: A Systematic Review: A Systematic Review. Chest. 2012;141(6):1554-67.

147. Simpson SJ, Champion Z, Hall GL, French N, Reynolds V. Upper airway pathology contributes to respiratory symptoms in children born very preterm. The Journal of Pediatrics. 2019;213:46-51.
148. Abu-Hasan M, Tannous B, Weinberger M. Exercise-induced dyspnea in children and adolescents: if not asthma then what? Annals of allergy, asthma & immunology. 2005;94(3):366-71.
149. Reynolds V, Meldrum S, Simmer K, Vijayasekaran S, French N. Laryngeal pathology at school age following very preterm birth. International Journal of Pediatric Otorhinolaryngology. 2015;79(3):398-404.
150. O'Dea CA, Logie K, Wilson AC, Pillow JJ, Murray C, Banton G, et al. Lung abnormalities do not influence aerobic capacity in school children born preterm. European Journal of Applied Physiology. 2021;121(2):489-98.
151. Duke JW, Lovering AT. Respiratory and cardiopulmonary limitations to aerobic exercise capacity in adults born preterm. Journal of Applied Physiology. 2020;129(4):718-24.
152. Welsh L, Kirkby J, Lum S, Odendaal D, Marlow N, Derrick G, et al. The EPICure study: maximal exercise and physical activity in school children born extremely preterm. Thorax. 2010;65(2):165-72.
153. MacLean J, DeHaan K, Fuhr D, Hariharan S, Kamstra B, Hendson L, et al. Altered breathing mechanics and ventilatory response during exercise in children born extremely preterm. Thorax. 2016;71(11):1012-9.
154. Smith LJ, van Asperen PP, McKay KO, Selvadurai H, Fitzgerald DA. Reduced exercise capacity in children born very preterm. Pediatrics. 2008;122(2):e287-e93.
155. Clemm H, Røksund O, Thorsen E, Eide GE, Markestad T, Halvorsen T. Aerobic capacity and exercise performance in young people born extremely preterm. Pediatrics. 2012;129(1):e97-e105.
156. Kriemler S, Keller H, Saigal S, Bar-Or O. Aerobic and lung performance in premature children with and without chronic lung disease of prematurity. Clinical journal of sport medicine. 2005;15(5):349-55.
157. Lombardi E, Fainardi V, Calogero C, Puglia M, Voller F, Cuttini M, et al. Lung function in a cohort of 5-year-old children born very preterm. Pediatric pulmonology. 2018;53(12):1633-9.
158. Roberts D, Brown J, Medley N, Dalziel SR. Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. Cochrane database of systematic reviews. 2017(3).
159. Yang J, Kingsford RA, Horwood J, Epton MJ, Swanney MP, Stanton J, et al. Lung function of adults born at very low birth weight. Pediatrics. 2020;145(2).
160. Halvorsen T, Torunn Skadberg B, Egil Eide G, Drange Røksund O, Markestad T. Better care of immature infants; has it influenced long-term pulmonary outcome? Acta Paediatrica. 2006;95(5):547-54.
161. Cheong JL, Doyle LW. Long-term pulmonary outcome of preterm infants. The Newborn Lung. 2019:161-70.
162. Jobe AH, Kemp M, Schmidt A, Takahashi T, Newnham J, Milad M. Antenatal corticosteroids: a reappraisal of the drug formulation and dose. Pediatric Research. 2021;89(2):318-25.
163. Jobe AH, Goldenberg RL. Antenatal corticosteroids: an assessment of anticipated benefits and potential risks. American journal of obstetrics and gynecology. 2018;219(1):62-74.
164. Kemp MW, Schmidt AF, Jobe AH, editors. Optimizing antenatal corticosteroid therapy. Seminars in Fetal and Neonatal Medicine; 2019: Elsevier.
165. Malmberg L, Mieskonen S, Pelkonen A, Kari A, Sovijarvi A, Turpeinen M. Lung function measured by the oscillometric method in prematurely born children with chronic lung disease. European Respiratory Journal. 2000;16(4):598-603.
166. Verheggen M, Wilson AC, Pillow JJ, Stick SM, Hall GL. Respiratory function and symptoms in young preterm children in the contemporary era. Pediatr Pulmonol. 2016;51(12):1347-55.
167. Gray DM, Czovek D, McMillan L, Turkovic L, Stadler JA, Vanker A, et al. Intra-breath measures of respiratory mechanics in healthy African infants detect risk of respiratory illness in early life. European Respiratory Journal. 2019;53(2).

168. Lorx A, Czövek D, Gingl Z, Makan G, Radics B, Bartusek D, et al. Airway dynamics in COPD patients by within-breath impedance tracking: effects of continuous positive airway pressure. European Respiratory Journal. 2017;49(2).
169. Riedel T, Kyburz M, Latzin P, Thamrin C, Frey U. Regional and overall ventilation inhomogeneities in preterm and term-born infants. Intensive care medicine. 2009;35(1):144-51.
170. Schulzke SM, Hall GL, Nathan EA, Simmer K, Nolan G, Pillow JJ. Lung volume and ventilation inhomogeneity in preterm infants at 15-18 months corrected age. The Journal of pediatrics. 2010;156(4):542-9. e2.
171. Kotecha SJ, Edwards MO, Watkins WJ, Lowe J, Henderson AJ, Kotecha S. Effect of bronchodilators on forced expiratory volume in 1 s in preterm-born participants aged 5 and over: a systematic review. Neonatology. 2015;107(3):231-40.
172. Thunqvist P, Tufvesson E, Bjermer L, Winberg A, Fellman V, Domellöf M, et al. Lung function after extremely preterm birth—A population-based cohort study (EXPRESS). Pediatric pulmonology. 2018;53(1):64-72.
173. Bui DS, Lodge CJ, Burgess JA, Lowe AJ, Perret J, Bui MQ, et al. Childhood predictors of lung function trajectories and future COPD risk: a prospective cohort study from the first to the sixth decade of life. The lancet Respiratory medicine. 2018;6(7):535-44.
174. Gibbons JT, Wilson AC, Simpson SJ. Predicting lung health trajectories for survivors of preterm birth. Frontiers in Pediatrics. 2020;8:318.

Chapter 6: Elevated neutrophilic inflammation and oxidative stress in survivors of preterm birth

INTRODUCTION

Emerging evidence suggests that preterm born children not only have increased respiratory symptoms and lower lung function than their term-born counterparts, but that lung function declines throughout childhood (62, 65). Ongoing inflammation and oxidative stress may play a role in this persistent and progressive respiratory morbidity in children born preterm (2). Preterm birth can initiate several pro-inflammatory events and can result in lung injury from preterm birth itself or subsequent stress from mechanical ventilation or prolonged oxygen supplementation (31, 33). Inflammation and oxidative stress have been shown to play a key role in the development of bronchopulmonary dysplasia (BPD) (31-33). Increased pro-inflammatory cytokines, chemokines, neutrophils and reactive oxygen species are observed in neonates who go on to develop BPD and are associated with decreased pulmonary vascularisation and more simplified alveoli (31, 32). Regardless of BPD diagnosis, the pro-inflammatory events associated with preterm birth may contribute to the persistent respiratory morbidity in this population.

Beyond the neonatal period, several small studies have identified a 16-fold increase in sputum neutrophils, a 3-fold increase in sputum interleukin-8 (98) and increased urinary leukotriene E4 (99) in school-aged children born preterm compared to those born at term. Fillipone *et al.* found increased 8-isoprostane, a marker of oxidative stress, in the exhaled breath condensate (EBC) of preterm-born adolescents both with and without a neonatal diagnosis of BPD in comparison to term-born controls (101). Despite only examining small numbers of preterm-born children, these studies together suggest that inflammation and oxidative stress can persist well beyond the neonatal period and may be contributing to the ongoing and declining lung function seen in this population as in other diseases such as asthma (175) and COPD (176). However, this potential link remains to be established since no studies have explored the link between lung function and inflammation in those born preterm. Additionally, these few limited studies have only examined those born in the pre-surfactant era, leaving the relevance to contemporary preterm birth unknown.

Quantification of inflammation and oxidative stress in preterm-born children is essential to better understanding the contribution of these processes to later lung disease. Eosinophilic inflammation is often elevated in childhood asthma (141), which is a common diagnosis amongst children who are born preterm (55). However, there is more evidence of elevated neutrophilic inflammation and oxidative stress processes in both neonatal and later lung disease in those born preterm (16, 32, 98, 101). Injury

from ventilatory support and inflammatory events may also result in airway remodelling and damage to pulmonary vasculature which could contribute to poorer lung function outcomes (33). Establishing the underlying pathways of lung disease in preterm-born children has implications for treatment, with many asthma medications targeting eosinophilic inflammation. Identifying markers of these different processes in the lungs of those born preterm can help elucidate the main drivers of persistent and progressive disease in this population. Inflammatory markers which have previously been identified in those born preterm, as well as those associated with other diseases like asthma and COPD, include 8-isoprostane (oxidative stress) (101), leukotriene B4 (neutrophilic inflammation) (16, 177), cysteinyl leukotrienes (eosinophilic inflammation) (141), interleukin-8 (inflammation and immunity) (178) and matrix metallo-proteinase 9 (airway remodelling) (179), among others. This chapter focuses on these particular markers, which I have chosen to ensure the balance between finite bio-sample size and a broad representation of mechanisms known to play an important role in the progression of other respiratory diseases.

Exhaled breath condensate is a bio-fluid specific to the airways that is non-invasive and relatively simple to collect, which is particularly useful in a paediatric population (180). Markers identified in EBC have distinguished between disease groups in asthma, COPD and lung cancer (181), however analysis methods for EBC are variable and not well-established (131). Analysis methods for urine are more established and while EBC is specific to the lungs, urine is not and may be reflective of systemic processes. Despite this, urinary markers have been associated with paediatric respiratory morbidity such as acute respiratory tract infection and persistent airway obstruction (182, 183). Analysis of these non-invasive, easily collected biofluids in childhood may help establish the inflammatory phenotype involved in respiratory morbidity after preterm birth which may be a potential target for future interventions.

AIMS & HYPOTHESES

This study aimed to identify markers of inflammation in exhaled breath condensate and urine from infants and children born preterm, and determine which inflammatory markers are associated with poorer lung health during childhood in survivors of preterm birth.

It was hypothesised that inflammatory markers will be increased in preterm-born infants and children compared to those born at term, and further increased in those with respiratory symptoms and/or lower lung function

METHODS

Participants

Preterm-born infants aged between 12-16 months corrected attended Princess Margaret (now known as Perth Children's) Hospital as part of the Preterm Infant Functional and Clinical Outcome (PIFCO) study. Term-born healthy infants aged between 9 and 18 months were recruited from the community and agreed to EBC sample collection during a home visit.

Preterm children and young adults between the ages of 6 and 23 years, with and without a neonatal diagnosis of BPD, and healthy term-born controls, attended Perth Children's Hospital for a study visit.

All preterm-born participants were born at 32 weeks gestation or less at King Edward Memorial Hospital (KEMH) in Perth, Western Australia. Participants born preterm were classified as having bronchopulmonary dysplasia if they received 28 days of oxygen supplementation or more, as assessed at 36 weeks postmenstrual age. Healthy term born participants were born at 37 weeks gestation or more and had no history of recurrent respiratory symptoms or lung disease. Written informed consent was obtained from participants over 18 years and from parents or guardians for participants under 18 years. Assent was obtained from all participants under 18 years. Ethical approval was obtained from the Child and Adolescent Health Service Human Research Ethics Committee (RGS367, RGS815) and Curtin University (HRE2018-0407).

Symptoms and lung function

For preterm-born participants, neonatal and maternal health data was obtained from medical records and the KEMH neonatal database. Current and past respiratory symptoms at rest and with exercise as well as previous and current treatment use was obtained as previously described using validated general and respiratory questionnaires adapted from the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaires (117). Anthropometric data collected at the 6-23-year-old participant's study visits included height, weight, oxygen saturation and blood pressure.

As previously described in Chapter 2, fractional exhaled nitric oxide, respiratory oscillometry and spirometry measures were collected during the study visit for 6-23-year-old participants. Oscillometry and spirometry were performed before and after delivery of 400 µg salbutamol. Spectral oscillometry and spirometry outcomes were expressed as z-scores.

Exhaled Breath Condensate (EBC)

Exhaled breath condensate (EBC) was collected using the R-Tube collection device (Respiratory Research Inc, Charlottesville, VA). In infant participants, exhaled breath condensate samples were collected using an R-Tube device (Respiratory Research Inc, Charlottesville, VA) adapted for use in infants as described in Chapter 4 (114). Participants breathed tidally through the device mouthpiece (or infant facemask) with a one-way valve connected to a collection tube cooled to approximately -20 °C for 15 minutes. The exhaled air cooled and condensed in the collection tube, with droplets of EBC aliquoted into 1 mL vials and stored at-80 °C until analysed. The EBC samples were analysed neat for all assays.

Urine

Participants aged 6-23 years provided a urine sample into a collection jar. Samples were aliquoted into 1 mL tubes and frozen at -80 °C. Urine samples were diluted 8-fold for 8-isoprostane analysis and 16-fold for cysteinyl leukotriene analysis and were added neat for all other assays as per manufacturer guidelines and following assay optimisation.

Sample Analysis

Samples were analysed for markers associated with eosinophilic inflammation, neutrophilic inflammation, oxidative stress, immunity and airway remodelling; respectively cysteinyl leukotrienes, leukotriene B4, 8-isoprostane, interleukin-8 (IL-8) and matrix-metalloproteinase-9 (MMP-9).

Urine and EBC samples were analysed using enzyme-linked immune-assays (ELISA). Due to the small volume of infant EBC samples, only leukotriene B4 and 8-isoprostane ELISAs were performed on these samples.

ELISA buffer, ELISA standards, samples, AChE tracers and ELISA antiserums for cysteinyl leukotrienes, leukotriene B4 and 8-isoprostane samples were added to the assay plates according to the protocol outlined by the Cayman Chemical Cysteinyl leukotrienes EIA Kit (Item No. 500390), Leukotriene B4 EIA Kit (Item No. 520111) and 8-Isoprostane EIA Kit (Item No. 516351). A standard curve was plotted using the absorbance readings of the maximum binding, non-specific binding and standard wells. The standard curve was then used to determine the concentrations of cysteinyl leukotrienes, leukotriene B4 and 8-isoprostane in the EBC samples.

Samples were analysed for interleukin-8 (IL-8) using the BD OptEIA human interleukin-8 set according to the manufacturer protocol. A standard curve was generated by plotting a linear best fit curve for the

log of standard absorbance readings against the log of known standards concentrations. This standard curve was then used to determine the IL-8 concentrations of samples.

MMP-9 levels were analysed using the R&D Systems Human MMP-9 Quantikine kit, a solid phase sandwich ELISA. Reagents, standards, samples and controls were added according to the protocol provided by R&D systems. A standard curve was generated using the standard absorbance readings and then used to determine the MMP-9 concentration in the samples.

Statistical Analysis

Differences between groups were analysed by one-way analysis of variance with post-hoc comparisons, independent samples t-test or Mann-Whitney U test depending on normality of the data. Normally distributed data are presented as means and standard deviations. Non-normally distributed data are presented as medians and interquartile ranges. The chi-squared test was used to compare proportions between groups. Bivariate correlation was used to assess associations between biomarker levels and lung function parameters.

RESULTS

Infants

Exhaled breath condensate samples were collected from 15 term-born infants and 33 preterm-born infants, 12 with a neonatal diagnosis of BPD. All infant EBC samples were analysed for 8-isoprostane, with enough sample for leukotriene B4 analysis for 9 term-born participants and 29 preterm-born participants, 9 with BPD. Anthropometric and birth information for infant participants are found in Table 1.

Infant EBC markers

At a median age of 14.4 months corrected for gestational age, leukotriene B4 and 8-isoprostane levels were elevated in the exhaled breath condensate of preterm-born infants compared to those born at term (Figure 1). There was no difference in either leukotriene B4 or 8-isoprostane levels between preterm infants with and without BPD, or those whose mother did or did not have chorioamnionitis. There were also no associations with days of mechanical ventilation, CPAP or oxygen supplementation (Figure 2).

Table 1. Demographic and anthropometric information for term and preterm-born infants.

	Term Controls	Preterm Infants	Preterm, no BPD	Preterm with BPD
Participants, n	15	33	21	12
Corrected age (months)	14.4 ± 2.9	14.4 ± 1.0	14.2 ± 1.0	14.7 ± 0.9
Male, n (%)	9 (60)	25 (75.7)	18 (85.7)	7 (58.3)
Weight (kg)	9.8 ± 1.4	9.9 ± 1.4	9.8 ±1.0	10.2 ± 1.9
Gestational age at birth (w)	39 ± 1.2	27.7 ± 2.2*	29.0 ± 1.4	25.5 ± 1.3ǂ
Birth weight (kg)	3.2 (3.0 – 3.5)	0.96 (0.78-1.21)*	1.13 (0.92-1.36)	0.82 (0.71-0.92)ǂ
Chorioamnionitis, n (%)	n/a	15 (45.5 %)	6 (28.6)	9 (75 %)ǂ
Days of ventilation	n/a	0.53 (0.29-3.83)	0.38 (0-0.58)	13.6 (2.55-42.5) ǂ
Days of CPAP	n/a	31.8 (6.25-49.8)	10.3 (5.42-30.3)	51.5 (47.2-56.6) ǂ
Days of oxygen	n/a	4.17 (0.54-68.1)	0.75 (0.25-2.54)	90.4 (64.3-125.6) ǂ
EBC Leukotriene B_4 (pg/mL)	0.3 (0.0-1.4)	1.9 (1.2-3.3)*	1.9 (1.1-3.2)	2.3 (1.5-2.7)
EBC 8-isoprostane (pg/mL)	0.2 (0.0-0.3)	0.9 (0.6-1.2)*	0.9 (0.6-1.3)	0.9 (0.7-1.0)

Data expressed as mean ± SD, median (IQR) or number (percentage).
*significantly different between term and preterm-born groups ($p<0.05$)
ǂ significantly different between preterm infants with and without BPD ($p<0.05$)

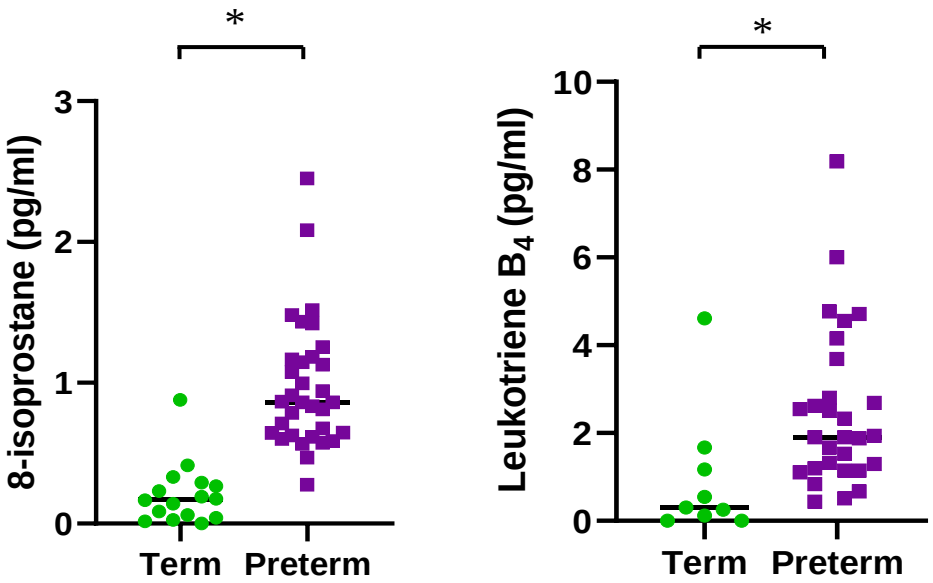


Figure 1. 8-isoprostane and leukotriene B4 levels detected in the exhaled breath condensate of term and preterm-born infants.

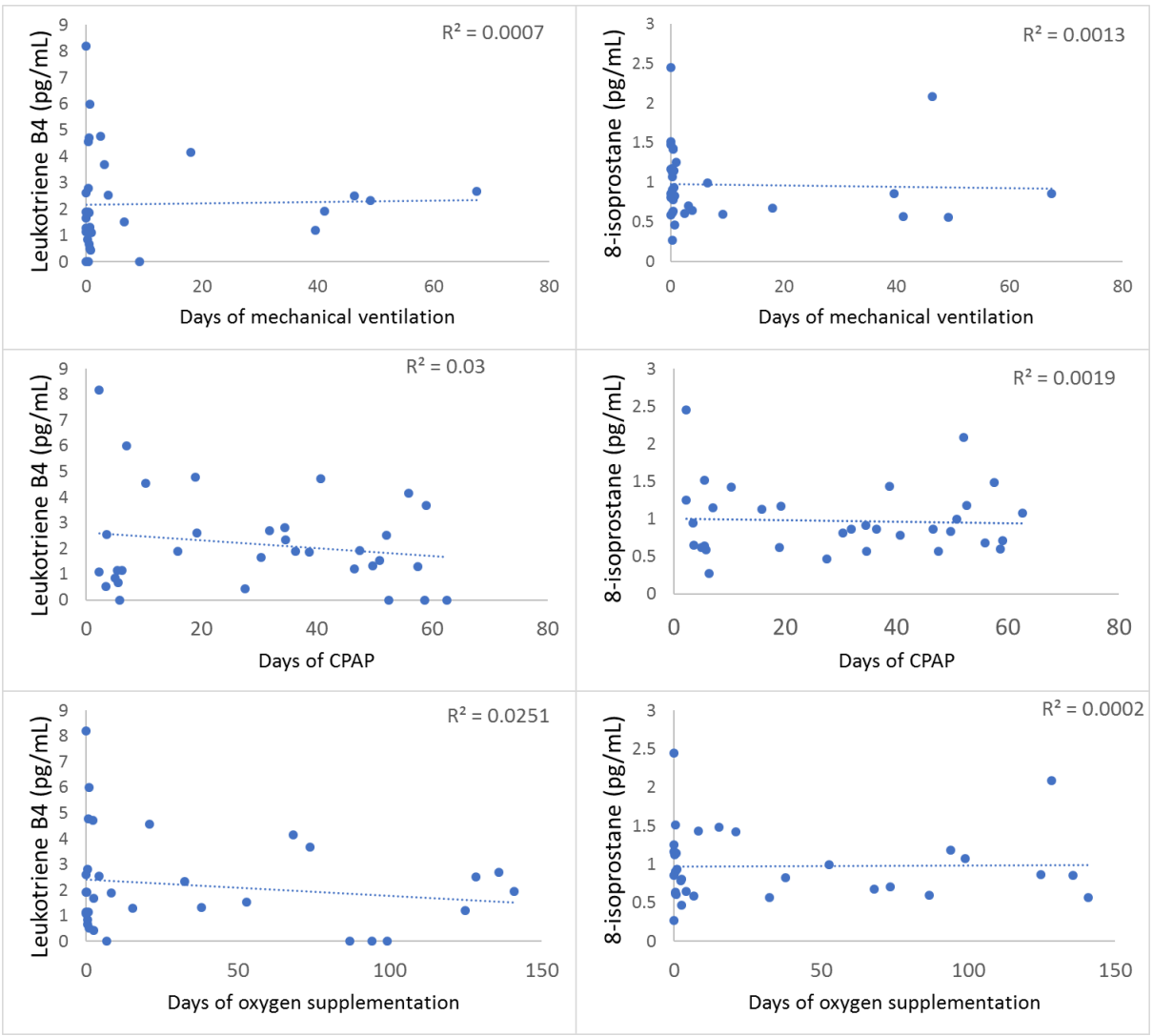


Figure 2. Association between days of mechanical ventilation, CPAP and oxygen supplementation in the NICU and levels of leukotriene B4 and 8-isoprostane in exhaled breath condensate from infants aged 12-15 months who were born at less than 32 weeks gestation. There were no significant correlations between days of respiratory support and levels of either leukotriene B4 or 8-isoprostane.

Children and young people

Thirty-four term-born and 94 preterm-born children and young adults, 34 of whom had a neonatal diagnosis of BPD, took part in the study. Preterm-born participants were slightly shorter and had poorer spirometry outcomes compared to those born at term (Table 2). FeNO, spectral and intra-breath oscillometry measures did not differ between those born preterm and those born at term (Table 2).

Table 2. Demographic, anthropometric and lung function information for participants aged 6-23 years

	Term Controls	Preterm	Preterm, no BPD	Preterm with BPD
Participants, n	34	94	60	34
Age (years)	16.0 ± 4.78	14.8 ± 4.83	14.5 ± 4.29	15.4 ± 5.70
GA (weeks)	40.0 (40.0-40.0)	29.0 (27.0-30.0)*	30.0 (28.0 – 31.0)	26.0 (24.8-28.3) ǂ
Male, n (%)	17 (50.0)	53 (56.4)	33 (55.0)	20 (58.8)
Height (cm)	162.7 ± 20.2	154.6 ± 17.8*	155.6 ± 17.2	152.7 ± 18.9
Weight (kg)	63.7 (35.4–98.9)	50.3 (32.8-66.5)	51.0 (34.5-65.2)	49.1 (26.0-70.2)
FEV_1 (z-score)	0.13 ± 1.26	-0.61 ± 1.19*	-0.35 ± 1.05	-1.06 ± 1.30ǂ
FVC (z-score)	0.25 ± 1.22	-0.05 ± 0.99	-0.05 ± 0.98	-0.07 ± 1.02
FEV_1/FVC (z-score)	-0.19 ± 1.05	-0.80 ± 1.22*	-0.51 ± 1.05	-1.35 ± 1.34ǂ
$FEF_{25-75\%}$ (z-score)	-0.07 ± 1.07	-0.66 ± 1.29*	-0.33 ± 1.05	-1.26 ± 1.48#
FeNO (ppb)	19.5 ± 17.1	21.6 ± 22.2	23.3 ± 26.2	18.7 ± 12.3
R_5 (z-score)	0.56 ± 1.32	0.56 ± 1.32	0.46 ± 1.30	0.74 ± 1.35
X_5 (z-score)	0.48 ± 1.12	0.06 ± 1.86	-0.44 ± 1.48**	0.93 ± 2.12#
AX (z-score)	0.97 ± 1.05	1.49 ± 1.39	1.34 ± 1.32	1.74 ± 1.49
Fres (z-score)	0.83 ± 1.07	1.29 ± 1.31	0.99 ± 1.10	1.82 ± 1.49**#
$R_{10in-ex}$ (Hz)	-0.27 (-0.42 - -0.12)	-0.23 (-0.56 – 0.10)	-0.26 (-0.61–0.09)	-0.19 (-0.5–0.21)
$X_{10in-ex}$ (Hz)	0.16 (0 – 0.33)	0.41 (-0.01 – 0.85)	0.41 (-0.17–0.99)	0.44 (0.03–0.85)

Data presented as mean ± standard deviation, median (interquartile range) or number (percentage).
*significantly different to term-born controls $p<0.05$
**significantly different to term-born controls $p<0.01$
ǂ significant difference to preterm participants with no BPD $p<0.05$
significant difference to preterm participants with no BPD $p<0.01$

Exhaled breath condensate

We were unable to detect leukotriene B4, 8-isoprostane or MMP-9 in any of the EBC samples from participants during later childhood/early adulthood. IL-8 and cysteinyl leukotrienes were detected in some (16.5 % and 77.8 % respectively), but not all samples (Table 3).

Cysteinyl leukotriene concentrations were not different between preterm and term-born participants. Interleukin-8 was decreased in EBC samples from preterm-born participants compared to those born at term (Figure 3). IL-8 and cysteinyl leukotriene levels in EBC were not associated with lung function outcomes.

Table 3. Detection rates and concentrations of inflammatory markers in exhaled breath condensate from term and preterm-born participants aged 6-23 years

EBC	Term Controls	Preterm	Preterm, no BPD	Preterm with BPD
LTB4 detected	0/32	0/77	0/55	0/22
LTB4 concentration (pg/mL)	0	0	0	0
8-isoprostane detected	0/32	0/77	0/55	0/22
8-isoprostane concentration (pg/mL)	0	0	0	0
Cysteinyl leukotriene detected	26/31 (83.9 %)	58/77 (75.3 %)	42/55 (76.4 %)	16/22 (72.7 %)
Cysteinyl leukotriene concentration (pg/mL)	227 (131-271)	168 (22-248)	173 (0-257)	131 (0-211)
IL-8 detected	11/32 (34.4 %)	9/89 (10.1 %)	5/56 (8.9 %)	4/33 (12.1 %)
IL-8 concentration (pg/mL)	0 (0-3.71)	0 (0-0) *	0 (0-0)	0 (0-0)
MMP-9 detected	0/16	0/46	0/29	0/17
MMP-9 concentration (ng/mL)	0	0	0	0

Data presented median (IQR) or number detected/number of participants in group.
*significantly different to term-born controls $p<0.05$

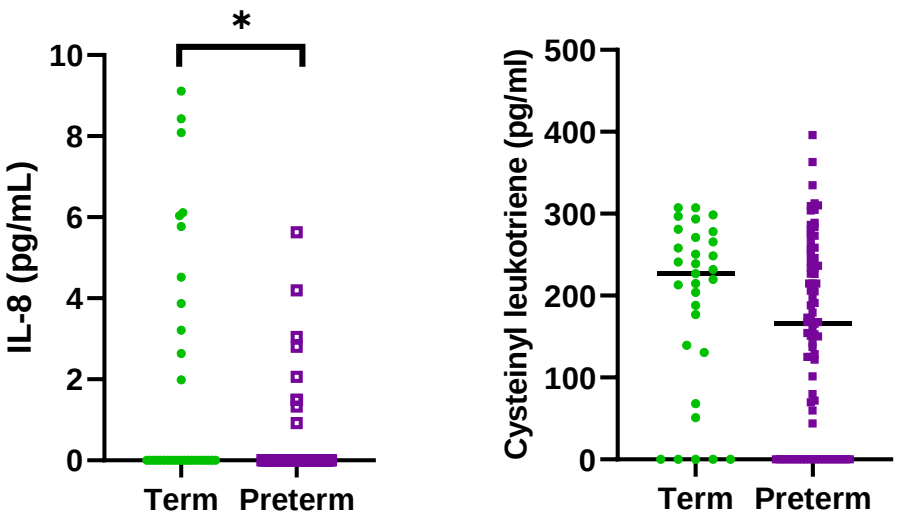


Figure 3. IL-8 and cysteinyl leukotriene concentrations detected in exhaled breath condensate samples from term and preterm-born participants aged between 6 and 23 years.

Urine

Leukotriene B4, 8-isoprostane, cysteinyl leukotrienes, IL-8 and MMP-9 were detected in urine. (Table 4).

Cysteinyl leukotriene, IL-8 and MMP-9 concentrations did not differ between preterm and term-born groups ($p>0.05$). However, Leukotriene B4 and 8-isoprostane levels were significantly elevated in those born preterm compared to those born at term ($p<0.05$) (Figure 4).

Urinary marker levels did not correlate with demographic factors like age or neonatal factors including GA and length of respiratory support ($p>0.05$) (Table 5). Markers also did not differ between those with and without BPD and were not associated with poorer spirometry outcomes ($p>0.05$), however there was a weak positive correlation between IL-8 levels and X_5 outcomes and between LTB4 levels and resonant frequency (Table 6). Marker levels were not significantly different between symptomatic and asymptomatic groups ($p>0.05$).

Table 4. Detection rates and concentrations of inflammatory markers in urine samples from term and preterm-born participants aged 6-23 years

Urine	Term Controls	Preterm	Preterm, no BPD	Preterm with BPD
LTB4 detected	26/27 (96.3 %)	60/60 (100 %)	45/45	15/15
LTB4 concentration (pg/mL)	83.3 (38.2-585)	132.4 (82-484.8)*	148.2 (68.6 – 197.9)	123.4 (82.6 – 156.7)
8-isoprostane detected	23/23 (100 %)	58/58 (100 %)	40/40	20/20
8-isoprostane concentration (pg/mL)	424.3 (191.1-639.9)	606.8 (389.2-968)*	528.9 (405.5 – 979.3)	657.2 (341.2-827.1)
Cysteinyl leukotriene detected	14/14 (100 %)	52/52 (100 %)	34/34	18/18
Cysteinyl leukotriene concentration (pg/mL)	4328 ± 2380	5455 ± 2696	5625 ± 2696	5134 ± 3194
IL-8 detected	20/28 (71.4 %)	53/69 (76.8 %)	34/46	19/23
IL-8 concentration (pg/mL)	11.7 (0-41.1)	8.9 (1.8-19.6)	7.93 (0 – 16.4)	17.5 (1.82 – 39.4)
MMP-9 detected	13/15 (86.7 %)	25/25 (100 %)	16/16	9/9
MMP-9 concentration (ng/mL)	0.83 (0.26-6.55)	0.43 (0.12-1.5)	0.34 (0.12 – 0.94)	0.45 (0.12 – 2.75)

Data presented as mean ± SD, median (IQR) or number (percentage).
*significantly different to term-born controls $p<0.05$

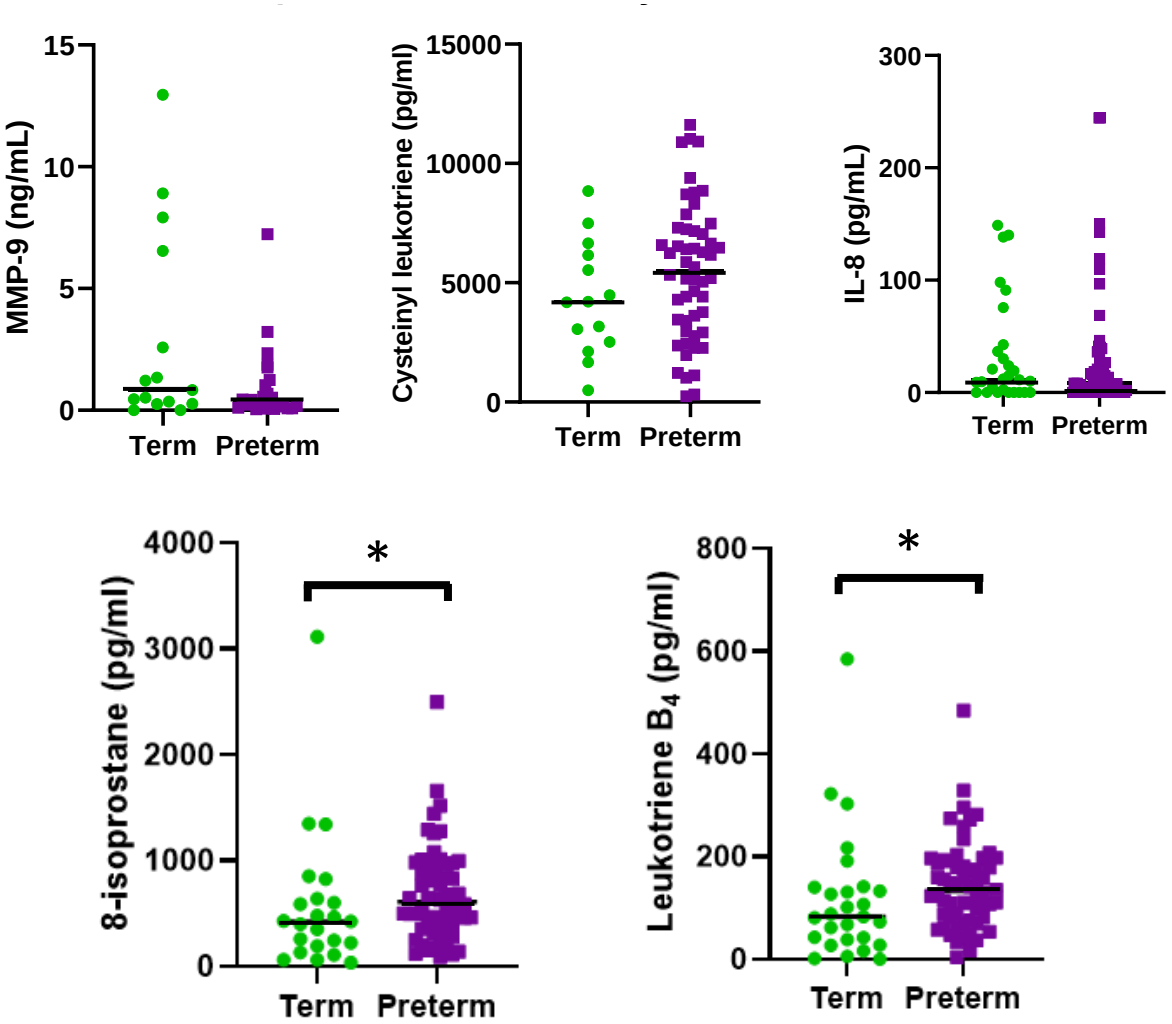


Figure 4. MMP-9, IL-8, cysteinyl leukotriene, 8-isoprostane and leukotriene B4 concentrations detected in urine samples from term and preterm-born participants aged between 6 and 23 years.
* $p<0.05$

Table 5. Correlation coefficients for urinary leukotriene B4 (LTB4), 8-isoprostane, cysteinyl leukotrienes, matrix metallo-proteinase 9 (MMP-9) and interleukin-8 (IL-8) with gestational age, birth weight and length of respiratory support in the neonatal intensive care unit.

		LTB4	8-isoprostane	Cysteinyl leukotrienes	MMP-9	IL-8
Gestational Age (weeks)	Spearman's rho[2]	-0.169	-0.032	-0.017	0.257	0.089
	Sig.	0.123	0.788	0.896	0.119	0.427
Birth weight (z-score)	Spearman's rho[2]	0.106	-0.015	-0.050	-0.039	-0.021
	Sig.	0.423	0.917	0.746	0.861	0.880
Supplemental oxygen (days)	Spearman's rho[2]	-0.233	-0.125	-0.239	-0.085	-0.053
	Sig.	0.075	0.382	0.114	0.699	0.703
Mechanical ventilation (days)	Spearman's rho[2]	-0.193	-0.091	-0.160	-0.086	-0.084
	Sig.	0.144	0.527	0.295	0.696	0.541
CPAP (days)	Spearman's rho[2]	-0.108	-0.038	-0.260	-0.107	-0.139
	Sig.	0.417	0.791	0.084	0.626	0.310

Table 6. Correlation coefficients for urinary leukotriene B4 (LTB4), 8-isoprostane, cysteinyl leukotrienes, matrix metallo-proteinase 9 (MMP-9) and interleukin-8 (IL-8) with exhaled nitric oxide (FeNO), spirometry and respiratory oscillometry outcomes.

		LTB4	8-isoprostane	Cysteinyl leukotrienes	MMP-9	IL-8
FeNO (ppb)	Spearman's rho[2]	0.008	0.096	0.027	-0.289	-0.118
	Sig.	0.941	0.427	0.842	0.079	0.302
FEV_1 (z-score)	Spearman's rho[2]	-0.084	-0.090	-0.069	0.031	-0.030
	Sig.	0.449	0.454	0.612	0.853	0.791
FVC (z-score)	Spearman's rho[2]	-0.202	-0.165	-0.050	-0.042	-0.098
	Sig.	0.067	0.168	0.716	0.802	0.388
FEV_1/FVC (z-score)	Spearman's rho[2]	0.186	0.154	-0.041	0.068	0.108
	Sig.	0.092	0.200	0.762	0.685	0.341
$FEF_{25-75\%}$ (z-score)	Spearman's rho[2]	0.127	0.188	-0.026	0.062	0.038
	Sig.	0.252	0.116	0.851	0.713	0.743
R_5 (z-score)	Spearman's rho[2]	-0.014	0.050	0.167	-0.160	0.138
	Sig.	0.901	0.676	0.211	0.337	0.219
X_5 (z-score)	Spearman's rho[2]	0.037	0.009	0.200	0.025	**.243***
	Sig.	0.735	0.937	0.133	0.879	**0.029**
AX (z-score)	Spearman's rho[2]	0.052	0.082	0.097	-0.227	0.012
	Sig.	0.637	0.489	0.469	0.170	0.913
Fres (z-score)	Spearman's rho[2]	**.222***	0.168	0.170	-0.216	0.062
	Sig.	**0.048**	0.172	0.218	0.213	0.594

* significant correlation at $p<0.05$

DISCUSSION

This study showed that preterm-born infants at 12-16 months have evidence of persistent airway inflammation with elevated leukotriene B4 and 8-isoprostane in EBC compared to those born at term. These markers were not elevated in the EBC of older children born preterm, however urinary 8-isoprostane and LTB4 were increased in preterm compared to term-born children. Neither airway or urinary cysteinyl leukotrienes were different between groups. Together, these results suggest that a neutrophilic (rather than eosinophilic) inflammatory profile persists through childhood in children born preterm, though may be less acute in the airway over time.

The increased levels of leukotriene B4, a marker of neutrophilic inflammation (184), and 8-isoprostane, a marker of oxidative stress (185), in EBC from preterm-born infants may be indicative of persisting lung injury from premature birth, subsequent intervention (16) or other insult in the first year of life. Leukotriene B4 is a potent neutrophil chemoattractant (184), while 8-isoprostane is a mediator and marker of oxygen radical injury (185). Neutrophilic inflammation and oxidative stress are associated with altered lung development which can lead to BPD diagnosis, with preterm neonates also lacking anti-inflammatory and antioxidant defences (31, 33). The presence of these markers in the exhaled breath of preterm-born infants aged 12 to 15 months suggests these inflammatory and oxidative stress processes continue beyond the first year of life. Studies have shown suppression of neutrophil apoptosis in the airways of preterm-born infants (186, 187), which may explain the presence of neutrophils into the first years of life. With elevated inflammation causing lung injury and altering development (188), and elevated airway neutrophilia associated with increased infection severity in diseases such as COPD (189), the presence of elevated airway neutrophils may make the lungs of preterm-born children susceptible to more severe respiratory infections, which in turn may contribute to the higher rate of respiratory hospitalisations in the first few years of life in this population (52). In the early years of life, alveolar development is still occurring, which may be altered if occurring in the presence of inflammation and oxidative stress (3). A recent study in mice found that neutrophilic inflammation during lung development prevents the normal assembly of elastin fibres around terminal airspaces leading to alveolar simplification and predisposing adult mice to COPD (190). In COPD, increased neutrophilic inflammation and oxidative stress with normal or reduced eosinophilic inflammation is often observed, leading to protease imbalance and alveolar cell apoptosis (191). When considered in the context of structural lung damage on CT and low and declining lung function observed during childhood in those born preterm, these biomarkers may indicate a predisposition to COPD development in this population. Alternatively, elevated neutrophilia and oxidative stress markers may indicate a pathway to the development of neutrophilic asthma (192), particularly when considering the higher

rates of asthma diagnosis in those born preterm compared to those born at term in childhood (56). Additionally, recurrent infection, an altered immune response and airway microbiota play a role in neutrophilic/non-eosinophilic asthma, and these factors are similarly altered in those born preterm (51, 92, 193). In the context of what is known about respiratory conditions like non-eosinophilic asthma and COPD, in addition to respiratory morbidity in preterm-born children, elevated markers of neutrophilic inflammation and oxidative stress may indicate the underlying disease process in this group. As such, clinical respiratory follow up of this population are warranted after discharge from the NICU, with the aim of identifying individuals at risk of persistent or progressive lung disease. As yet, there remains no co-ordinated respiratory follow-up for those surviving preterm birth in Australia.

Our study found no direct correlations with neonatal factors and no difference in biomarker levels between those with and without BPD, which may indicate that regardless of BPD diagnosis, preterm-born infants exhibit ongoing lung inflammation and oxidative stress. However, we had limited sample numbers where larger studies may more powered to assess any associations with potential predictors of inflammatory marker levels. The variability in biomarker levels that we observed may suggest that perhaps aside from BPD diagnosis, any interruption of normal foetal lung development, as well as factors beyond the neonatal period, such as respiratory infections in the first year of life, may have a more pronounced influence on persistent lung inflammation. The lack of correlation with neonatal factors may also be explained by the inability to normalise EBC for variable dilution of any airway surface liquid in water vapour may result in inexact quantification of biomarkers (194), which in turn may mask any associations with a more severe neonatal course. This presents a problem as we still have no way to predict which infants are at highest risk of future respiratory morbidity. Obtaining longitudinal lung health information from these infants would allow us to assess if exceeding a threshold concentration of these markers in EBC is associated with future respiratory morbidity. Data collected weekly throughout the neonatal course could help predict those infants most at risk of poor long-term lung health, with data across the first year of life potentially identifying lifestyle or exposure factors which may contribute to respiratory morbidity in this population. Additionally, further standardisation of EBC analysis will allow for more reliable biomarker quantification. Despite these limitations, increased inflammation and oxidative stress in the airways of infants born preterm regardless of BPD diagnosis may be an important contributor to the high rates of recurrent respiratory infection and hospitalisation in the first years observed in this population. Interventions to reduce inflammation and oxidative stress in this population may include probiotic treatment which may ameliorate dysregulated gut and airway microbiome in preterm infants which have been associated with BPD development – however the benefit of probiotics in lung disease remains poorly understood (94, 193). Additionally, superoxide dismutase and melatonin supplementation suggest beneficial effects on lung oxidative

stress, but have not reduced the risk of BPD in trials (195). Surfactant replacement may help ameliorate lung inflammation and oxidative stress (195). However, more research is needed into potential anti-oxidant therapy to prevent damage from chronic inflammation.

Levels of leukotriene B4 and 8-isoprostane were not detected in EBC of older children, perhaps indicating a 'growing out' of lung inflammation when further away from the injurious events of the neonatal phase. Alternatively, these findings might also be explained by larger volumes of EBC collected at this age potentially being more dilute, therefore reducing the concentration of these markers below detectable limits. However, although previous studies found that minute ventilation was directly related to EBC volume in infants (133), we did not find this association in the present study. Despite this, the variable dilution of EBC minimising biomarker detection may be a possible explanation for our results considering that we did observe elevated levels of leukotriene B4 and 8-isoprostane detected in urine from preterm-born participants compared to those born at term. Analysis methods for urine are more established than EBC, and most commercial ELISA kits have specific recommendations for urine samples. We had higher rates of detection for all ELISAs attempted in urine compared to EBC. Despite urine not being lung-specific, markers detected in urine may be more reliable than those detected in EBC. Although detectable urinary levels of inflammatory markers may arise from the activation of inflammatory pathways in other body systems, urine has been shown to be useful in detecting biomarkers of childhood asthma and may also prove to be useful in chronic lung disease after prematurity (182, 183, 196). Elevated urinary leukotriene B4 and 8-isoprostane again suggests persistent neutrophilic inflammation and oxidative stress in individuals born preterm into later childhood and even early adulthood.

MMP-9 is present in low quantities in healthy adult lungs, and sustained, elevated levels have been associated with lung remodelling in asthma, COPD and pulmonary fibrosis (179). We were not able to detect MMP-9 in any EBC samples, which again could mean that MMP-9 is either not present in the airways in this population or is undetectable in EBC. However, the latter may be less likely considering previous studies have detected MMP-9 in EBC (197) and observed elevated levels in asthmatics (198) and during COPD exacerbation (199). Additionally, MMP-9 levels in urine were not different between groups, which implies that airway remodelling is not occurring in later childhood in survivors of preterm birth. This could suggest that the evidence of structural lung disease in those born preterm (45) is a result of early lung injury during neonatal intervention, which may not actively worsen throughout childhood or be associated with inflammatory processes enhancing MMP-9 activity. Currently, there are no longitudinal imaging studies in survivors of preterm birth to investigate whether structural lung

damage is fixed or progressive. Further studies into structural lung changes and any associations with ongoing inflammation after preterm birth is clearly warranted.

IL-8 in EBC was lower in survivors of preterm birth compared to term controls and was not different between groups in urine. This finding was somewhat unexpected, given our observation of increased LTB4, since IL-8 plays a key role in neutrophil recruitment (200). IL-8 is involved in immune response to acute injury and infection (201) and is seen in higher levels in COPD patients during exacerbations (201), perhaps more reflective an acute inflammatory response. All participants seen were otherwise well and free from any infection within the 6 weeks prior to their study visit so we were unlikely to observe acute response, which may explain the absence of elevated IL-8. IL-8 also can originate from multiple aetiologies and can be produced by macrophage, epithelial, airway smooth muscle and endothelial cells (201), whereas leukotriene B4 is predominantly produced by macrophages and neutrophils (202). Our results may suggest that macrophage activity may be a dominant pathway for inflammation after preterm birth. However, considering that IL-8 was only detectable in a small proportion of EBC samples, this may be a chance finding or it may be that this biofluid may not be the most appropriate for quantifying biomarkers particularly if it cannot be normalised for variable water vapour to airway surface liquid ratio. Further investigation into the role of IL-8 and other cytokines in the lungs of preterm-born children is warranted, particularly in the presence of other elevated markers of inflammation.

Cysteinyl leukotrienes detected in both EBC and urine were not different between preterm and term-born participants. Cysteinyl leukotrienes are markers of eosinophilic inflammation and have been shown to be elevated in asthmatic children compared to non-asthmatics (141, 203). We did not find that cysteinyl leukotriene levels were directly correlated to lung function outcomes, which may be explained by the heterogeneity of EBC dilution or the systemic nature of urine. However, these results are consistent with our other marker of eosinophilic inflammation, exhaled nitric oxide, which was also not different between preterm and term-born groups as reported in Chapter 1. These results are supported by a recent review which demonstrates that exhaled nitric oxide levels are not elevated in children born prematurely in comparison to term-born counterparts (142). Together this reinforces the idea that the underlying pathways of respiratory morbidity in preterm-born children is distinct from that of typical eosinophilic childhood asthma. In light of elevated neutrophilic inflammation and oxidative stress markers, it may potentially indicate the development of neutrophilic asthma or COPD in preterm-born individuals instead.

The weak positive correlations between urinary IL-8 with reactance and urinary leukotriene B4 with resonant frequency may suggest that neutrophilic inflammation is associated with increased airway

stiffness. Although our current study did not observe any correlations between biomarker levels and other lung function outcomes, measuring these biomarkers can still complement lung function testing to provide insight into underlying pathophysiology of lung disease. The lack of association between biomarker levels and lung function may be explained by the unknown heterogeneity of EBC dilution or systemic nature of urine sampling. To identify markers that reflect lung function or clinical status, further exploration of the neutrophilic and oxidative stress pathways that seem to be associated with chronic lung disease after preterm birth is necessary. There may also be a role in for measuring plasma markers of inflammation and oxidative stress alongside anti-oxidant or anti-inflammatory markers, which may suggest reduced anti-oxidant balance as found by Wood et al. in asthmatics (204). Longitudinal studies of this cohort will be particularly useful to determine if individuals with increased markers have accelerated lung function decline in the future. Additionally, investigating the roles of other potential biomarkers, such as prostaglandins and cytokines may provide more insight into the underlying mechanisms of lung disease in those born preterm.

Together, the results of the present study reinforce emerging evidence that respiratory morbidity and decreased lung function in survivors of preterm birth is not driven by eosinophilic inflammation, but likely neutrophilic inflammation and oxidative stress. Regardless of BPD diagnosis, these processes appear to persist well into childhood and even young adulthood. This has implications for treatment, especially considering that children born preterm are more likely to be diagnosed with asthma and are treated with common asthma medications like inhaled corticosteroids (55, 56) which target eosinophilic inflammation and may not be the most effective treatment in this group. Treatments that target the neutrophilic inflammatory and oxidative stress processes may be more useful in ameliorating the respiratory morbidity observed in survivors of preterm birth. If persistent neutrophilic inflammation and oxidative stress predispose those born preterm to diseases such as neutrophilic asthma or COPD, further establishing the pathways of lung disease to enable early, targeted intervention is crucial to preserving lung health in this population.

REFERENCES

2. Urs R, Kotecha S, Hall GL, Simpson SJ. Persistent and progressive long-term lung disease in survivors of preterm birth. Paediatric respiratory reviews. 2018.
3. Smith LJ, McKay KO, van Asperen PP, Selvadurai H, Fitzgerald DA. Normal development of the lung and premature birth. Paediatric respiratory reviews. 2010;11(3):135-42.
16. Speer CP. Inflammation and bronchopulmonary dysplasia: a continuing story. Semin Fetal Neonatal Med. 2006;11(5):354-62.
31. Speer C. Pulmonary inflammation and bronchopulmonary dysplasia. Journal of Perinatology. 2006;26(S1):S57.
32. Perrone S, Tataranno ML, Buonocore G. Oxidative stress and bronchopulmonary dysplasia. Journal of clinical neonatology. 2012;1(3):109.
33. Speer CP, Groneck P, editors. Oxygen radicals, cytokines, adhesion molecules and lung injury in neonates. Seminars in Neonatology; 1998: Elsevier.
45. Simpson SJ, Logie KM, O'Dea CA, Banton GL, Murray C, Wilson AC, et al. Altered lung structure and function in mid-childhood survivors of very preterm birth. Thorax. 2017.
51. Pramana IA, Latzin P, Schlapbach LJ, Hafen G, Kuehni CE, Nelle M, et al. Respiratory symptoms in preterm infants: burden of disease in the first year of life.(Clinical report). 2011;16(197).
52. Greenough A, Alexander J, Burgess S, Chetcuti P, Cox S, Lenney W, et al. Home oxygen status and rehospitalisation and primary care requirements of infants with chronic lung disease. Archives of disease in childhood. 2002;86(1):40-3.
55. Fawke J, Lum S, Kirkby J, Hennessy E, Marlow N, Rowell V, et al. Lung function and respiratory symptoms at 11 years in children born extremely preterm: the EPICure study. American journal of respiratory and critical care medicine. 2010;182(2):237-45.
56. Korhonen P, Laitinen J, Hyoudynmaa E, Tammela O. Respiratory outcome in school-aged, very-low-birth-weight children in the surfactant era. Acta Pædiatrica. 2004;93(3):316-21.
62. Simpson SJ, Turkovic L, Wilson A, Verheggen M, Logie KM, Pillow JJ, et al. Lung function trajectories throughout childhood in survivors of very preterm birth: A longitudinal cohort study. The Lancet Child and Adolescent Health. 2018.
65. Doyle LW, Adams A-M, Robertson C, Ranganathan S, Davis NM, Lee KJ, et al. Increasing airway obstruction from 8 to 18 years in extremely preterm/low-birthweight survivors born in the surfactant era. Thorax. 2017;72(8):712.
92. Melville JM, Moss TJ. The immune consequences of preterm birth. Frontiers in neuroscience. 2013;7.
94. Gentle SJ, Lal CV. Predicting BPD: lessons learned from the airway microbiome of preterm infants. Frontiers in pediatrics. 2020;7:564.
98. Teig N, Allali M, Rieger C, Hamelmann E. Inflammatory markers in induced sputum of school children born before 32 completed weeks of gestation. J Pediatr. 2012;161(6):1085-90.
99. Halvorsen T, Skadberg BT, Eide GE, Roksund O, Aksnes L, Oymar K. Characteristics of asthma and airway hyper-responsiveness after premature birth. Pediatr Allergy Immunol. 2005;16(6):487-94.
101. Filippone M, Bonetto G, Corradi M, Frigo AC, Baraldi E. Evidence of unexpected oxidative stress in airways of adolescents born very pre-term. The European respiratory journal. 2012;40(5):1253-9.
114. Urs R, Stoecklin B, Pillow JJ, Hartmann B, Hall GL, Simpson SJ. Collecting exhaled breath condensate from non-ventilated preterm-born infants: a modified method. Pediatric Research. 2021:1-3.
117. Asher M, Keil U, Anderson H, Beasley R, Crane J, Martinez F, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. European respiratory journal. 1995;8(3):483-91.

131. Horváth I, Barnes PJ, Loukides S, Sterk PJ, Högman M, Olin A-C, et al. A European Respiratory Society technical standard: exhaled biomarkers in lung disease. European Respiratory Journal. 2017;49(4):1600965.
133. Moeller A, Franklin P, Hall GL, Horak Jr F, Wildhaber JH, Stick SM. Measuring exhaled breath condensates in infants. Pediatric pulmonology. 2006;41(2):184-7.
141. Ferraro V, Carraro S, Bozzetto S, Zanconato S, Baraldi E. Exhaled biomarkers in childhood asthma: old and new approaches. Asthma research and practice. 2018;4(1):1-7.
142. Course CW, Kotecha S, Kotecha SJ. Fractional exhaled nitric oxide in preterm-born subjects: A systematic review and meta-analysis. Pediatric pulmonology. 2019;54(5):595-601.
175. Robroeks C, Van De Kant K, Jöbsis Q, Hendriks H, Van Gent R, Wouters E, et al. Exhaled nitric oxide and biomarkers in exhaled breath condensate indicate the presence, severity and control of childhood asthma. Clinical & Experimental Allergy. 2007;37(9):1303-11.
176. Borrill Z, Roy K, Singh D. Exhaled breath condensate biomarkers in COPD. European Respiratory Journal. 2008;32(2):472-86.
177. Groneck P, Gotze-Speer B, Oppermann M, Eiffert H, Speer CP. Association of pulmonary inflammation and increased microvascular permeability during the development of bronchopulmonary dysplasia: a sequential analysis of inflammatory mediators in respiratory fluids of high-risk preterm neonates. Pediatrics. 1994;93(5):712-8.
178. Huang A-X, Lu L-W, Liu W-J, Huang M. Plasma inflammatory cytokine IL-4, IL-8, IL-10, and TNF-α levels correlate with pulmonary function in patients with asthma-chronic obstructive pulmonary disease (COPD) overlap syndrome. Medical science monitor: international medical journal of experimental and clinical research. 2016;22:2800.
179. Chakrabarti S, Patel KD. Matrix metalloproteinase-2 (MMP-2) and MMP-9 in pulmonary pathology. Experimental lung research. 2005;31(6):599-621.
180. Rosias PP, Dompeling E, Hendriks HJ, Heijnens JW, Donckerwolcke RA, Jöbsis Q. Exhaled breath condensate in children: pearls and pitfalls. Pediatric allergy and immunology. 2004;15(1):4-19.
181. Davis MD, Montpetit A, Hunt J. Exhaled breath condensate—an overview. Immunology and allergy clinics of North America. 2012;32(3):363.
182. Adamko DJ, Bear M, Regush S, Robinson JL. Urine metabolomic profiling of children with respiratory tract infections in the emergency department: a pilot study. BMC infectious diseases. 2016;16(1):1-11.
183. Rabinovitch N, Zhang L, Gelfand EW. Urine leukotriene E4 levels are associated with decreased pulmonary function in children with persistent airway obstruction. Journal of allergy and clinical immunology. 2006;118(3):635-40.
184. Yokomizo T, Izumi T, Shimizu T. Leukotriene B4: metabolism and signal transduction. Archives of biochemistry and biophysics. 2001;385(2):231-41.
185. van't Erve TJ, Kadiiska MB, London SJ, Mason RP. Classifying oxidative stress by F2-isoprostane levels across human diseases: A meta-analysis. Redox biology. 2017;12:582-99.
186. Kotecha S, Mildner R, Prince L, Vyas J, Currie A, Lawson R, et al. The role of neutrophil apoptosis in the resolution of acute lung injury in newborn infants. Thorax. 2003;58(11):961-7.
187. Oei J, Lui K, Wang H, Henry R. Decreased neutrophil apoptosis in tracheal fluids of preterm infants at risk of chronic lung disease. Archives of Disease in Childhood-Fetal and Neonatal Edition. 2003;88(3):F245-F9.
188. Kallapur S, Jobe A. Contribution of inflammation to lung injury and development. Archives of Disease in Childhood-Fetal and Neonatal Edition. 2006;91(2):F132-F5.
189. Papi A, Bellettato CM, Braccioni F, Romagnoli M, Casolari P, Caramori G, et al. Infections and airway inflammation in chronic obstructive pulmonary disease severe exacerbations. American journal of respiratory and critical care medicine. 2006;173(10):1114-21.
190. Benjamin JT, Plosa EJ, Sucre JM, van der Meer R, Dave S, Gutor S, et al. Neutrophilic inflammation during lung development disrupts elastin assembly and predisposes adult mice to COPD. The Journal of clinical investigation. 2021;131(1).

191. Fischer BM, Pavlisko E, Voynow JA. Pathogenic triad in COPD: oxidative stress, protease–antiprotease imbalance, and inflammation. International journal of chronic obstructive pulmonary disease. 2011;6:413.
192. Esteban-Gorgojo I, Antolín-Amérigo D, Domínguez-Ortega J, Quirce S. Non-eosinophilic asthma: current perspectives. Journal of asthma and allergy. 2018;11:267.
193. Pammi M, Lal CV, Wagner BD, Mourani PM, Lohmann P, Luna RA, et al. Airway microbiome and development of bronchopulmonary dysplasia in preterm infants: a systematic review. The Journal of Pediatrics. 2019;204:126-33. e2.
194. Hunt J. Exhaled breath condensate: an evolving tool for noninvasive evaluation of lung disease. Journal of Allergy and Clinical Immunology. 2002;110(1):28-34.
195. Poggi C, Dani C. Antioxidant strategies and respiratory disease of the preterm newborn: an update. Oxidative medicine and cellular longevity. 2014;2014.
196. Tao JL, Chen YZ, Dai QG, Tian M, Wang SC, Shan JJ, et al. Urine metabolic profiles in paediatric asthma. Respirology. 2019;24(6):572-81.
197. Maskey-Warzęchowska M, Gorska K, Nejman-Gryz P, Paplińska-Goryca M, Grzela T, Krejner A, et al. Matrix metalloproteinase 9 in exhaled breath condensate in patients with stable chronic obstructive pulmonary disease: an observational study. Polish archives of internal medicine. 2018;128(7-8):427-33.
198. Karakoc GB, Yukselen A, Yilmaz M, Altintas DU, Kendirli SG. Exhaled breath condensate MMP-9 level and its relationship wıth asthma severity and interleukin-4/10 levels in children. Annals of Allergy, Asthma & Immunology. 2012;108(5):300-4.
199. Kwiatkowska S, Noweta K, Zieba M, Nowak D, Bialasiewicz P. Enhanced exhalation of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in patients with COPD exacerbation: a prospective study. Respiration. 2012;84(3):231-41.
200. Harada A, Sekido N, Akahoshi T, Wada T, Mukaida N, Matsushima K. Essential involvement of interleukin-8 (IL-8) in acute inflammation. Journal of leukocyte biology. 1994;56(5):559-64.
201. Pease JE, Sabroe I. The role of interleukin-8 and its receptors in inflammatory lung disease. American Journal of Respiratory Medicine. 2002;1(1):19-25.
202. Ko FW, Leung T-F, Wong GW, Ngai J, To KW, Ng S, et al. Measurement of tumor necrosis factor-α, leukotriene B4, and interleukin 8 in the exhaled breath condensate in patients with acute exacerbations of chronic obstructive pulmonary disease. International journal of chronic obstructive pulmonary disease. 2009;4:79.
203. Karrasch S, Linde K, Rücker G, Sommer H, Karsch-Völk M, Kleijnen J, et al. Accuracy of FENO for diagnosing asthma: a systematic review. Thorax. 2017;72(2):109-16.
204. Wood LG, Fitzgerald DA, Gibson PC, Cooper DM, Garg ML. Lipid peroxidation as determined by plasma isoprostanes is related to disease severity in mild asthma. Lipids. 2000;35(9):967-74.

Chapter 7: Urinary metabolomics of bronchopulmonary dysplasia in preterm-born neonates at 36 weeks postmenstrual age

INTRODUCTION

The development of bronchopulmonary dysplasia (BPD) is one of the most significant consequences of preterm birth and can have long term effects on respiratory morbidity (2). BPD is characterised by fewer, larger alveoli and decreased pulmonary vasculature secondary to chronic inflammatory processes following prolonged treatment with oxygen supplementation, mechanical ventilation and continuous positive airway pressure (CPAP) (15). BPD is defined by the need for and extent of respiratory support the neonate requires at 28 days or 36 weeks postmenstrual age (PMA) (22). A limitation of the current definition is its retrospective nature which is based on treatment rather than the underlying pathophysiology (205). Assessing the capacity for pulmonary gas exchange through shift of the oxyhaemoglobin saturation curve may allow better understanding of the underlying pathophysiology of BPD (205). However, risk factors for BPD are multifactorial and can involve highly variable antenatal and postnatal events (2). As a result of its complexity, there is no current effective prognostic risk model for BPD, which could enable closer monitoring and early intervention in high-risk infants (103). Markers of disease detectable in biofluids will play a vital role in better understanding the underlying pathophysiology of BPD and identifying infants most at risk.

Metabolomics is the analysis of the end-products of metabolism in biological systems. Through chromatography and mass spectrometry methods, metabolomics can identify compounds that differ in health and disease including proteins, carbohydrates, fatty acids, lipids, hormones, vitamins, drugs, and other small molecules (206). A few small metabolomics studies analysing maternal amniotic fluid, tracheal aspirates and urine from neonates at birth were able to discriminate between those who would and would not go on to develop BPD (105-107, 207). These studies show metabolomic patterns associated with oxidative stress and altered lipid and amino acid metabolism as potentially predictive of BPD development (103). Together these studies suggest that the trajectory to BPD may be set even before birth and highly influenced by inflammatory obstetric events, such as chorioamnionitis which is associated with a higher risk of BPD development (208).

Aside from the underpowered nature of existing metabolomics studies in BPD development, these studies tend to only examine the influence of prematurity itself or prenatal events on the development of BPD by using samples collected in the first days of life. The study of tracheal aspirates found 41 statistically different metabolites between samples collected on day 1 and day 7 of life, which suggests that metabolism of the preterm neonate changes significantly over the first few days and possibly weeks

of life (105). In this time, neonates are exposed to many different events, drugs and interventions in the neonatal intensive care unit (NICU) such as prolonged respiratory support which are associated with the development of BPD (2). It may be more informative to assess samples at the point of BPD diagnosis which may reflect metabolic changes that occur as a result of various exposures and treatments in the NICU which subsequently lead to the development of BPD.

AIM

This study aimed to identify metabolomic differences between neonates with and without BPD at 36 weeks postmenstrual age.

Hypothesis:

Neonates with and without BPD will have different metabolomic profiles. Metabolomic differences will indicate metabolic pathways which are altered due to neonatal intensive care treatment and subsequent respiratory morbidity.

METHODS

Participants

Neonates (n=113) born very preterm (<32 weeks gestation) at King Edward Memorial Hospital participated in this study as part of the larger Preterm Infant Functional and Clinical Outcome (PIFCO) study. Participants included 50 neonates who received a diagnosis of BPD while 63 who did not. Urine samples were collected from neonates at 36 weeks postmenstrual age (PMA) by extracting urine from cotton wool placed in the infant's nappy. Samples were aliquoted into 1 mL tubes and stored at -80 degrees Celsius. Neonatal information was obtained from medical records and the PIFCO study database. Shunt, shift and ventilation-perfusion measures were obtained from shunt-shift tests performed using a head-box as part of the larger PIFCO study (209). This study was approved by the Women and Newborn Health Service (Approval #2013091EW) and Curtin University Human Research Ethics Committees (HRE2020-0097). All parents/guardians of the participants provided informed written consent.

Urine sample analysis

The specific gravity of each sample was measured by placing 100 µL of the sample on a refractometer (ATAGO-UGα, Atago, Tokyo, Japan). On the day of analysis, urine sample concentration was normalised with dilution in water based on the sample's specific gravity measurement.

Further details of the liquid chromatography/mass-spectrometry are described in the methodology chapter. Samples were analysed using a ThermoFisher Triple quadrupole liquid chromatography-mass spectrometry (LC/MS) system. Metabolites were identified by matching mass and retention time to an in-house and the mzCloud chemical reference databases (https://www.mzcloud.org/, HighChem LLC, Slovakia).

Data processing

The data were cleaned based on the Quality Control-Relative Standard Deviation (QC-RSD), a metric of analytical precision and D-Ratio, the ratio of analytical variance to overall biological variance. Metabolites with a QC-RSD of >30 % and D-Ratio above 35 % were excluded from analysis, as per guidelines for untargeted clinical metabolomic studies (127). A principal component analysis was performed and identified no sample outliers.

Statistical analysis

The relative abundance of metabolites was not normally distributed, and therefore univariate comparison between groups was performed using Mann-Whitney U tests. Correlations between metabolite abundances and neonatal factors were tested using pairwise Spearman's rank correlation coefficients (r).

Principal component-canonical variate analysis (PC-CVA) of the relationship between metabolomic profiles and neonatal diagnosis of BPD was performed. Subsequent loadings plots were created to display the metabolites that significantly contributed to any separation in metabolomic profile between neonates with and without BPD. Metabolites that were found to significantly contribute to separation between the groups after PC-CVA analysis were analysed for correlation with neonatal factors using pairwise Spearman's rank correlation coefficients. Circle plots were created to display metabolites with similar pathways and spring plots display metabolite associations with neonatal variables.

Analysis was performed using the Python programming language in Jupyter notebooks (128).

RESULTS

Participants

Demographic, intensive care and ventilation-perfusion information for neonates are displayed in Table 1. When compared to neonates without BPD, those with BPD were born significantly earlier and at a lower birth weight (however birth weight z-scores were not different), required more respiratory support and exhibited impaired ventilation-perfusion as measured by shunt and shift testing (Table 1).

Table 1. Demographic and neonatal information for preterm-born neonates with and without a diagnosis of bronchopulmonary dysplasia.

	No BPD (n=63)	BPD (n=50)
Gestational age at birth (weeks)	28.9 (27.7–30.1)	25 (24–26)**
Male, n (%)	36 (57.1)	35 (70.0)
Birth weight (g)	1224.83 (± 294.12)	806.48 (± 181.98)**
Birth weight z-score	-0.05 (± 0.81)	0.09 (± 0.92)
Mechanical ventilation duration (days)	0.46 (0-6)	13.5 (0-30.2)**
CPAP duration (days)	17.0 (0.06–33.9)	48.3 (36.6-60.0)**
Oxygen supplementation duration (days)	1.21 (0-5.25)	75.1 (49.3-101.0)**
Shunt (%)	1.60 (0-5.2)	6.35 (0.25-12.5)**
Ventilation-perfusion (V/Q)	0.62 (0.55-0.69)	0.50 (0.42-0.58)**
Shift (kPa)	10.1 (9.1-11.1)	13.1 (10.7-15.5)**

Data presented as mean (± SD), median (IQR) or number (percentage).
*significantly different between groups $p<0.05$; **significantly different between groups $p<0.01$

Metabolomic analysis

Liquid chromatography-mass spectrometry analysis of urine samples detected 152 MS/MS identity-confirmed metabolites. Univariate comparison of those with and without BPD found 19 metabolites with a significant ($p<0.05$) fold change between groups (Table 2). The significantly different metabolites were primarily amino acid and lipid metabolites. A correlation plot (Figure 1) displays highly correlated metabolites in clusters.

Table 2. Median fold change of metabolites whose relative abundance was significantly different between preterm-born neonates with and without BPD.

Metabolite	**Median Fold Change**		**Significance**	
	No BPD	**BPD**	**p-value**	**q-value**
Tryptophan	1 (0.87, 1.16)	1.21 (0.93, 1.32)	0.009	0.221
trans-3-Indoleacrylic acid	1 (0.87, 1.09)	1.24 (0.89, 1.32)	0.010	0.221
Phenylalanine	1 (0.88, 1.11)	1.25 (0.88, 1.26)	0.006	0.221
Histidine	1 (0.85, 1.11)	1.2 (0.84, 1.23)	0.023	0.272
Tyrosine	1 (0.92, 1.08)	1.29 (0.82, 1.17)	0.004	0.221
Homoarginine	1 (0.76, 1.20)	1.59 (0.71, 1.34)	0.012	0.221
sn-glycero-3-Phosphocholine	1 (0.87, 1.12)	1.24 (0.84, 1.2)	0.046	0.370
Jasmonic acid	1 (0.74, 1.22)	1.26 (0.79, 1.55)	0.034	0.324
Proline	1 (0.79, 1.12)	0.69 (0.85, 1.24)	0.034	0.324
5-Hydroxylysine	1 (0.76, 1.08)	1.2 (0.87, 1.12)	0.022	0.272
Glutamine	1 (0.88, 1.48)	0.6 (0.88, 1.31)	0.000	0.065
Choline	1 (0.92, 1.23)	0.93 (0.82, 1.06)	0.050	0.370
Citrulline	1 (0.79, 1.43)	1.46 (0.86, 1.24)	0.006	0.221
Cystine	1 (0.85, 1.11)	1.19 (0.89, 1.25)	0.026	0.285
Methionine sulfoxide	1 (0.84, 1.16)	1.43 (0.86, 1.12)	0.023	0.272
Nicotinic acid	1 (0.73, 1.30)	0.57 (0.69, 1.36)	0.014	0.231
p-Aminobenzoic acid	1 (0.84, 1.09)	0.78 (0.72, 1.15)	0.018	0.272
N-dodecanoyl-L-Homoserine lactone	1 (0.86, 1.12)	1.16 (0.88, 1.15)	0.038	0.342
N-alpha-Acetyllysine	1 (0.85, 1.29)	1.45 (0.83, 1.16)	0.011	0.221

Data presented as the fold-change of the median (95 % CI).

Univariate correlation of significantly different metabolites against demographic and intensive care factors identified that earlier gestational age was significantly associated with greater histidine (r^2=-0.364; p=0.023), citrulline (r^2=-0.392; p=0.006), 5-hydroxylysine (r^2=-0.354; p=0.038) and cystine (r^2=-0.39; p=0.007) levels (Figure 2). More mechanical ventilation was associated with increased histidine (r^2=0.389; p=0.007), 5-hydroxylysine (r^2=0.367; p=0.021) and tyrosine (r^2=0.396; p=0.005). More CPAP was associated with greater citrulline levels (r^2=0.413; p=0.002) and more supplemental oxygen was associated with increased 5-hydroxylysine (r^2=0.356; p=0.049) and homoarginine (r^2=0.439; p=0.001)

When adjusted for multicollinearities, only gestational age and length of oxygen supplementation (corrected for gestational age) had significant correlations with metabolites. Earlier gestation was associated with increased histidine (r^2=-0.674; p=0.029), citrulline (r^2=-0.392, p=0.007) and 5-Hydroxylysine (r^2=-0.354; p=0.048). Increased O_2 supplementation was associated with increased 5-hydroxylysine (r^2=0.366; p=0.039) and increased homoarginine (r^2=0.453; p=0.000).

Multivariate analysis using PC-CVA Scores plot (Figure 3) found the difference in metabolomic profiles between neonates with and without BPD was only slight and not significantly different. Loadings plots (Figure 4) display the contribution of individual metabolites to the separation between groups. This analysis found that 52/152 metabolites significantly contributed to the slight, although non-significant, difference between the metabolomic profiles of each group. Correlation analysis of these 52 metabolites and neonatal factors (Figure 5) found only 1 significant correlation between homoarginine and length of oxygen supplementation (r^2=0.439, p=0.004), similar to the univariate correlation analyses.

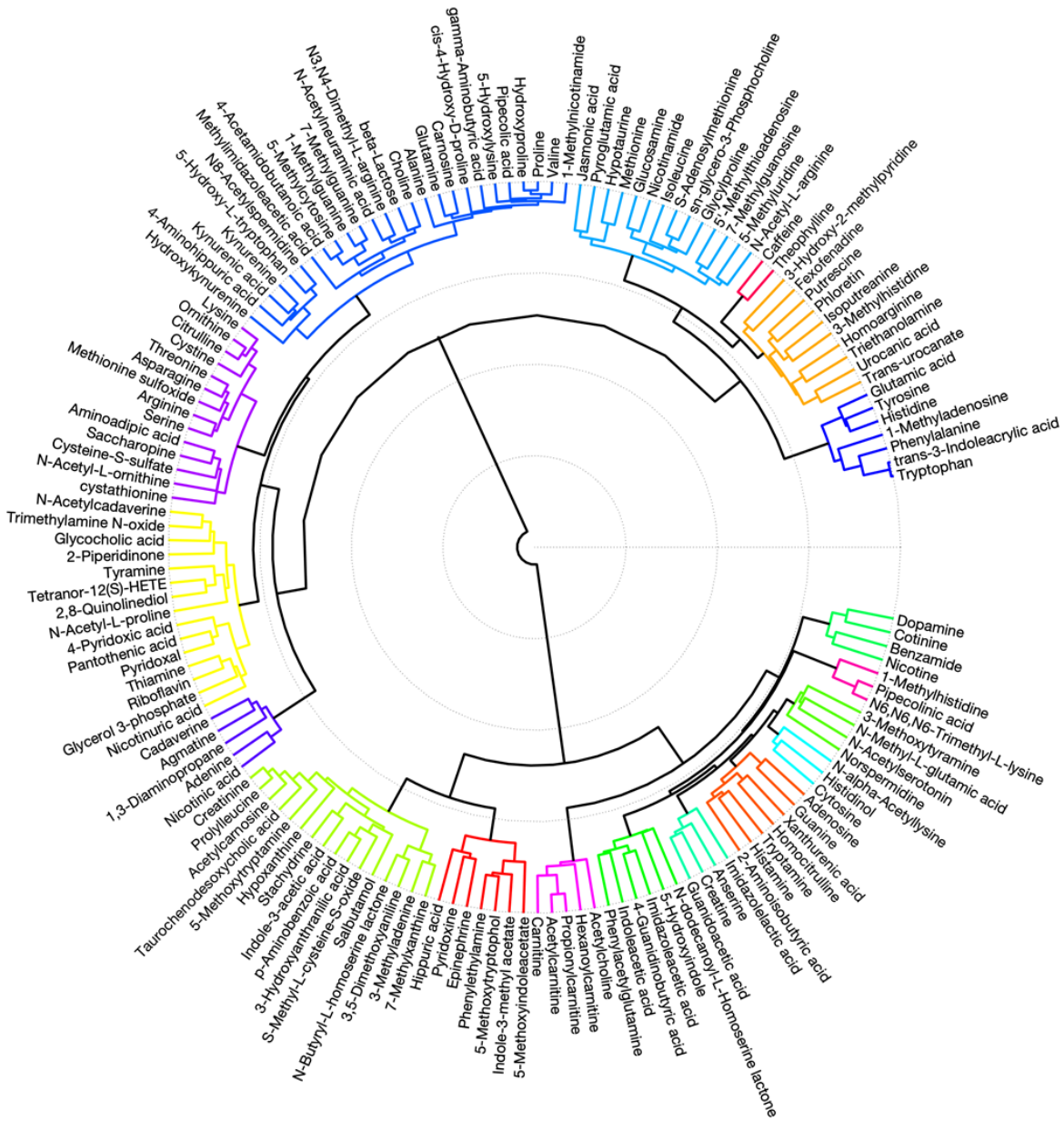


Figure 1. Circle plot displaying all identified metabolites found in the urine of preterm-born neonates. Each set of coloured branches represent separate clusters of metabolites which were correlated with each other.

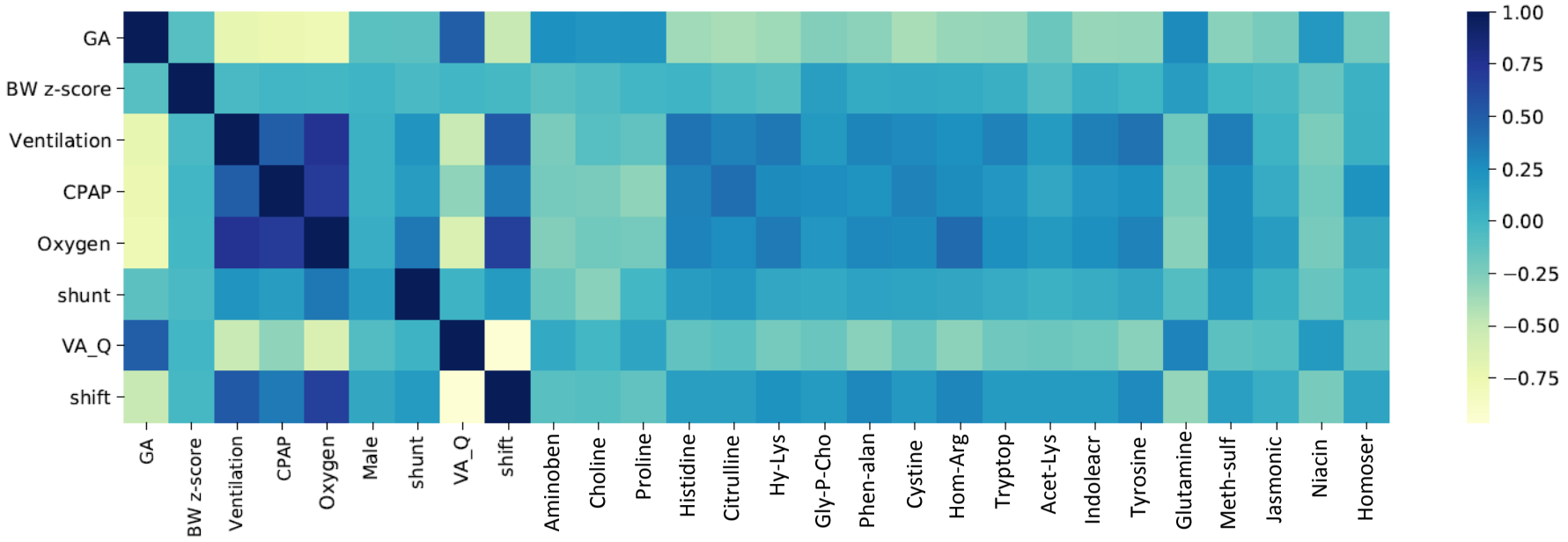


Figure 2. Heatmap illustrating the univariate correlation coefficients between demographic, respiratory support, ventilation-perfusion measures and metabolites that significantly differed ($p<0.05$) between neonates with and without BPD. Block colour is representative of the correlation coefficient between variables as per the colour bar to the right of the figure.

GA: Gestational age; BW z-score: birth weight z-score; Ventilation: length of mechanical ventilation; CPAP: length of continuous positive airway pressure delivery; Oxygen: length of oxygen supplementation; shunt: measure of physiological shunt; VA_Q: Ventilation-perfusion coefficient; shift: measure of physiological shift; Aminoben: p-Aminobenzoic acid; Hy-Lys: 5-Hydroxylysine; Gly-P: sn-glycero-3-Phosphocholine; Phen-alan: Phenylalanine; Hom-Arg: Homoarginine; Tryptop: Tyrptophan; Acet-Lys: N-alpha-Acetyllysine; Indoleacr: trans-3-Indoleacrylic acid; Meth-sulf: Methionine sulfoxide; Jasmonic: Jasmonic acid; Homoser: N-dodecanoyl-L-Homoserine lactone.

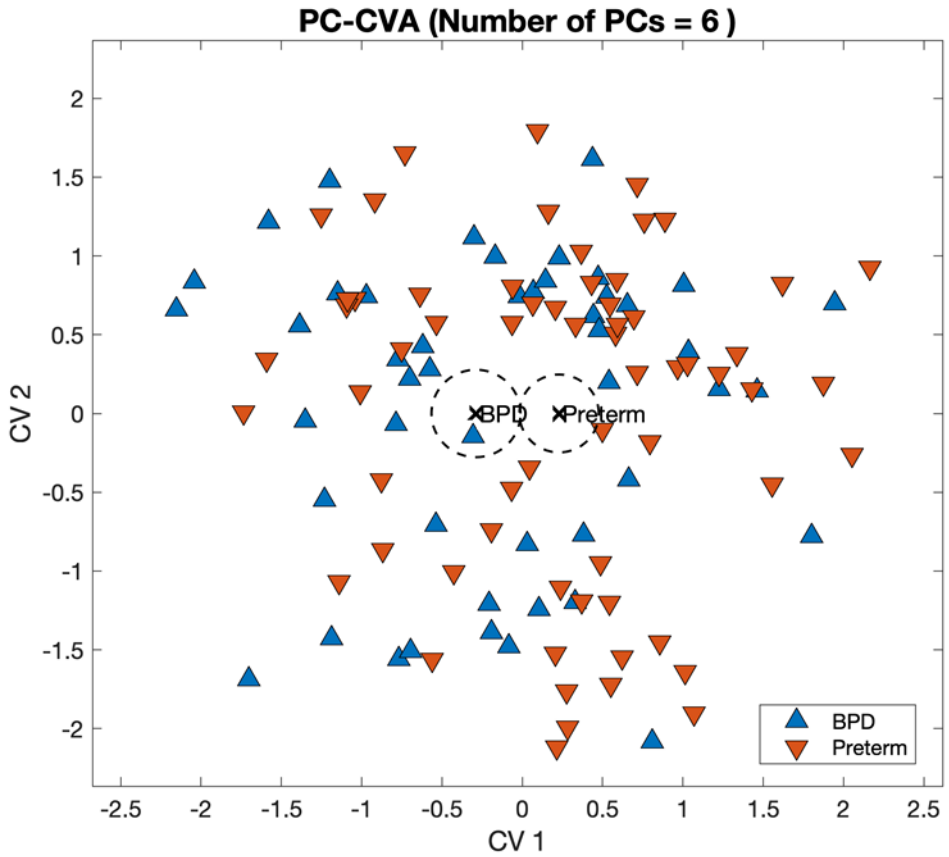


Figure 3. Principal component-canonical variate analysis (PC-CVA) of the relationship between metabolite profile and diagnosis of BPD. Blue triangles represent preterm neonates with a BPD diagnosis ('BPD') and red triangles represent preterm neonates without a diagnosis of BPD ('Preterm'). The black X marks correspond to the mean of each group with dashed circles representing the 95 % confidence intervals for each group. The PC-CVA model was constructed using 6 PCs.

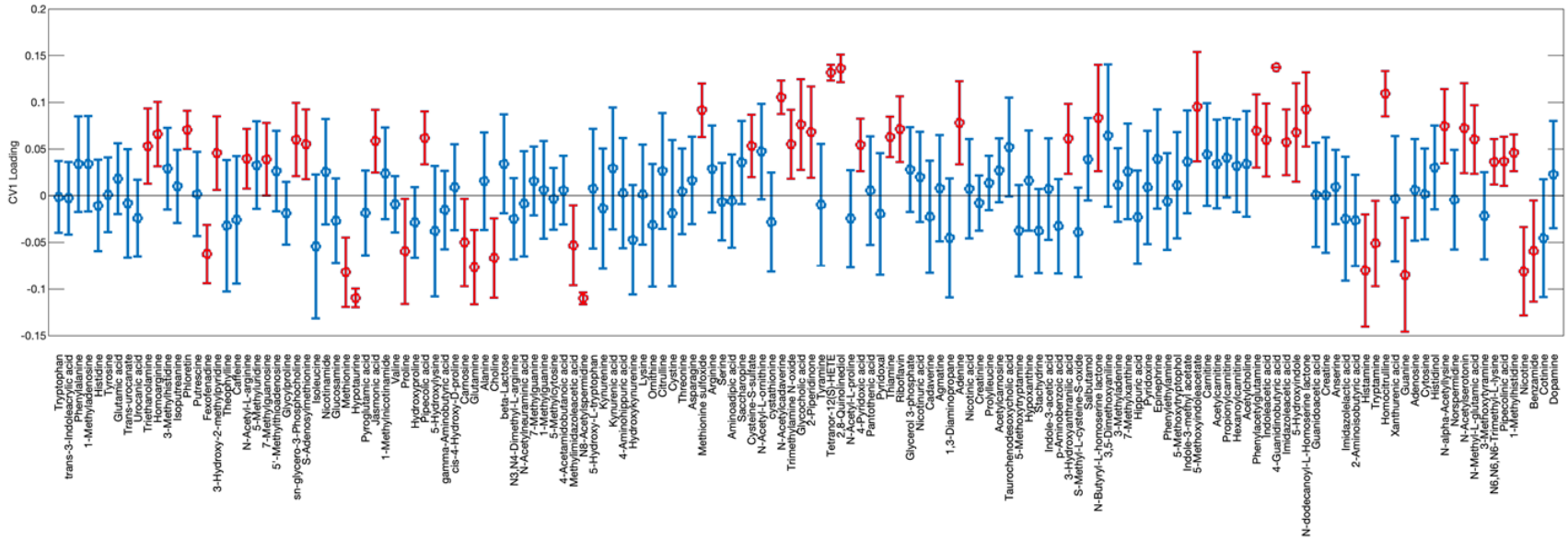


Figure 4. Loading plot displaying the contribution of each identified metabolite to the separation between the metabolomic profiles of neonates with and without BPD. Circles represent the mean loading values and the whiskers illustrate the 95 % confidence intervals. Those in red signify metabolites that significantly ($p<0.05$) contribute to separation between groups and blue denotes metabolites that do not significantly contribute to separation.

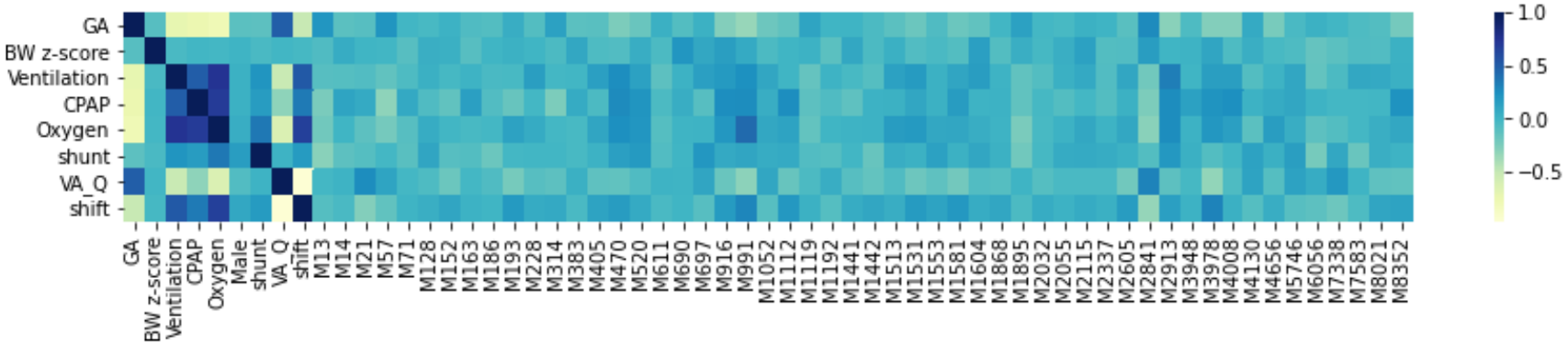


Figure 5. Heatmap illustrating correlation coefficients between demographic, respiratory support, ventilation-perfusion measures and metabolites that significantly ($p<0.05$) contributed to separation between metabolomic profiles of neonates with and without BPD after multivariate analysis. Block colour is representative of the correlation coefficient between variables as per the colour bar to the right of the figure. Only 1 significant metabolite correlation was found; M991 Homoarginine with length of oxygen supplementation ($r^2=0.439$, $p=0.004$).

GA: Gestational age; BW z-score: birth weight z-score; Ventilation: length of mechanical ventilation; CPAP: length of continuous positive airway pressure delivery; Oxygen: length of oxygen supplementation; shunt: measure of physiological shunt; VA_Q: Ventilation-perfusion coefficient; shift: measure of physiological shift; M13-M8352 correspond to the metabolites which significantly contributed to any differentiation between groups.

DISCUSSION

This study demonstrated that the overall urinary metabolomic profile of preterm-born neonates with and without BPD are not significantly different. However, certain individual metabolites contributed to some separation between those with and without BPD, and 19 metabolites were found to be significantly different between groups. Metabolites that differed in abundance between groups primarily consisted of amino acid and fatty acid products. Correlation analyses revealed select metabolites which were associated with gestational age and length of respiratory support.

The heterogeneity of the urinary metabolomic 'fingerprint' across preterm infants at 36 weeks postmenstrual age, regardless of BPD diagnosis, implies that there is no distinct systemic metabolomic alteration that occurs with the development of BPD. Considering that urine analysis is not specific to the lung and more reflective of systemic processes, it may not be the optimal biofluid to assess differences in lung health. However, previous smaller studies by Fanos *et al.* (107) and Pintus *et al.* (106) were able to distinguish between the metabolomic profile of preterm-born neonates who did and did not go on to develop BPD using urine samples collected within the first week after birth. These studies suggest that urinary metabolomics at birth can anticipate BPD development (106, 107). The individual metabolites which differed between groups in these studies together suggested altered oxidative stress pathways and resulting destruction of airway structure in those with BPD (106, 107). Subsequently, Fanos *et al*. propose that BPD is a congenital disease detectable at birth, likely due to intrauterine epigenetic alteration from increased inflammation and oxidative stress, with treatments in the NICU only modulating disease severity, not the incidence of the disease itself (107).

Our study did not observe these metabolomic differences in neonates at 36 weeks postmenstrual age, which may be because the multitude of interventions and events that occur in the NICU from birth to 36 weeks of age results in variable metabolomic profiles, adding 'noise' to the metabolomic signature and making it difficult to distinguish between those with and without BPD. Alternatively, as proposed by Fanos *et al.*, neonatal treatments may ameliorate effects of inflammatory prenatal events and birth at earlier gestational ages, which may be the strongest predictors of BPD development (107). Effective treatments in the NICU such as respiratory support and medications may facilitate the development of the immature lung resulting in less inflammation and oxidative stress than at birth. Piersigilli *et al.* (105) found that the temporal profile of metabolites in tracheal aspirates changed over the first week of life in preterm neonates, with Fabiano *et al.* (210) reporting differences in the metabolomic profile of bronchoalveolar lavage fluid pre- and post-surfactant administration. Both studies reflect metabolomic changes after medical intervention, which may also explain the heterogeneity of metabolomic profiles observed in our study and resultingly, no differences in overall metabolomic profiles between those

with and without BPD. Considering that length of respiratory support is often dependent on local practice and variable clinical decision-making, the characteristics of neonates diagnosed with BPD may vary between centres and countries, which may also explain why our study found no differences between neonates with and without BPD, whereas studies such as those by Fanos *et al.* (107) and Pintus *et al.* (106) did. The complexity, heterogeneity and variability of BPD risk factors such as antenatal events, genetics, altered immunity, dysbiotic microbiome, gestational age and variable clinical management (2) may also mean that the mechanisms leading to a BPD diagnosis are too variable to be detected through systemic metabolomic analysis. Additionally, the larger sample size of our study compared to previous studies may reduce the chances of underpowered findings. As more work is done in the field of neonatal metabolomics, it may become clearer what metabolomic pathways, if any, are altered in those with chronic lung disease. Regardless, our study provides some insight into the evolution of the metabolome in preterm infants with and without BPD after the many neonatal events that occur as they grow in the NICU.

Our data highlight the complex nature of BPD as a disease influenced by multiple interactions of genomics, metabolomics and the microbiome (103), such that the systemic metabolome at 36 weeks is not different between those with and without BPD. At this stage, a more specific biofluid such as tracheal aspirate or bronchoalveolar lavage may better reflect any ongoing metabolomic changes in the lung that are no longer detectable systemically and which result in infants requiring ongoing respiratory support. Our BPD group were born significantly earlier than those without BPD, due to the nature of the BPD definition. Subsequently we saw a significant correlation of gestational age with certain individual metabolites, however no associations with the functional measures of shunt, shift or ventilation-perfusion. In future, stratifying participants based on functional outcomes like shunt-shift cut-offs rather than BPD diagnosis may reveal metabolites associated with functional changes.

Our results suggest that the extent of prematurity, rather than BPD diagnosis, has a greater effect on the systemic metabolomic profile at 36 weeks PMA. Similarly, Piersigilli *et al*. (105) and Verder *et al.* (211) note the association between metabolomic profile and gestational age at birth, with Verder *et al.* also noting birth weight and need for surfactant administration as the most important predicative clinical data in an artificial intelligence predictor of BPD development (211). This latter study was able to combine many clinical factors along with lamellar body counts from gastric aspirates at birth into a predictive algorithm with a sensitivity of 88 % and specificity of 91 % for the prediction of BPD development in preterm infants (211). As per the urine metabolomic studies at birth, Verder *et al.* suggest that BPD development can be predicted at birth, and lamellar body count, gestational age, birth weight and surfactant treatment are the best predictors of BPD (211). More longitudinal studies

examining metabolomic changes across the first few weeks of life will be useful examine other predictive factors for BPD such as nutrition, respiratory support and blood gases, in addition to linking these factors to long-term respiratory outcomes.

Despite not observing differences in the overall metabolomic profile between neonates with and without BPD, correlation analyses showed dietary-related changes associated with parenteral feeding supplementation commonly received by preterm neonates. When adjusted for multicollinearities, earlier gestation was associated with increased histidine, citrulline and 5-hydroxylysine. Increased length of oxygen supplementation was associated with increased 5-hydroxylysine and homoarginine. These metabolites are different to the discriminant metabolites found in the previous neonatal urine studies (106, 107), but similar to those found by Piersigilli *et al.* (105) using tracheal aspirates. This study also found that gestational age at birth effected the metabolomic profile of the neonates involved (105). This study reported higher levels of histidine, glutamic acid, citrulline, glycine and isoleucine levels in neonates with BPD compared to those without (105). The raised levels of these amino acids observed in the present study are likely due to the amino-acid supplementation in parenteral nutrition received by preterm-born neonates in the NICU (212).

Preterm neonates are born with immature metabolomic systems, with parenteral amino acid supplementation common practice to ensure synthesisation of essential amino acids and promote normal cellular growth and development (212). Arginine and citrulline are two such amino acids which are significantly lower at birth in preterm compared to term born infants (213). Citrulline and arginine supplementation have been found to have protective effects against necrotising enterocolitis and pulmonary hypertension (213, 214), with animal studies suggesting that citrulline also attenuates arrested alveolar growth in newborn rats with oxygen-induced lung injury (215). Children who underwent cardiac surgery had lesser pulmonary hypertension when citrulline was administered (214, 216) and preterm neonates who developed pulmonary hypertension had lower plasma citrulline than those who did not (217). The metabolism of L-arginine to L-citrulline produces endogenous nitric oxide (218), which acts as a vasodilator and promotes lung growth (219). Current trials are underway to demonstrate the effectiveness of oral L-citrulline supplementation for the prevention of BPD-associated pulmonary hypertension. Our study, which reports elevated citrulline and arginine levels in neonates born earlier, is less likely to reflect a disease process than these amino acids being supplemented via parenteral nutrition, of which the more premature neonates are likely to have received for longer. A 2017 study found that in comparison to term-born controls, very preterm-born children at preschool age had significantly higher levels of arginine in plasma, suggesting a long-term elevation of these amino acids (220). With arginine and citrulline pathways being protective in cardiovascular health (219), it

remains to be understood whether this early supplementation is beneficial to cardiovascular outcomes in later life for individuals born very prematurely. Prospective metabolomic studies examining arginine and citrulline supplementation throughout NICU management would be helpful in understanding their potentially protective role in pulmonary hypertension and perhaps other respiratory outcomes as a result.

Our study also found greater levels of histidine and 5-hydroxylysine associated with neonates born at an earlier gestation and who required more ventilation, with greater levels of 5-hydroxylysine also associated with longer oxygen supplementation requirement. Lysine and histidine are considered nutritionally essential amino acids in humans (221). Histidine is particularly essential amino acid for infants up to 6 months old, and a lack of histidine in the diet may cause weight gain restriction and nitrogen retention (222). Similarly, lysine is considered indispensable for preterm infants (221), known as a component of collagen with a major impact on child growth and functional development (223). As such, these amino acids are included in parenteral nutrition on which very premature neonates are usually dependent. Our observations of higher levels of histidine and lysine in the urine of more premature infants is again likely due to these infants receiving more parenteral nutrition supplementation. Hao *et al.* (224) found that preterm-born infants who did not receive parenteral nutrition had lower urinary histidine levels than term-born infants, possibly due to less protein deposition of endogenous amino acids or a lack of metabolic enzymes. Preterm infants receiving full enteral feeding were found to have lower whole body lysine oxidisation and a higher lysine balance compared to those who were partially parenterally fed in a 2004 study, suggesting that orally fed infants utilise amino acids for synthesis more efficiently than parenterally fed infants (225) which may explain why we saw greater lysine levels excreted in urine by our more parenterally fed premature infants. Longitudinal studies would be useful for investigating the long-term impact of this early supplementation of lung and overall health in this vulnerable population.

Clearly, nutrition has an important effect on the metabolomic profile of preterm-born infants which may also have implications for lung health. The existing metabolomics studies in bronchopulmonary dysplasia suggest changes in lipid and amino acid metabolism play a role in the development of chronic lung disease of prematurity (103, 106, 226). The decreased urinary lactate and TMAO in neonates with BPD observed by Pintus *et al.* suggests an alteration of the intestinal microbiota (106). The gut microbiome is known to be an important modulator of metabolism and overall health, which in turn may influence lung health (103). Recent studies also implicate alterations in the airway microbiome in the development of BPD (193) with bacterial infection a well-known inflammatory trigger and risk factor for BPD, particularly with Ureaplasma Urealyticum colonisation (227).

CONCLUSIONS

BPD development is complex and its long-term effect on the lungs requires a multifactorial approach to identifying biomarkers of disease utilising analysis across microbiome, genomics, proteomics, and metabolomics platforms. Our results demonstrate that the urinary metabolomic profile of neonates with and without BPD at 36 weeks postmenstrual age are not different and suggests that nutritional supplementation in preterm infants has long-lasting effects on metabolism, which may 'hide' information about any disease processes related to the lung. Further work is needed to better understand the mechanisms involved in BPD development for effective intervention for infants at risk of ongoing respiratory morbidity.

REFERENCES

2. Urs R, Kotecha S, Hall GL, Simpson SJ. Persistent and progressive long-term lung disease in survivors of preterm birth. Paediatric respiratory reviews. 2018.
15. Jobe AH. The new bronchopulmonary dysplasia. Current opinion in pediatrics. 2011;23(2):167.
22. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. Am J Respir Crit Care Med. 2001;163(7):1723-9.
103. Piersigilli F, Bhandari V. Metabolomics of bronchopulmonary dysplasia. Clinica Chimica Acta. 2020;500:109-14.
105. Piersigilli F, Lam TT, Vernocchi P, Quagliariello A, Putignani L, Aghai ZH, et al. Identification of new biomarkers of bronchopulmonary dysplasia using metabolomics. Metabolomics. 2019;15(2):20.
106. Pintus MC, Lussu M, Dessì A, Pintus R, Noto A, Masile V, et al. Urinary 1H-NMR metabolomics in the first week of life can anticipate bpd diagnosis. Oxidative medicine and cellular longevity. 2018;2018.
107. Fanos V, Cristina Pintus M, Lussu M, Atzori L, Noto A, Stronati M, et al. Urinary metabolomics of bronchopulmonary dysplasia (BPD): preliminary data at birth suggest it is a congenital disease. The Journal of Maternal-Fetal & Neonatal Medicine. 2014;27(sup2):39-45.
127. Broadhurst D, Goodacre R, Reinke SN, Kuligowski J, Wilson ID, Lewis MR, et al. Guidelines and considerations for the use of system suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies. Metabolomics. 2018;14(6):1-17.
128. Mendez KM, Pritchard L, Reinke SN, Broadhurst DI. Toward collaborative open data science in metabolomics using Jupyter Notebooks and cloud computing. Metabolomics. 2019;15(10):1-16.
193. Pammi M, Lal CV, Wagner BD, Mourani PM, Lohmann P, Luna RA, et al. Airway microbiome and development of bronchopulmonary dysplasia in preterm infants: a systematic review. The Journal of Pediatrics. 2019;204:126-33. e2.
205. Stoecklin B, Simpson SJ, Pillow JJ. Bronchopulmonary dysplasia: rationale for a pathophysiological rather than treatment based approach to diagnosis. Paediatric respiratory reviews. 2019;32:91-7.
206. Nambiar S, Bong How S, Gummer J, Trengove R, Moodley Y. Metabolomics in chronic lung diseases. Respirology. 2020;25(2):139-48.
207. Baraldi E, Giordano G, Stocchero M, Moschino L, Zaramella P, Tran MR, et al. Untargeted metabolomic analysis of amniotic fluid in the prediction of preterm delivery and bronchopulmonary dysplasia. PLoS One. 2016;11(10):e0164211.

208. Villamor-Martinez E, Álvarez-Fuente M, Ghazi AM, Degraeuwe P, Zimmermann LJ, Kramer BW, et al. Association of chorioamnionitis with bronchopulmonary dysplasia among preterm infants: a systematic review, meta-analysis, and metaregression. JAMA network open. 2019;2(11):e1914611-e.
209. Svedenkrans J, Stoecklin B, Jones JG, Doherty DA, Pillow JJ. Physiology and predictors of impaired gas exchange in infants with bronchopulmonary dysplasia. American journal of respiratory and critical care medicine. 2019;200(4):471-80.
210. Fabiano A, Gazzolo D, Zimmermann LJ, Gavilanes AW, Paolillo P, Fanos V, et al. Metabolomic analysis of bronchoalveolar lavage fluid in preterm infants complicated by respiratory distress syndrome: preliminary results. The Journal of Maternal-Fetal & Neonatal Medicine. 2011;24(sup2):55-8.
211. Verder H, Heiring C, Ramanathan R, Scoutaris N, Verder P, Jessen TE, et al. Bronchopulmonary dysplasia predicted at birth by artificial intelligence. Acta Paediatrica. 2020.
212. Burattini I, Bellagamba MP, Biagetti C, Carnielli VP. Amino Acid Intake in Preterm Infants. Protein in Neonatal and Infant Nutrition: Recent Updates. 86: Karger Publishers; 2016. p. 151-60.
213. Contreras MT, Gallardo MJ, Betancourt LR, Rada PV, Ceballos GA, Hernandez LE, et al. Correlation between plasma levels of arginine and citrulline in preterm and full-term neonates: Therapeutical implications. Journal of clinical laboratory analysis. 2017;31(6):e22134.
214. Fike CD, Dikalova A, Kaplowitz MR, Cunningham G, Summar M, Aschner JL. Rescue treatment with L-citrulline inhibits hypoxia-induced pulmonary hypertension in newborn pigs. American journal of respiratory cell and molecular biology. 2015;53(2):255-64.
215. Vadivel A, Aschner JL, Rey-Parra GJ, Magarik J, Zeng H, Summar M, et al. L-citrulline attenuates arrested alveolar growth and pulmonary hypertension in oxygen-induced lung injury in newborn rats. Pediatric research. 2010;68(6):519-25.
216. Smith HA, Canter JA, Christian KG, Drinkwater DC, Scholl FG, Christman BW, et al. Nitric oxide precursors and congenital heart surgery: a randomized controlled trial of oral citrulline. The Journal of thoracic and cardiovascular surgery. 2006;132(1):58-65.
217. Montgomery AM, Bazzy-Asaad A, Asnes JD, Bizzarro MJ, Ehrenkranz RA, Weismann CG. Biochemical screening for pulmonary hypertension in preterm infants with bronchopulmonary dysplasia. Neonatology. 2016;109(3):190-4.
218. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. New England Journal of Medicine. 1993;329(27):2002-12.
219. Oliveira-Paula GH, Lacchini R, Tanus-Santos JE. Clinical and pharmacogenetic impact of endothelial nitric oxide synthase polymorphisms on cardiovascular diseases. Nitric Oxide. 2017;63:39-51.
220. Posod A, Müller S, Komazec IO, Dejaco D, Peglow UP, Griesmaier E, et al. Former very preterm infants show alterations in plasma amino acid profiles at a preschool age. Pediatric research. 2017;81(5):787-94.
221. Pencharz P, House J, Wykes L, Ball R. What are the essential amino acids for the preterm and term infant? Recent Developments in Infant Nutrition: Springer; 1996. p. 278-96.
222. Synderman S, Boyer A, Roitman E, Jr HL, Prose P. The histidine requirement of the infant. Pediatrics. 1963;31:786-801.
223. Huang L, Hogewind-Schoonenboom JE, de Groof F, Twisk JW, Voortman GJ, Dorst K, et al. Lysine requirement of the enterally fed term infant in the first month of life. The American journal of clinical nutrition. 2011;94(6):1496-503.
224. Hao H, Li S, Zhou W, Wang H, Liu M, Shi C, et al. Metabolic products in urine of preterm infants characterized via gas chromatography-mass spectrometry. International journal of clinical and experimental medicine. 2015;8(9):16454.
225. van der Schoor SR, Reeds PJ, Stellaard F, Wattimena J, Sauer P, Büller HA, et al. Lysine kinetics in preterm infants: the importance of enteral feeding. Gut. 2004;53(1):38-43.

226. Carraro S, Giordano G, Pirillo P, Maretti M, Reniero F, Cogo PE, et al. Airway metabolic anomalies in adolescents with bronchopulmonary dysplasia: new insights from the metabolomic approach. The Journal of pediatrics. 2015;166(2):234-9. e1.
227. Lowe J, Watkins WJ, Edwards MO, Spiller OB, Jacqz-Aigrain E, Kotecha SJ, et al. Association between pulmonary ureaplasma colonization and bronchopulmonary dysplasia in preterm infants: updated systematic review and meta-analysis. The Pediatric infectious disease journal. 2014;33(7):697-702.

Chapter 8: Differences in urinary metabolomic profile between term- and preterm-born children and young-adults

INTRODUCTION

Survivors of preterm birth have persistently poorer respiratory outcomes than their term-born counterparts, which is more pronounced in those with a neonatal diagnosis of bronchopulmonary dysplasia (BPD) (2). Emerging evidence suggests that lung function is not only persistently low in preterm-born children, but may be declining throughout childhood (62, 65). Those with a neonatal diagnosis of BPD have been shown to have lung function trajectories which decline by 0.1 z-scores per year throughout childhood (62). There is therefore a need to develop ways of detecting those most at risk of persistently low or declining lung function. The underlying mechanisms of persistent respiratory morbidity and functional deficit after preterm birth are poorly understood and are further complicated by the influence of multifactorial biological and environmental processes that occur prenatally, postnatally and throughout childhood (2).

Metabolomics offers a way to understand these underlying pathways of disease by detecting individual metabolites and metabolite profiles that differ between groups (103). Metabolomics methods have been able to identify altered pathways in other respiratory diseases such as acute respiratory distress syndrome (109), COPD (110) and asthma (111, 112). Several studies report that pathways associated with oxidative stress, inflammation, immunity and lipid metabolism differ between asthmatic and non-asthmatic groups, in childhood and adulthood (112, 113). By identifying these pathways, the mechanisms driving disease are better understood, enable identification of those most at risk of poorer respiratory outcomes and may serve as potential targets for treatment.

Beyond the neonatal period, only one metabolomics study has been reported in survivors of preterm birth. This study delineated between the metabolomic profile of exhaled breath condensate in healthy term-born adolescents and those with BPD (108). This study suggested differing surfactant lipid profiles between groups, despite the small number of adolescents (all with BPD) exhibiting heterogeneous clinical symptoms and inhaled corticosteroid usage (108). However, this study did not examine individuals who were born preterm without BPD, in order to examine whether these metabolomic changes were related to prematurity or current respiratory disease.

Similar metabolomics methods in survivors of preterm birth may identify altered pathways that contribute to the lung function decline observed in this vulnerable group. The discovery of a panel of biomarkers associated with poorer lung function throughout childhood may help identify which children

are most at risk of ongoing respiratory problems. Importantly, these methods may also help to identify potential therapeutic targets and capture the metabolic response to pharmaceutical interventions.

AIM

This study aimed to determine if the metabolome differs between survivors of very preterm birth and those born at term, and identify any markers associated with poorer respiratory outcomes in later childhood.

METHODS

Participants

Children between the ages of 6 and 12 and young adults between the ages of 16 and 23 years took part in this study, including participants who were born very preterm (<32 weeks gestation) at King Edward Memorial Hospital, and age-matched healthy term-born individuals. Preterm-born children included those with and without a neonatal diagnosis of BPD. Participants attended Perth Children's Hospital for comprehensive lung function testing and biological samples collection as part of the larger Preterm Paediatric Inhaled Corticosteroid Intervention (PICSI) and West Australian Lung Health in Prematurity (WALHIP) studies. Urine samples were collected from participants at their baseline study visits. Samples were aliquoted into 1 mL tubes and stored at -80 degrees Celsius.

As described in Chapter 3: Methodology, neonatal and maternal health data was obtained from medical records and the KEMH neonatal database. Respiratory symptoms history was obtained using validated general and respiratory questionnaires. Anthropometric data collected at the participant's study visit included height, weight, oxygen saturation and blood pressure. Spirometry, FeNO and respiratory oscillometry measures were also collected as part of the study visit.

This study was approved by the Child and Adolescent Health Service Human Research Ethics Committee (Approvals RGS367 and RGS815) and reciprocal approval from Curtin University Human Research Ethics Committees (HRE2018-0385 and HRE2019-0723). Participants provided written informed consent if aged 18 or older and parents/guardians of participants under 18 years of age provided informed written consent.

Urine sample analysis

The specific gravity of each sample was measured by placing 100 µL of the sample on a refractometer (ATAGO-UGα, Atago, Tokyo, Japan). On the day of analysis, urine sample concentration was normalised with dilution in water based on the sample's specific gravity measurement.

Samples were prepared for mass-spectrometry and analysed using a ThermoFisher Triple quadrupole LC/MS system as described in detail in the Methodology chapter. Metabolites were identified by matching mass and retention time to an in-house and the mzCloud chemical reference databases (https://www.mzcloud.org/, HighChem LLC, Slovakia).

Data processing

The data were cleaned based on the Quality Control-Robust Spline Correction (QC-RSC), a metric of analytical precision and D-Ratio, the ratio of analytical variance to overall biological variance. Metabolites with a QC-RSD of >30 % and D-Ratio above 35 % were excluded from analysis.

A principal component analysis was performed and identified no sample outliers.

Statistical analysis

Principal component-canonical variate anlaysis (PC-CVA) was used for multivariate analyses of metabolite differences between groups. The relative abundance of metabolites was not normally distributed, and therefore univariate comparison between groups was performed using Kruskal Wallis rank tests. Correlations between metabolite abundances and neonatal factors were tested using pairwise Spearman's rank correlation coefficients (r). Analysis was performed using Python in Jupyter notebooks (128).

RESULTS

Participants

Urine samples were analysed for 36 term-born participants and 91 preterm-born participants, 44 of whom had a neonatal diagnosis of BPD. Neonatal information for participants is displayed in Table 1. As expected, due to the definition of BPD, participants with a neonatal diagnosis of BPD received longer mechanical ventilation, CPAP and oxygen supplementation. Participants with BPD also had lower birth weight z-scores. Table 2 details the demographic, anthropometric and lung function data for participants at their study visit. Preterm-born participants had lower FEV_1, FEV_1/FVC and FEF_{25-75} outcomes than their term-born counterparts, with worse outcomes in those with a neonatal diagnosis of BPD. Preterm-born participants also had increased AX and Fres outcomes, with these measures further increased in those with BPD.

Table 1. Neonatal information for term-born and preterm-born participants with and without BPD.

Neonatal Factors	Term (n=36)	Preterm (n=91)	No BPD (n=47)	BPD (n=44)
Gestational age at birth (weeks)	40 (38-40)	28 (25.5–29.8)	30 (28.9–31)	26 (24.6–27.8)
Male, n (%)	16 (44 %)	53 (58 %)	28 (60 %)	25 (57 %)
Birth weight (z-score)	-	-0.01 ± 0.83	0.19 ± 0.75	-0.21 ± 0.86[ǂ]
Mechanical ventilation duration (days)	-	1.83 (0.02–22.5)	0 (0-0.84)	12.8 (2.3-36) [#]
CPAP duration (days)	-	5.63 (0.98–25.2)	1 (0-3.6)	19.7 (5.7–40.6) [#]
Oxygen supplementation duration (days)	-	30 (0.63–84.0)	0.2 (0-3.3)	73 (36.2-97.3) [#]

Data presented as mean ± standard deviation, median (interquartile range) or number (percentage).
[ǂ] significant difference to preterm participants with no BPD $p<0.05$
[#] significant difference to preterm participants with no BPD $p<0.01$

Table 2. Demographic and lung function information for term-born and preterm-born participants with and without BPD.

	Term (n=37)	Preterm (n=90)	No BPD (n=47)	BPD (n=43)
Age (years)	18.4 (10.4–19.9)	18.3 (12.5–20.3)	13.2 (12.7–19.7)	19.5 (12.9–20.7)
Height (cm)	167.3 (137.6–177.7)	159.5 (148.3–172.3)	164.0 (148.8–175.6)	162.0 (154.6–172.6)
Weight (kg)	56.5 ± 21.9	55.5 ± 20.8	55.0 ± 21.5	56.1 ± 20.1
BMI	21.5 (18.1–22.9)	21.1 (17.2–24.0)	21.2 (17.4–24.3)	21.8 (18.0 24.2)
FEV_1 (z-score)	0.20 ± 1.08	-0.64 ± 1.19**	-0.32 ± 0.98*	-0.97 ± 1.29**[#]
FVC (z-score)	0.28 ± 1.03	-0.04 ± 0.93	0 ± 0.89	-0.11 ± 0.99
FEV_1/FVC (z-score)	-0.13 ± 1.04	-0.86 ± 1.24**	-0.50 ± 1.15	-1.19 ± 1.27**[#]
$FEF_{25-75\%}$ (z-score)	0.04 ± 1.07	-0.84 ± 1.31**	-0.41 ± 1.07	-1.26 ± 1.41**
FeNO (ppb)	19 (11.5–22.5)	14 (9.9–27.4)	16.3 (10–35.8)	13.3 (9.9–23.5)
R_5 (z-score)	0.51 ± 1.17	0.71 ± 1.42	0.65 ± 1.54	0.78 ± 1.28
X_5 (z-score)	0.34 (-0.43–0.90)	0.19 (-0.47–1.31)	-0.17 (-0.71–0.63)	0.87 (-0.04–1.55) [#]
AX (z-score)	0.80 ± 0.91	1.56 ± 1.36**	1.45 ± 1.43*	1.68 ± 1.28**
Fres (z-score)	0.57 ± 0.84	1.43 ± 1.35**	1.05 ± 1.29	1.85 ± 1.31**[#]
$R_{10.in-ex}$ (Hz)	-0.23 (-0.39 – -0.04)	-0.14 (-0.46–0)	-0.20 (-0.70 – -0.04)	-0.11 (-0.23–0.02) [ǂ]
$X_{10.in-ex}$ (Hz)	0.12 (-0.02–0.26)	0.12 (-0.07–0.61)	0.39 (-0.01–0.99)	0.01 (-0.12–0.19) [ǂ]

Data presented as mean ± standard deviation, median (interquartile range) or number (percentage).
*significantly different to term-born controls $p<0.05$
**significantly different to term-born controls $p<0.01$
[ǂ] significant difference to preterm participants with no BPD $p<0.05$
[#] significant difference to preterm participants with no BPD $p<0.01$

Metabolomic analysis

LC/MS analysis of urine samples detected 152 MS/MS identity-confirmed metabolites. PC-CVA analysis revealed a clear distinction in the metabolomic profile between samples collected from school-aged participants between 6-12 years old and young-adult participants between the ages of 16 and 23 years (Figure 1). Subsequently, univariate analysis was split between school-age and young-adult groups and conducted separately for each of these age groups.

Multivariate analysis using PC-CVA allowed for a visual representation of the separation in metabolomic profiles between groups (Figure 1). The first canonical variate (CV1, Figure 1) described a significant mean difference between school-aged and young-adult groups. The second canonical variate (CV2, Figure 1) described the mean separation between those born preterm with and without BPD, and the term-born controls.

Univariate analysis of our school-age cohort found 13 metabolites with a significant fold change between preterm and term-born children (Table 3). There was a marginal significant difference of 8 metabolites between school-age participants born at term, those born preterm without BPD and those with BPD, however none passed the false discovery rate (FDR) or Benjamini-Hochberg correction (Table 5).

In the young adult cohort, 12 metabolites were found to be significantly different between term and preterm born participants (Table 4). 10 metabolites were found to be significantly different between participants who were term-born, preterm-born with BPD and preterm-born without BPD. However, similarly to the school-age cohort, none passed the FDR or Benjamini-Hochberg correction (Table 6).

As none of the individual metabolites passed the FDR or Benjamini-Hochberg correction, we were unable to perform correlation or regression analysis to assess which, if any, of these metabolites were associated with neonatal factors or poorer lung function.

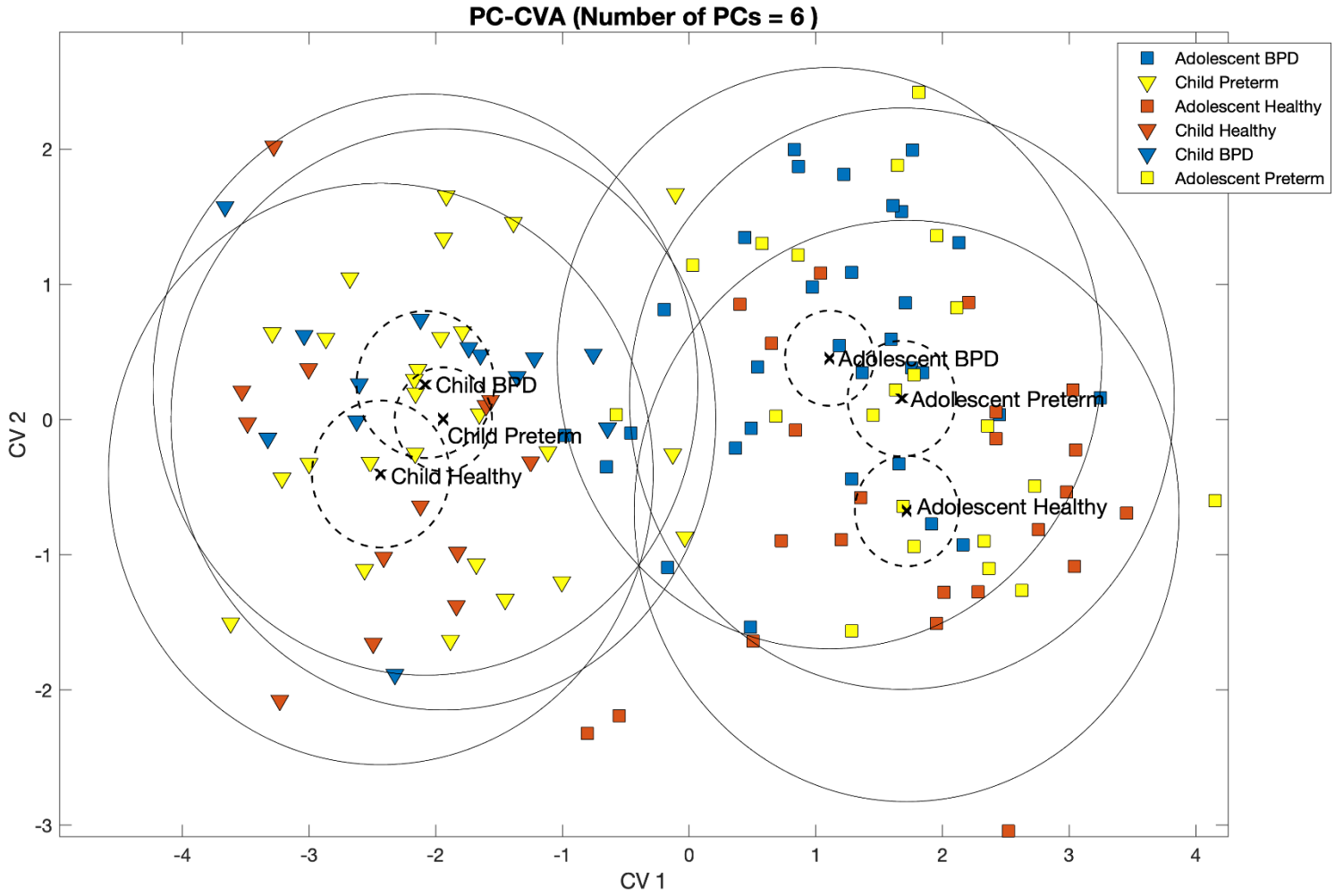


Figure 1. Principal component-canonical variate analysis (PC-CVA) of the relationship between metabolite profile and diagnosis of BPD. Triangles represent school aged participants and squares represent young-adult participants. Blue triangles and squares represent preterm participants with a BPD diagnosis ('BPD') and red triangles and squares represent preterm participants without a diagnosis of BPD ('Preterm'). Yellow triangles and squares represent participants born at term ('Healthy'). The black X marks correspond to the mean of each group with dashed circles representing the 95 % confidence intervals for each group. The PC-CVA model was constructed using 6 PCs.

Table 3. Fold change in abundance for selected metabolites that significantly differed between term- and preterm-born school-aged participants between the ages of 6 and 12 years.

Metabolite	Fold Change		Significance	
	Term	Preterm	p-value	q-value
Histidine	1 (0.70, 1.33)	0.62 (0.57, 0.74)	0.006	0.214
Theophylline	1 (0.55, 1.86)	4.80 (2.66, 6.44)	0.028	0.472
Caffeine	1 (0.75, 1.60)	1.34 (1.21, 1.66)	0.024	0.472
Methionine	1 (0.78, 1.20)	0.74 (0.70, 0.95)	0.038	0.472
Valine	1 (0.82, 1.14)	1.21 (1.06, 1.36)	0.031	0.472
Lysine	1 (0.44, 1.08)	0.40 (0.29, 0.55)	0.005	0.214
Methionine sulfoxide	1 (0.68, 1.54)	0.77 (0.61, 0.86)	0.040	0.472
Pyridoxal	1 (0.67, 1.26)	0.59 (0.42, 0.80)	0.040	0.472
Salbutamol	1 (0.92, 5.76)	7.18 (2.97, 13.76)	0.003	0.214
3,5-Dimethoxyaniline	1 (0.50, 1.56)	2.82 (1.45, 3.88)	0.018	0.472
7-Methylxanthine	1 (0.31, 2.01)	4.03 (2.51, 5.50)	0.004	0.214
Hexanoylcarnitine	1 (0.60, 1.13)	1.28 (1.07, 1.39)	0.028	0.472
Histidinol	1 (0.64, 2.13)	0.62 (0.51, 0.84)	0.028	0.472

Data are presented as the fold-change of the median (95 % confidence interval). Displayed data were selected based upon $p<0.05$ and corrected for multiple comparisons according to Benjamini and Hochberg (q-value).

Table 4. Fold change in abundance for selected metabolites that significantly differed between term- and preterm-born young-adult participants between the ages of 16 and 23 years.

Metabolite	Fold Change		Significance	
	Term	Preterm	p-value	q-value
Tryptophan	1 (0.90, 1.28)	1.23 (1.14, 1.43)	0.049	0.477
Nicotinamide	1 (0.71, 1.23)	0.77 (0.67, 0.83)	0.024	0.477
Valine	1 (0.87, 1.22)	1.20 (1.07, 1.45)	0.028	0.477
γ-Aminobutyric acid	1 (0.84, 1.52)	1.49 (1.37, 1.94)	0.011	0.477
Alanine	1 (0.85, 1.17)	1.29 (1.06, 1.49)	0.038	0.477
Stachydrine	1 (0.33, 1.97)	0.23 (0.10, 0.47)	0.015	0.477
Indole-3-acetic acid	1 (0.28, 2.41)	0.24 (0.18, 0.31)	0.017	0.477
p-Aminobenzoic acid	1 (0.45, 1.46)	0.41 (0.32, 0.53)	0.008	0.477
Hippuric acid	1 (0.88, 1.29)	0.74 (0.61, 0.84)	0.023	0.477
Pyridoxine	1 (0.72, 1.60)	0.58 (0.38, 0.77)	0.045	0.477
Epinephrine	1 (0.75, 1.27)	0.69 (0.59, 0.82)	0.045	0.477
N-Methyl-L-glutamic acid	1 (0.52, 1.54)	0.40 (0.33, 0.46)	0.034	0.477

Data are presented as the fold-change of the median (95 % confidence interval). Displayed data were selected based upon $p<0.05$ and corrected for multiple comparisons according to Benjamini and Hochberg (q-value).

Table 5. Fold change in abundance for selected metabolites that significantly differed between term- and preterm-born school-aged participants with and without BPD between the ages of 6 and 12 years.

Metabolite	Fold Change			Significance		
	Healthy	Preterm	BPD	p-value	FDR	q-value
Lysine	1 (0.56,1.78)	0.44 (0.3,0.89)	0.33 (0.23,0.84)	0.04	0.48	0.78
Histidine	1 (0.6,1.75)	0.65 (0.45,0.97)	0.55 (0.35,0.97)	0.01	0.60	0.60
Pyridoxal	1 (0.57,1.82)	0.48 (0.29,0.85)	0.91 (0.5,1.77)	0.01	0.38	0.60
Salbutamol	1 (0.2,5.23)	5.53 (0.88,11.88)	7.32 (1.24,17.23)	0.02	0.36	0.69
Hexanoylcarnitine	1 (0.58,1.75)	1.23 (0.97,2.12)	1.61 (0.79,2.88)	0.04	0.42	0.78
7-Methylxanthine	1 (0.28,3.55)	3.1 (1,9.48)	6.59 (1.84,21.32)	0.01	0.31	0.60
Theophylline	1 (0.2,4.74)	4.38 (0.81,10.39)	5.08 (0.93,16.94)	0.02	0.37	0.71
Jasmonic acid	1 (0.71,1.4)	1.22 (0.81,1.54)	1.56 (0.96,2.01)	0.03	0.39	0.74

Data are presented as the fold-change of the median (95 % confidence interval). Displayed data were selected based upon $p<0.05$ and corrected for multiple comparisons according to Benjamini and Hochberg (q-value).

Table 6. Fold change in abundance for selected metabolites that significantly differed between term- and preterm-born young adult participants with and without BPD between the ages of 16 and 23 years.

Metabolite	Fold Change			Significance		
	Healthy	Preterm	BPD	p-value	FDR	q-value
Stachydrine	1 (0.21,5.18)	0.4 (0.09,1.77)	0.15 (0.05,0.74)	0.02	0.30	0.45
p-Aminobenzoic acid	1 (0.42,2.62)	0.48 (0.23,1.67)	0.38 (0.16,1.03)	0.01	0.50	0.42
γ-Aminobutyric acid	1 (0.55,1.81)	1.45 (0.88,2.16)	1.54 (0.9,2.65)	0.01	0.28	0.42
Propionylcarnitine	1 (0.26,3.68)	3.06 (1.07,7.45)	1.62 (0.49,4.57)	0.02	0.30	0.51
Fexofenadine	1 (0.65,1.55)	1.74 (0.95,2.62)	1.06 (0.69,1.23)	0.01	0.25	0.42
Nicotinamide	1 (0.67,1.48)	0.77 (0.4,1.11)	0.8 (0.6,1.15)	0.02	0.26	0.45
Indole-3-acetic acid	1 (0.22,3.89)	0.22 (0.08,0.82)	0.25 (0.1,0.78)	0.04	0.39	0.55
Phloretin	1 (0.64,1.6)	1.11 (0.71,1.47)	0.88 (0.47,1.22)	0.04	0.41	0.55
3-Methoxytyramine	1 (0.7,1.34)	1.16 (0.74,1.73)	0.81 (0.59,1.02)	0.01	0.28	0.42
Histidinol	1 (0.5,2)	1.56 (0.71,2.92)	0.63 (0.37,1.11)	0.03	0.34	0.55

Data are presented as the fold-change of the median (95 % confidence interval). Displayed data were selected based upon $p<0.05$ and corrected for multiple comparisons according to Benjamini and Hochberg (q-value).

DISCUSSION

Our results suggest that there are subtle differences in urine metabolomic profiles between children and young people born preterm with BPD, those born preterm without BPD and those born at term. These differences are only evident when applying multivariate analysis and are not evident at the individual metabolite level. Univariate analysis found individual metabolites that were marginally different between groups but did not pass the false discovery rate or Benjamini-Hochberg correction. Our results also found a clear difference in the metabolomic profiles of participants in childhood (6-12 years old) and those in young-adulthood (16-23 years old). These large differences between groups meant that we could not reliably examine associations between individual metabolites and lung function. We may hypothesise that the overall metabolite difference between those born at term and those born preterm with and without BPD could be associated with the reduced and altered lung function outcomes observed in our preterm-born participants. However, as urine is not a lung-specific biofluid, the metabolomic differences may alternatively reflect differences in metabolism of other body systems.

The most distinct difference in urinary metabolomic profiles in this study was between childhood participants and those in adolescence or young adulthood. This age-related difference in systemic metabolism is most likely due to changes pre- and post-puberty. It is also important to consider that this was a cross-sectional study examining 2 different cohorts. Differences in the extent of separation across BPD, non-BPD and term-born groups between the age groups may also reflect advances in neonatal care, perhaps resulting in a smaller gap in metabolomic profile for those born prematurely in more recent years compared to those born at term. Despite the distinction between age groups, there was a similar pattern of separation across the CV2 axis (Figure 1) between those with and without BPD and term-born controls in both childhood and young-adulthood. This indicates that despite the significant changes during puberty, there are differences in the metabolome of individuals born at term, those born preterm without BPD and those with BPD in both childhood and young-adulthood.

These results suggest that a neonatal diagnosis of BPD, as well as being born very prematurely, can be associated with alterations in the metabolome that persists into childhood and even adulthood. Metabolomics studies in preterm neonates suggest that the progression to BPD can be predicted at birth, with alterations in metabolomic patterns associated with oxidative stress, lipid and amino acid metabolism (103, 106). Recently, artificial intelligence-generated prediction tools also suggest BPD development can be predicated at birth with certain neonates predisposed to BPD development because of a range of prenatal factors, as well as gestational age, surfactant need and epigenetic changes as a result of inflammatory and oxidative stress exposures (211). Beyond the neonatal period, Carraro *et al.* (226) found the metabolomic profiles of exhaled breath condensate (EBC) to be completely distinct

between adolescents born at term and those with a neonatal diagnosis of BPD. We similarly attempted to determine the metabolomic profile in EBC during this study, however, were unable to optimise the assay to a level where we were confident in the outputted results. Analysis methods for EBC are not well established and the variation of sample volume and dilution (131) meant that our attempts to develop a metabolomics approach to analysing EBC resulted in any metabolites remaining unquantifiable due to a large noise to signal ratio. Carraro *et al.* (226) found that pathways related to lung inflammation, oxidative injury and impaired pulmonary circulation, as well as potentially surfactant lipid composition to differ between healthy adolescents and those with BPD. These results, with our results in urine, imply that there are long-term changes in metabolomic profile with preterm birth.

As metabolomics in survivors of preterm birth is a new field of research, the present study serves as an untargeted exploration of potential pathways contributing to disease in those born preterm. However, we could not identify any individual metabolites that were significantly different between groups which also passed the false discovery rate. Those metabolites that were significantly different between groups mostly consisted of amino acids. These amino acids may reflect changes in dietary metabolism in those born preterm and those with BPD. There is some evidence that dietary metabolites linked to nutrition in the NICU and both the gut and airway microbiome play a role in lung health and the development of BPD (94, 103, 106, 226), but it remains unclear why we would be detecting these changes many years after initial discharge. Additionally, decreased microbial diversity and increased amounts of pathogenic microbial species have been detected in the microbiome of adult survivors of preterm birth, which may be contributing to the metabolomic differences we observe. Further, an altered microbiome has been associated with low lung function in this population (95). However, considering none of these identified metabolites in the present study passed the false discovery rate or Benjamini-Hochberg correction, we cannot reliably associate these metabolites with other clinical features.

There are several limitations to this exploratory study which restricted our ability to see differences in individual metabolites between groups. Considering BPD is a respiratory diagnosis, we may have seen more discrimination between groups by using a lung-specific biofluid, as used by Carraro *et al.* in individuals born preterm (226), and as has been recently explored in other diseases such as asthma and COPD (110, 206, 228). However, we have been unsuccessful in reliably analysing EBC using metabolomics methods, and other lung-specific samples are difficult and invasive to obtain. Our untargeted approach using urine reflected systemic metabolism, which is perhaps why we could identify differences in overall metabolite profiles but were unable to detect differences in specific metabolites.

Other methods of analysing urine such as nuclear magnetic resonance have been able to detect biomarkers specific to lung disease (229, 230) and may be a more sensitive method for future studies.

Although our untargeted design allowed us to identify differences in metabolomic profile between those born at term, those born preterm and those with a neonatal diagnosis of BPD, this approach minimised the specificity of our analysis. In order to better identify specific pathways contributing to these differences, future studies could take a more targeted approach to analysis. Emerging evidence implicates neutrophilic inflammation, oxidative stress, (101, 106, 107) (as also described in previous chapters) and alterations in surfactant lipid composition in children born preterm (211, 226), which may also contribute to worse respiratory outcomes. Targeting these pathways using metabolomics techniques such as liquid-chromatography or nuclear magnetic resonance may further establish these pathways as contributors to poor lung function in those born preterm, and potentially identify other metabolites involved in these pathways that could serve as biomarkers of disease.

Alternatively, we may not have observed differences in individual metabolites between groups as our population exhibited relatively mild lung disease. Our groups were based on premature birth and a neonatal diagnosis of BPD, which both had a heterogenous spread of respiratory outcomes in participants who were otherwise well. Our untargeted metabolomics approach may not have been sensitive to smaller differences in disease pathways resulting in mild disease. As our methods were not sensitive enough to detect differences in individual metabolites across groups, we were unable to assess for any metabolite associations with respiratory outcomes. Stratifying groups according to current clinical or functional features such as lung function may allow future studies to more directly investigate associations between metabolites pathways and respiratory outcomes. Ideally, this would lead to the development of a panel of biomarkers which could help identify those individuals most at risk of persistent respiratory disease after preterm birth and enable earlier, more targeted intervention.

As survivors of extremely premature birth in the post-surfactant era are only just now entering early adulthood, it is increasingly important to understand the long-term effects of preterm birth on lung function and the mechanisms underpinning persistent lung function deficits. The present study suggests that despite changes in metabolism from childhood to young adulthood, the metabolome remains different between individuals born at term, those born preterm and those with a neonatal diagnosis of BPD. This study was an important first-look, discovery study which suggests there are differences in metabolism with preterm birth and BPD diagnosis, Further studies are needed to better understand which specific pathways are associated with these differences, Ideally, this will lead to the development of a panel of biomarker which could predict those individuals at risk of function decline and help identify potential avenues for early intervention.

REFERENCES

2. Urs R, Kotecha S, Hall GL, Simpson SJ. Persistent and progressive long-term lung disease in survivors of preterm birth. Paediatric respiratory reviews. 2018.

62. Simpson SJ, Turkovic L, Wilson A, Verheggen M, Logie KM, Pillow JJ, et al. Lung function trajectories throughout childhood in survivors of very preterm birth: A longitudinal cohort study. The Lancet Child and Adolescent Health. 2018.

65. Doyle LW, Adams A-M, Robertson C, Ranganathan S, Davis NM, Lee KJ, et al. Increasing airway obstruction from 8 to 18 years in extremely preterm/low-birthweight survivors born in the surfactant era. Thorax. 2017;72(8):712.

94. Gentle SJ, Lal CV. Predicting BPD: lessons learned from the airway microbiome of preterm infants. Frontiers in pediatrics. 2020;7:564.

95. Rofael SA, McHugh TD, Troughton R, Beckmann J, Spratt D, Marlow N, et al. Airway microbiome in adult survivors of extremely preterm birth: the EPICure study. European Respiratory Journal. 2019;53(1).

101. Filippone M, Bonetto G, Corradi M, Frigo AC, Baraldi E. Evidence of unexpected oxidative stress in airways of adolescents born very pre-term. The European respiratory journal. 2012;40(5):1253-9.

103. Piersigilli F, Bhandari V. Metabolomics of bronchopulmonary dysplasia. Clinica Chimica Acta. 2020;500:109-14.

106. Pintus MC, Lussu M, Dessì A, Pintus R, Noto A, Masile V, et al. Urinary 1H-NMR metabolomics in the first week of life can anticipate bpd diagnosis. Oxidative medicine and cellular longevity. 2018;2018.

107. Fanos V, Cristina Pintus M, Lussu M, Atzori L, Noto A, Stronati M, et al. Urinary metabolomics of bronchopulmonary dysplasia (BPD): preliminary data at birth suggest it is a congenital disease. The Journal of Maternal-Fetal & Neonatal Medicine. 2014;27(sup2):39-45.

108. Carraro S, Giordano G, Pirillo P, Maretti M, Reniero F, Cogo PE, et al. Airway metabolic anomalies in adolescents with bronchopulmonary dysplasia: new insights from the metabolomic approach. J Pediatr. 2015;166(2):234-9 e1.

109. Evans CR, Karnovsky A, Kovach MA, Standiford TJ, Burant CF, Stringer KA. Untargeted LC–MS metabolomics of bronchoalveolar lavage fluid differentiates acute respiratory distress syndrome from health. Journal of proteome research. 2014;13(2):640-9.

110. Adamko DJ, Nair P, Mayers I, Tsuyuki RT, Regush S, Rowe BH. Metabolomic profiling of asthma and chronic obstructive pulmonary disease: a pilot study differentiating diseases. Journal of Allergy and Clinical Immunology. 2015;136(3):571-80. e3.

111. Reinke SN, Gallart-Ayala H, Gómez C, Checa A, Fauland A, Naz S, et al. Metabolomics analysis identifies different metabotypes of asthma severity. European Respiratory Journal. 2017;49(3).

112. Kelly RS, Dahlin A, McGeachie MJ, Qiu W, Sordillo J, Wan ES, et al. Asthma metabolomics and the potential for integrative omics in research and the clinic. Chest. 2017;151(2):262-77.

113. Mattarucchi E, Baraldi E, Guillou C. Metabolomics applied to urine samples in childhood asthma; differentiation between asthma phenotypes and identification of relevant metabolites. Biomedical Chromatography. 2012;26(1):89-94.

128. Mendez KM, Pritchard L, Reinke SN, Broadhurst DI. Toward collaborative open data science in metabolomics using Jupyter Notebooks and cloud computing. Metabolomics. 2019;15(10):1-16.

131. Horváth I, Barnes PJ, Loukides S, Sterk PJ, Högman M, Olin A-C, et al. A European Respiratory Society technical standard: exhaled biomarkers in lung disease. European Respiratory Journal. 2017;49(4):1600965.

206. Nambiar S, Bong How S, Gummer J, Trengove R, Moodley Y. Metabolomics in chronic lung diseases. Respirology. 2020;25(2):139-48.

211. Verder H, Heiring C, Ramanathan R, Scoutaris N, Verder P, Jessen TE, et al. Bronchopulmonary dysplasia predicted at birth by artificial intelligence. Acta Paediatrica. 2020.
226. Carraro S, Giordano G, Pirillo P, Maretti M, Reniero F, Cogo PE, et al. Airway metabolic anomalies in adolescents with bronchopulmonary dysplasia: new insights from the metabolomic approach. The Journal of pediatrics. 2015;166(2):234-9. e1.
228. Ntontsi P, Ntzoumanika V, Loukides S, Benaki D, Gkikas E, Mikros E, et al. EBC metabolomics for asthma severity. Journal of breath research. 2020;14(3):036007.
229. McClay JL, Adkins DE, Isern NG, O'Connell TM, Wooten JB, Zedler BK, et al. 1H nuclear magnetic resonance metabolomics analysis identifies novel urinary biomarkers for lung function. Journal of proteome research. 2010;9(6):3083-90.
230. Carrola J, Rocha CM, Barros AS, Gil AM, Goodfellow BJ, Carreira IM, et al. Metabolic signatures of lung cancer in biofluids: NMR-based metabonomics of urine. Journal of proteome research. 2011;10(1):221-30.

Chapter 9: General Discussion

SUMMARY OF FINDINGS

This thesis investigated the underlying inflammatory and altered metabolomic pathways contributing to persistently deficient respiratory function in survivors of preterm birth. The studies within this thesis demonstrated altered respiratory mechanics and flows in survivors of preterm birth compared to those born at term, with worse outcomes associated with increased prematurity and more respiratory intervention in the neonatal stage of life. Additionally, poorer respiratory function was not associated with markers of airway eosinophilic inflammation, but instead markers of neutrophilic inflammation and oxidative stress. Metabolomic analysis in very preterm neonates suggested that altered dietary metabolites were associated with birth at an earlier gestation, but the overall metabolomic profile was not different in those with and without BPD at 36 weeks postmenstrual age. However, in comparison to their term-born counterparts. children and young adults who were born preterm displayed an altered metabolomic profile, with some distinction between those with and without a history of BPD. The findings of this thesis suggest that individuals born prematurely exhibit an underlying neutrophilic inflammatory profile and a dysregulated metabolome which persists into childhood and adolescence, as does deficits in airway flow and mechanics. Although more work is needed to develop and expand analytical techniques, these findings provide some insight into some of the underlying process contributing to respiratory morbidity in survivors of preterm birth. These processes may also serve as potential avenues of intervention to alleviate the high burden of respiratory disease in this population.

RESPIRATORY FUNCTION PHENOTYPE AND NEONATAL PREDICTORS

This thesis demonstrated the preterm-born children had an obstructive pattern of spirometry and oscillometry outcomes suggestive of altered peripheral airway mechanics and stiffness, in the absence of elevated eosinophilic inflammation compared to term-born controls. The rate of bronchodilator responsiveness was only elevated in those with a neonatal diagnosis of BPD, of whom about one-third had a significant bronchodilator response. More abnormal lung function measures in childhood were associated with the receival of more neonatal respiratory intervention, highlighting the long-term impact of neonatal events. These results add to the growing body of literature showing similar lung function deficiencies in survivors of preterm birth in the post-surfactant era (54-56). Longitudinal studies have shown a trend of lung function decline throughout childhood in survivors of preterm birth (62, 65). Together these studies prompted the development of European Respiratory Society guidelines on the long-term management of children with bronchopulmonary dysplasia, which made conditional recommendations based on what was determined to be very low to low quality of existing evidence (231). These guidelines highlight the need for new evidence surrounding the long-term respiratory impacts of prematurity, the underlying pathophysiology of disease and response to treatments (231). As more evidence of abnormal lung function in survivors of preterm birth arises, it raises questions about

the underlying pathways of disease. There is very limited evidence around whether long-term respiratory morbidity is a result of ongoing inflammatory processes and environmental insults beyond the neonatal phase or perhaps fixed airway damage from preterm birth and subsequent respiratory intervention. It is important to understand the factors that predispose preterm neonates to develop BPD as well as the effect of interventions and ongoing processes that may be modifiable in order to prevent lung function decline and further respiratory morbidity. This thesis aimed to address these questions further by taking both a targeted approach to look for known markers of airway inflammation and oxidative stress and an untargeted approach using metabolomics to identify altered pathways in survivors of preterm birth.

IMPORTANCE OF FINDING BIOMARKERS

As respiratory morbidity in survivors of preterm birth begins in the earliest stages of life and even prenatally, it is vital to be able to identify biomarkers in neonates, infants and young children to enable early detection and intervention in the most high-risk infants and children. Markers that can be detected in biofluids that are easily and non-invasively collected from infants and young children will enable improved understanding of pathways of disease in early life and will be most useful in a clinical setting. Exhaled breath condensate is specific to the lung and non-invasive to collect. This thesis has shown that exhaled breath condensate is feasible to collect even in neonates and infants using an adapted commercially available device (114). However, the analysis of EBC is not well-established and it is therefore limited in how it can be analysed. Urine is also collected easily and non-invasively, however it is not specific to the lungs. Despite this, it has been shown to be useful in distinguishing groups with and without lung disease (106, 107), including in the present study. Analysis methods for urine are better established across both immunoassay and metabolomics techniques. This thesis found that biomarkers were more detectable in urine in comparison to EBC, and that metabolomics techniques were much more feasible using urine compared to EBC. This body of work suggests that biomarkers, such as leukotriene B4 or 8-isoprostane, may predict disease to enable earlier detection of high-risk survivors of preterm birth and monitoring of treatment effectiveness. However, more work to establish clinical correlates is required. As investigations into biomarkers of BPD are fairly limited, future work may necessitate measures from both invasive and non-invasive samples such as blood, induced sputum and bronchoalveolar lavage fluid to initially establish the most important pathway to be targeted. Subsequent studies may then build on this work to identify biomarkers using non-invasive techniques suitable for infants and young children and for regular monitoring purposes.

TARGETED ANALYSIS

The targeted analysis of EBC in infants at 12-16 months old demonstrated elevated neutrophilic inflammation and oxidative stress processes persisting in the lungs of preterm-born infants beyond the first year of life, regardless of BPD diagnosis. These results could highlight the significance of preterm

birth itself on lung health or perhaps be linked to other factors such as respiratory infections over the first year of life, to which infants born preterm are more susceptible. Elevated airway inflammatory and oxidative stress processes in infants whose lungs are still developing may have implications for lung growth which may contribute to the long-term lung function deficits seen in this population.

In older children and young adults, the lung function deficits seen in the absence of elevated eosinophilia measured by FeNO, EBC and urinary cysteinyl leukotrienes further reinforces the distinction of chronic lung disease after prematurity from typical childhood eosinophilic asthma (142), although commonly diagnosed in this population (56). This is particularly relevant to the clinical management of preterm-born children who present with asthma-like symptoms, as common treatments such as inhaled corticosteroids which target eosinophilic inflammation may not be as effective in preterm-born children compared to asthmatics. MMP-9 was also shown to not differ between those born preterm and those born at term children. With MMP-9 as a marker of airway remodelling (179), this finding suggests that airway remodelling may not be actively occurring in older children. Some studies have shown that children born preterm have structural changes on CT (45, 232), implying that airway remodelling and structural differences may play a role in poorer long-term respiratory outcomes. However, there have been no longitudinal studies to show if these changes are static damage from the neonatal stage of life or progressive throughout childhood to support the MMP-9 data in the present study. Longitudinal analysis of EBC becomes difficult however, due to the collection method changing from nasal breathing to oral breathing from infancy to older children. It is therefore difficult to compare samples from infants to older children and adults as contamination with nasal secretion may occur and interfere with targets from the lower respiratory tract (180). The use of a facemask to compartmentalise nasal air and oral air in older participants may allow for some consistency with infant EBC samples, however the contamination with nasal secretions remains a challenge in this instance as well. Elevated urinary markers of neutrophilic inflammation and oxidative stress suggest these processes persist systemically beyond the neonatal period and may influence lung function. The decreased IL-8 in EBC and no difference in urinary IL-8 between groups, in the presence of elevated neutrophilia, warrants more investigation of the role of cytokines in the airways of preterm-born children. Together, the results of targeted analysis of a few known inflammatory markers suggest more neutrophilic inflammatory and oxidative stress, rather than eosinophilic, pathways of disease which persist in survivors of preterm birth. It may be that targeting these pathways with treatments such as macrolide antibiotics, which are reported to be beneficial in both eosinophilic and neutrophilic asthma sub-types (233), might be more effective than treatments such as inhaled corticosteroids. Beyond the known inflammatory markers that we investigated, it is important to understand other potentially modifiable pathways which are significantly altered between preterm and term-born children, and which influence lung function.

METABOLOMICS

Untargeted metabolomics analysis can serve as a way to discover alterations in metabolism which contribute to disease. As this method measures the end products of metabolism, it potentially measures more modifiable disease factors compared to other techniques like genomics and proteomics. BPD is a disease influenced by a variety of external factors and not just innate predisposition, and so metabolomics may be a useful tool for identifying some of these contributors to respiratory morbidity. In concordance with other studies, our results highlighted the influence of nutrition on the metabolome and how this differs with increasing prematurity and the resulting supplementation received (103, 107). This early supplementation might have long-lasting effects on the metabolome which may have both protective and detrimental effects. As implied by our findings, emerging evidence also highlights the importance of diet and the microbiome, as well the airway microbiome, on lung health. Our results also found that the overall urinary metabolomic profile of neonates with and without BPD at 36 weeks postmenstrual age are not different. This contrasted with previous studies which analysed samples collected in the first days of life and were able to predict which infants would go on to develop BPD (103, 211). These studies implied that BPD is a congenital disease, resulting from epigenetic changes as a result of inflammatory events in the antenatal and early postnatal period (103, 211). However, our study suggests that these distinctions in the metabolome between neonates with and without BPD are not present at 36 weeks postmenstrual age, which may be a result of effective treatments in the NICU. The implications for long-term respiratory outcomes in these infants is yet to be understood, but our results might imply that all preterm infants regardless of BPD diagnosis are vulnerable to ongoing respiratory morbidity. This also highlights the limitations with the current definition of BPD, which is a retrospective diagnosis based on the length of respiratory support received by the neonate (22). The length of respiratory support does not reflect the underlying physiological state of the lungs, and BPD diagnosis is based on a somewhat arbitrary cut-off that is subject to variable clinical decision-making, failing to capture the heterogeneity of lung disease in preterm-born neonates (205). By stratifying neonates according to a more objective measures such as lung physiology, future studies may be able to better describe metabolomic changes that effect clinical outcome. Other methodological limitations of our study, such as the use of a non-airway specific sample and our broad untargeted approach, may also have minimised our ability to distinguish between those with and without BPD and future studies should attempt to ameliorate these limitations. Despite these limitations, our metabolomics results from our older children and young adults demonstrated that despite age-related changes in the metabolome, distinctions in the metabolome between individuals born at term and those born preterm with and without BPD persist, similarly to deficits in lung function. Metabolites related to reduced lung function have been identified in asthma, with dysregulated lipid metabolism products associated with spirometry results (112) and COPD (229). Further studies are needed in preterm-born individuals to better understand the specific pathways associated with lung function decline, and to identify potential avenues for early intervention. BPD development is complex and its long-term effect on the lungs

requires a multifactorial approach to identifying biomarkers of disease utilising analysis across microbiome, genomics, proteomics, and metabolomics platforms.

LIMITATIONS AND FUTURE DIRECTIONS

There are several limitations to the studies within this thesis. Ideally, the use of airway specific biofluids with more established analysis methods, such as bronchoalveolar lavage or induced sputum, may have provided better insight into the specific processes occurring in the lung and allowed for quantification of biomarkers. However, these samples are difficult to obtain from this population, and as such we utilised the simplicity and non-invasive collection techniques for exhaled breath condensate and urine. Further work is needed to optimise the analysis of exhaled breath condensate for both targeted and untargeted analysis. Although analysis methods for urine are better established, and differences can be detected in urine, it is not specific to the lung. Those born preterm are also likely to have comorbidities such as cerebral palsy or neurodevelopmental deficits which may also be producing some signal in the systemic biofluid of urine (234).

The samples used in our metabolomics analysis were collected as part of larger studies where metabolomics results were not the primary outcome, and as a result we were unable to control for confounders like age, height, weight and gestational age. As a result of the extra signal 'noise' created from these variables, the ability to distinguish between groups may have been diminished. Future studies will be more discrete and control for these confounders.

Although we examined preterm-born individuals of different ages, these measures were a cross-sectional examination of different individuals and not a longitudinal analysis of the same individuals. Therefore there should be some caution in interpreting correlations of neonatal risk factors with early biomarkers or mechanisms of disease. These correlations – such as the finding of lower FEV_1/FVC with antenatal steroid administration – may be confounded due to potential differences between cohorts based on birth era and may perhaps reflect changes in neonatal practice over time. Ideally, prospective longitudinal studies will be best placed to examine the neonatal and early-life predictors of ongoing respiratory morbidity in this population.

The limitations in the diagnostic criteria for BPD may also mean that it is not necessarily representative of worse long-term respiratory outcomes. More objective measures of lung function, like spirometry or respiratory mechanics, would be allow for more direct associations of potential risk factors with functional outcomes and perhaps future studies could be designed with this hypothesis in mind (i.e. comparing those with normal lung function to those with low lung function).

Longitudinal studies are necessary to determine the persistence of inflammatory and metabolomic changes in children born preterm and following up the later lung function of infants with increased EBC markers of inflammation should be a priority. Additionally, prospective examination of maternal

biomarkers or biomarkers in samples taken soon after birth and throughout the neonatal period will allow for further exploration of predictive risk factors for BPD and potentially modifiable risk factors in the neonatal period. Clearly, BPD and continued respiratory deficits after preterm birth are complex and multifactorial, so biomarkers should be considered in the context of functional and structural changes in the lung. Untargeted approaches to metabolomic analysis may be useful for biomarker discovery, but for lung function outcomes resulting from such multi-factorial interactions as after prematurity, future work may benefit from exploring more targeted pathways. By targeting potentially modifiable pathways of disease such as inflammatory and oxidative stress pathways, the roles of these processes will become clearer and targets for intervention may be developed.

CLINICAL IMPLICATIONS

Reduced respiratory outcomes in childhood and young-adulthood in survivors of preterm birth is of concern, particularly considering that lung function tracks through life and low FEV_1 is linked to all-cause mortality (143) in addition to emerging evidence of worsening lung function throughout childhood (62, 65). This places these individuals at risk of early-onset respiratory morbidity in adulthood. With changes to neonatal practice in the 1990s (235), survivors of extremely preterm birth are only just now in early adulthood, and it may be that we are likely to see more cases of respiratory morbidity in this population in the coming years. The reduced lung function and altered respiratory mechanics observed in children who were born preterm occurs in the absence of elevated eosinophilia, distinguishing the underlying mechanism from typical childhood asthma. This is of relevance considering that preterm-born children are more likely to be diagnosed with asthma and twice as likely to prescribed asthma medications than their term-born counterparts (55, 56). Treating physicians should take a thorough medical history which asks about preterm birth in order to further investigate underlying contributors to disease in order to effectively target treatment. Although these observed lung function deficits are concerning, the research in this thesis provides some insights into underlying processes which may serve as targets for intervention, particularly evidence of ongoing neutrophilic inflammation and oxidative stress.

Potential treatments and interventions

Prevention of long-term lung disease in preterm children has been focussed to intervention in the NICU, with the aim of decreasing lung injury. Corticosteroids have anti-inflammatory properties, with systemic dexamethasone administration in the NICU becoming popular for the prevention of BPD in the 1980s. The respiratory benefits of dexamethasone treatment include decreased ventilator requirements, earlier extubation, improved lung function, decreased lung inflammation, and ultimately improved survival with reduced BPD incidence (236-238). However, adverse long term neurodevelopmental effects and increased risk of gastro-intestinal perforation have led to the early termination of some clinical trials and cautious use of postnatal systemic corticosteroids during the

neonatal period after preterm birth (239). Nevertheless, systemic corticosteroids continue to be used especially to aid extubation of chronically ventilator-dependent preterm-born infants (240). Inhaled corticosteroids (ICS) administered in the neonatal period have not shown effectiveness in preventing BPD (241), and although one recent trial showed no association with adverse neurodevelopmental outcome, rates of mortality were higher in neonates who received ICS (242). Azithromycin, which has been shown to reduce disease severity in other inflammatory lung diseases, may be effective in both the short- and long-term in preterm infants but further studies are needed to determine its efficacy in reducing lung injury in preterm infants (243). Other potential anti-inflammatory therapies that have shown improvements in respiratory outcomes in animal models include curcumin and mesenchymal stromal cell therapy, but these are yet to be assessed in humans (244).

The benefits of anti-inflammatory treatment in survivors of preterm birth after the neonatal period have been less well studied: in part because the inflammatory contribution to lung disease in survivors of preterm birth remains poorly described. A follow-up at 8-11 years of very preterm children randomised to receive postnatal dexamethasone or placebo in the newborn period demonstrated long-lasting effects of corticosteroid administration, with fewer children exhibiting abnormal spirometry measures in the treated group (40 %; N=35) than the placebo group (68 %; N=28) (245). Smaller studies of no more than 18 participants each have shown inconsistent results, with no improvements in lung function or symptoms observed in children aged 7-13 years after ICS; however, a decrease in the variability of the peak expiratory flow was observed (246, 247), suggesting a reduction in bronchial reactivity. One study reported significant improvements in symptoms, lung function and reduced bronchodilator usage in ex-preterm infants aged around 10 months who used ICS (248). Although there is evidence that ICS may be of some benefit in the preterm population, its clinical use in this population is not clear and larger studies are needed to answer this question.

Potential anti-oxidant therapies have found limited benefit in BPD prevention, with some studies reporting superoxide dismutase and melatonin supplementation in infants reducing measures of oxidative stress but without reducing the risk of BPD development (195). Diet may play an important role in anti-oxidant defences and improving the gut-airway microbiome axis in those born preterm. Probiotic treatment in neonates may help improve the gut microbiome but their benefit in lung disease remains poorly understood (94). In asthma, lower plasma levels of antioxidant minerals such as zinc and selenium have been associated with higher levels of oxidative stress markers (204). Small trials suggest specific dietary antioxidants might improve asthma control, however the impact of diet modification on asthma remains poorly understood (249), with no evidence in chronic lung disease after prematurity.

Our study suggests that interventions targeted towards neutrophilic inflammation or those that promote lung growth may be more appropriate considering the phenotype of chronic lung disease after preterm

birth. Treatments with macrolide antibiotics, such as azithromycin, may also be useful in treating older children and young-adults who were born prematurely. The anti-inflammatory properties of azithromycin have been shown to be effective in adults with severe asthma (233) while targeting both eosinophilic and non-eosinophilic inflammation, which may be useful in the heterogeneous disease observed in survivors of preterm birth.

Identifying modifiable pathways

The long-term outcomes of preterm birth are widely heterogenous and influenced by many factors prenatally, in the neonatal period and throughout childhood. Although there are some risk factors for worse long-term respiratory outcomes, it remains difficult to predict those on the trajectory for early-onset respiratory morbidity (250). The development of a biomarker panel that could identify those most at risk of poor respiratory outcomes will enable targeted intervention. Identifying these markers in non-invasive, easily collectable biofluids such as EBC and urine will also enable early identification if able to be collected in young infants. This was our hope with our untargeted metabolomics studies. As a first-pass look at metabolomics methods in survivors of preterm birth, these discovery studies were important to establish improved protocols for future studies with the eventual goal of developing a biomarker panel. Future metabolomics studies should control well for confounders and perhaps utilise targeted techniques for pathways of neutrophilic inflammation and oxidative stress. Other techniques such as nuclear magnetic resonance and volatile organic compound analysis may also be more sensitive to differences in urine or exhaled breath.

The findings within this thesis also highlight the heterogeneity of phenotypes observed in preterm-born children with chronic lung disease. Reported symptoms, inflammatory profiles, bronchodilator responsiveness and functional deficits vary in their characterisation within this population. This may reflect the highly complex interaction between genetics, antenatal factors, neonatal trajectory and respiratory exposures in childhood and their effect on the resulting pulmonary functional or structural changes observed. Similarly to phenotypic variability within asthma, value will be found in more comprehensive characterisation of the various respiratory phenotypes within the preterm population and perhaps a more personalised approach in order to identify modifiable pathways and determine the most appropriate treatment. Considering the lack of research into the underlying mechanisms of ongoing respiratory disease after preterm birth, there is a wide scope for this type discovery research to ultimately improve outcomes in this population.

CONCLUDING REMARKS

With the survival of more individuals born very and extremely preterm, understanding the long-term respiratory outcomes of this population is of increasing importance. It is becoming well-established that lung function is diminished in survivors of preterm birth throughout childhood into adulthood, with the results from this thesis further supporting these findings and implicating altered peripheral mechanics

and reduced airway elasticity. The mounting evidence of impaired long-term respiratory outcomes put these individuals on a trajectory for early-onset respiratory morbidity. It is therefore crucial to understand the underlying mechanisms in order to intervene with effective treatments. Despite this, there has been little research undertaken to explore the mechanisms underlying poor and/or declining lung function in this population. Use of non-invasive biofluids such as EBC and urine will enable early identification and easy monitoring of disease progression. With further studies, we hope this will lead to the identification of a biomarker panel to identify those most at risk of respiratory morbidity, in addition to identifying the mechanisms of disease at play. This thesis has provided evidence for the persistence of neutrophilic inflammation and oxidative stress processes in survivors of preterm birth, with elevated markers in infancy, childhood and young-adulthood. These findings put forward neutrophilic and oxidative stress processes as potential targets for intervention, which may be treatable with already-approved medications such as macrolide antibiotics. Together these findings not only provide insights into the contributors to chronic lung disease after preterm birth, but also widens the scope for further mechanistic analysis and identifies potential pathways for treatment. Hopefully these findings contribute to early intervention and better respiratory outcomes for life in survivors of preterm birth.

REFERENCES

22. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. Am J Respir Crit Care Med. 2001;163(7):1723-9.
45. Simpson SJ, Logie KM, O'Dea CA, Banton GL, Murray C, Wilson AC, et al. Altered lung structure and function in mid-childhood survivors of very preterm birth. Thorax. 2017.
54. Simpson SJ, Hall GL, Wilson AC. Lung function following very preterm birth in the era of 'new' bronchopulmonary dysplasia. Respirology. 2015;20(4):535-40.
55. Fawke J, Lum S, Kirkby J, Hennessy E, Marlow N, Rowell V, et al. Lung function and respiratory symptoms at 11 years in children born extremely preterm: the EPICure study. American journal of respiratory and critical care medicine. 2010;182(2):237-45.
56. Korhonen P, Laitinen J, Hyoudynmaa E, Tammela O. Respiratory outcome in school-aged, very-low-birth-weight children in the surfactant era. Acta Pædiatrica. 2004;93(3):316-21.
62. Simpson SJ, Turkovic L, Wilson A, Verheggen M, Logie KM, Pillow JJ, et al. Lung function trajectories throughout childhood in survivors of very preterm birth: A longitudinal cohort study. The Lancet Child and Adolescent Health. 2018.
65. Doyle LW, Adams A-M, Robertson C, Ranganathan S, Davis NM, Lee KJ, et al. Increasing airway obstruction from 8 to 18 years in extremely preterm/low-birthweight survivors born in the surfactant era. Thorax. 2017;72(8):712.
94. Gentle SJ, Lal CV. Predicting BPD: lessons learned from the airway microbiome of preterm infants. Frontiers in pediatrics. 2020;7:564.
103. Piersigilli F, Bhandari V. Metabolomics of bronchopulmonary dysplasia. Clinica Chimica Acta. 2020;500:109-14.
106. Pintus MC, Lussu M, Dessì A, Pintus R, Noto A, Masile V, et al. Urinary 1H-NMR metabolomics in the first week of life can anticipate bpd diagnosis. Oxidative medicine and cellular longevity. 2018;2018.
107. Fanos V, Cristina Pintus M, Lussu M, Atzori L, Noto A, Stronati M, et al. Urinary metabolomics of bronchopulmonary dysplasia (BPD): preliminary data at birth suggest it is a congenital disease. The Journal of Maternal-Fetal & Neonatal Medicine. 2014;27(sup2):39-45.
112. Kelly RS, Dahlin A, McGeachie MJ, Qiu W, Sordillo J, Wan ES, et al. Asthma metabolomics and the potential for integrative omics in research and the clinic. Chest. 2017;151(2):262-77.
114. Urs R, Stoecklin B, Pillow JJ, Hartmann B, Hall GL, Simpson SJ. Collecting exhaled breath condensate from non-ventilated preterm-born infants: a modified method. Pediatric Research. 2021:1-3.
142. Course CW, Kotecha S, Kotecha SJ. Fractional exhaled nitric oxide in preterm-born subjects: A systematic review and meta-analysis. Pediatric pulmonology. 2019;54(5):595-601.
143. Magnussen C, Ojeda FM, Rzayeva N, Zeller T, Sinning CR, Pfeiffer N, et al. FEV1 and FVC predict all-cause mortality independent of cardiac function—Results from the population-based Gutenberg Health Study. International journal of cardiology. 2017;234:64-8.
179. Chakrabarti S, Patel KD. Matrix metalloproteinase-2 (MMP-2) and MMP-9 in pulmonary pathology. Experimental lung research. 2005;31(6):599-621.
180. Rosias PP, Dompeling E, Hendriks HJ, Heijnens JW, Donckerwolcke RA, Jöbsis Q. Exhaled breath condensate in children: pearls and pitfalls. Pediatric allergy and immunology. 2004;15(1):4-19.
195. Poggi C, Dani C. Antioxidant strategies and respiratory disease of the preterm newborn: an update. Oxidative medicine and cellular longevity. 2014;2014.
204. Wood LG, Fitzgerald DA, Gibson PC, Cooper DM, Garg ML. Lipid peroxidation as determined by plasma isoprostanes is related to disease severity in mild asthma. Lipids. 2000;35(9):967-74.
205. Stoecklin B, Simpson SJ, Pillow JJ. Bronchopulmonary dysplasia: rationale for a pathophysiological rather than treatment based approach to diagnosis. Paediatric respiratory reviews. 2019;32:91-7.
211. Verder H, Heiring C, Ramanathan R, Scoutaris N, Verder P, Jessen TE, et al. Bronchopulmonary dysplasia predicted at birth by artificial intelligence. Acta Paediatrica. 2020.

229. McClay JL, Adkins DE, Isern NG, O'Connell TM, Wooten JB, Zedler BK, et al. 1H nuclear magnetic resonance metabolomics analysis identifies novel urinary biomarkers for lung function. Journal of proteome research. 2010;9(6):3083-90.
231. Duijts L, van Meel ER, Moschino L, Baraldi E, Barnhoorn M, Bramer WM, et al. European Respiratory Society guideline on long-term management of children with bronchopulmonary dysplasia. European respiratory journal. 2020;55(1).
232. Aukland SM, Halvorsen T, Fosse KR, Daltveit AK, Rosendahl K. High-resolution CT of the chest in children and young adults who were born prematurely: findings in a population-based study. American Journal of Roentgenology. 2006;187(4):1012-8.
233. Gibson PG, Yang IA, Upham JW, Reynolds PN, Hodge S, James AL, et al. Effect of azithromycin on asthma exacerbations and quality of life in adults with persistent uncontrolled asthma (AMAZES): a randomised, double-blind, placebo-controlled trial. The Lancet. 2017;390(10095):659-68.
234. Raju TN, Pemberton VL, Saigal S, Blaisdell CJ, Moxey-Mims M, Buist S. Long-term healthcare outcomes of preterm birth: an executive summary of a conference sponsored by the National Institutes of Health. The Journal of pediatrics. 2017;181:309-18. e1.
235. Kamath BD, MacGuire ER, McClure EM, Goldenberg RL, Jobe AH. Neonatal mortality from respiratory distress syndrome: lessons for low-resource countries. Pediatrics. 2011;127(6):1139-46.
236. Doyle LW, Ehrenkranz RA, Halliday HL. Early (< 8 days) postnatal corticosteroids for preventing chronic lung disease in preterm infants. Cochrane Database Syst Rev. 2014(5):CD001146.
237. Bassler D, Plavka R, Shinwell ES, Hallman M, Jarreau PH, Carnielli V, et al. Early Inhaled Budesonide for the Prevention of Bronchopulmonary Dysplasia. N Engl J Med. 2015;373(16):1497-506.
238. Baud O, Maury L, Lebail F, Ramful D, El Moussawi F, Nicaise C, et al. Effect of early low-dose hydrocortisone on survival without bronchopulmonary dysplasia in extremely preterm infants (PREMILOC): a double-blind, placebo-controlled, multicentre, randomised trial. Lancet. 2016;387(10030):1827-36.
239. Committee on F, Newborn. Postnatal corticosteroids to treat or prevent chronic lung disease in preterm infants. Pediatrics. 2002;109(2):330-8.
240. Jobe AH. Postnatal corticosteroids for preterm infants--do what we say, not what we do. N Engl J Med. 2004;350(13):1349-51.
241. Shah VS, Ohlsson A, Halliday HL, Dunn M. Early administration of inhaled corticosteroids for preventing chronic lung disease in very low birth weight preterm neonates. The Cochrane Library. 2017.
242. Bassler D, Shinwell ES, Hallman M, Jarreau P-H, Plavka R, Carnielli V, et al. Long-Term Effects of Inhaled Budesonide for Bronchopulmonary Dysplasia. New England Journal of Medicine. 2018;378(2):148-57.
243. Nair V, Loganathan P, Soraisham AS. Azithromycin and other macrolides for prevention of bronchopulmonary dysplasia: a systematic review and meta-analysis. Neonatology. 2014;106(4):337-47.
244. Madurga A, Mizíková I, Ruiz-Camp J, Morty R. Recent advances in late lung development and the pathogenesis of bronchopulmonary dysplasia. American Journal of Physiology. 2013;305(12):L893.
245. Nixon PA, Washburn LK, Schechter MS, O'Shea TM. Follow-up study of a randomized controlled trial of postnatal dexamethasone therapy in very low birth weight infants: effects on pulmonary outcomes at age 8 to 11 years. The Journal of pediatrics. 2007;150(4):345-50.
246. Chan KN, Silverman M. Increased airway responsiveness in children of low birth weight at school age: effect of topical corticosteroids. Archives of disease in childhood. 1993;69(1):120-4.
247. Pelkonen AS, Hakulinen AL, Hallman M, Turpeinen M. Effect of inhaled budesonide therapy on lung function in schoolchildren born preterm. Respir Med. 2001;95(7):565-70.
248. Yuksel B, Greenough A. Randomised trial of inhaled steroids in preterm infants with respiratory symptoms at follow up. Thorax. 1992;47(11):910-3.
249. Moreno-Macias H, Romieu I. Effects of antioxidant supplements and nutrients on patients with asthma and allergies. Journal of Allergy and Clinical Immunology. 2014;133(5):1237-44.

250. Simpson SJ, Hallberg J. The PELICAN (Prematurity's Effect on the Lungs In Children and Adults Network) ERS Clinical Research Collaboration: understanding the impact of preterm birth on lung health throughout life. Eur Respiratory Soc; 2021.

Complete reference list

1. Phases of lung development.: University of Fribourg; 2005 [Available from: http://www.embryology.ch/dutch/rrespiratory/phasen01.html.
2. Urs R, Kotecha S, Hall GL, Simpson SJ. Persistent and progressive long-term lung disease in survivors of preterm birth. Paediatric respiratory reviews. 2018.
3. Smith LJ, McKay KO, van Asperen PP, Selvadurai H, Fitzgerald DA. Normal development of the lung and premature birth. Paediatric respiratory reviews. 2010;11(3):135-42.
4. Kotecha S. Lung growth: implications for the newborn infant. Archives of Disease in Childhood-Fetal and Neonatal Edition. 2000;82(1):F69-F74.
5. Burri PH. Fetal and postnatal development of the lung. Annual review of physiology. 1984;46(1):617-28.
6. Burri PH. Structural aspects of postnatal lung development–alveolar formation and growth. Neonatology. 2006;89(4):313-22.
7. Otto-Verberne CJ, Ten Have-Opbroek AA, Balkema JJ, Franken C. Detection of the type II cell or its precursor before week 20 of human gestation, using antibodies against surfactant-associated proteins. Anatomy and embryology. 1988;178(1):29-39.
8. Butler JP, Loring SH, Patz S, Tsuda A, Yablonskiy DA, Mentzer SJ. Evidence for adult lung growth in humans. New England Journal of Medicine. 2012;367(3):244-7.
9. Zeltner TB, Burri PH. The postnatal development and growth of the human lung. II. Morphology. Respiration physiology. 1987;67(3):269-82.
10. Northway WH, Jr., Rosan RC, Porter DY. Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. N Engl J Med. 1967;276(7):357-68.
11. Kamath BD, Macguire ER, McClure EM, Goldenberg RL, Jobe AH. Neonatal mortality from respiratory distress syndrome: lessons for low-resource countries. Pediatrics. 2011;127(6):1139-46.
12. Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller AB, Narwal R, et al. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. Lancet. 2012;379(9832):2162-72.
13. Australian, Network NZN, Chow SS, Creighton P, Kander V, Haslam R, et al. Report of the Australian and New Zealand Neonatal Network, 20162018.
14. Abman SH. Bronchopulmonary Dysplasia: "A Vascular Hypothesis". American journal of respiratory and critical care medicine. 2001;164(10):1755-6.
15. Jobe AH. The new bronchopulmonary dysplasia. Current opinion in pediatrics. 2011;23(2):167.
16. Speer CP. Inflammation and bronchopulmonary dysplasia: a continuing story. Semin Fetal Neonatal Med. 2006;11(5):354-62.
17. Lal MK, Manktelow BN, Draper ES, Field DJ. Chronic lung disease of prematurity and intrauterine growth retardation: a population-based study. Pediatrics. 2003;111(3):483-7.
18. Hoo A-F, Henschen M, Dezateux C, Costeloe K, Stocks J. Respiratory function among preterm infants whose mothers smoked during pregnancy. American journal of respiratory and critical care medicine. 1998;158(3):700-5.
19. Costeloe KL, Hennessy EM, Haider S, Stacey F, Marlow N, Draper ES. Short term outcomes after extreme preterm birth in England: comparison of two birth cohorts in 1995 and 2006 (the EPICure studies). Bmj. 2012;345:e7976.
20. Avery ME, Tooley WH, Keller JB, Hurd SS, Bryan MH, Cotton RB, et al. Is chronic lung disease in low birth weight infants preventable? A survey of eight centers. Pediatrics. 1987;79(1):26-30.
21. Somaschini M, Castiglioni E, Volonteri C, Cursi M, Ferrari M, Carrera P. Genetic predisposing factors to bronchopulmonary dysplasia: preliminary data from a multicentre study. The Journal of Maternal-Fetal & Neonatal Medicine. 2012;25(sup4):119-22.
22. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. Am J Respir Crit Care Med. 2001;163(7):1723-9.
23. Saugstad OD, editor Bronchopulmonary dysplasia—oxidative stress and antioxidants. Seminars in Neonatology; 2003: Elsevier.

24. Schmalisch G, Wilitzki S, Roehr CC, Proquitté H, Bührer C. Differential effects of immaturity and neonatal lung disease on the lung function of very low birth weight infants at 48–52 postconceptional weeks. Pediatric pulmonology. 2013;48(12):1214-23.
25. Hislop AA, Wigglesworth JS, Desai R, Aber V. The effects of preterm delivery and mechanical ventilation on human lung growth. Early Human Development. 1987;15(3):147-64.
26. Schmalisch G, Wilitzki S, Fischer HS, Bührer C. Effect of intubation and mechanical ventilation on exhaled nitric oxide in preterm infants with and without bronchopulmonary dysplasia measured at a median postmenstrual age of 49 weeks. BMC research notes. 2014;7(1):389.
27. Dennery PA. Role of redox in fetal development and neonatal diseases. Antioxidants and Redox Signaling. 2004;6(1):147-53.
28. Gerber C, Bruchelt G, Stegmann H, Schweinsberg F, Speer CP. Presence of bleomycin-detectable free iron in the alveolar system of preterm infants. Biochemical and biophysical research communications. 1999;257(1):218-22.
29. Schock BC, Sweet DG, Halliday HL, Young IS, Ennis M. Oxidative stress in lavage fluid of preterm infants at risk of chronic lung disease. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2001;281(6):L1386-L91.
30. Clerch LB, Wright AE, Coalson JJ. Lung manganese superoxide dismutase protein expression increases in the baboon model of bronchopulmonary dysplasia and is regulated at a posttranscriptional level. Pediatric research. 1996;39(2):253-8.
31. Speer C. Pulmonary inflammation and bronchopulmonary dysplasia. Journal of Perinatology. 2006;26(S1):S57.
32. Perrone S, Tataranno ML, Buonocore G. Oxidative stress and bronchopulmonary dysplasia. Journal of clinical neonatology. 2012;1(3):109.
33. Speer CP, Groneck P, editors. Oxygen radicals, cytokines, adhesion molecules and lung injury in neonates. Seminars in Neonatology; 1998: Elsevier.
34. Kotecha SJ, Edwards MO, Watkins WJ, Henderson AJ, Paranjothy S, Dunstan FD, et al. Effect of preterm birth on later FEV1: a systematic review and meta-analysis.(forced expiratory volume)(Report). Thorax. 2013;68(8):760.
35. Husain AN, Siddiqui NH, Stocker JT. Pathology of arrested acinar development in postsurfactant bronchopulmonary dysplasia. Human Pathology. 1998;29(7):710-7.
36. Coalson JJ, Winter VT, Siler-Khodr T, Yoder BA. Neonatal chronic lung disease in extremely immature baboons. American journal of respiratory and critical care medicine. 1999;160(4):1333-46.
37. Warner BB, Stuart LA, Papes RA, Wispé JR. Functional and pathological effects of prolonged hyperoxia in neonatal mice. American Journal of Physiology-Lung Cellular and Molecular Physiology. 1998;275(1):L110-L7.
38. Yee M, Chess PR, McGrath-Morrow SA, Wang Z, Gelein R, Zhou R, et al. Neonatal oxygen adversely affects lung function in adult mice without altering surfactant composition or activity. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2009;297(4):L641-L9.
39. Yee M, White RJ, Awad HA, Bates WA, McGrath-Morrow SA, O'Reilly MA. Neonatal hyperoxia causes pulmonary vascular disease and shortens life span in aging mice. The American journal of pathology. 2011;178(6):2601-10.
40. Tonson La Tour A, Spadola L, Sayegh Y, Combescure C, Pfister R, Barazzone Argiroffo C, et al. Chest CT in bronchopulmonary dysplasia: Clinical and radiological correlations. Pediatric Pulmonology. 2013;48(7):693-8.
41. Mahut B, De Blic J, Emond S, Benoist M-R, Jarreau P-H, Lacaze-Masmonteil T, et al. Chest computed tomography findings in bronchopulmonary dysplasia and correlation with lung function. Archives of Disease in Childhood - Fetal and Neonatal Edition. 2007;92(6):F459.
42. Broström EB, Thunqvist P, Adenfelt G, Borling E, Katz-Salamon M. Obstructive lung disease in children with mild to severe BPD. Respir Med. 2010;104(3):362-70.
43. Aukland SM, Halvorsen T, Fosse KR, Daltveit AK, Rosendahl K. High-resolution CT of the chest in children and young adults who were born prematurely: findings in a population-based study. AJR American journal of roentgenology. 2006;187(4):1012.
44. Aukland SM, Rosendahl K, Owens CM, Fosse KR, Eide GE, Halvorsen T. Neonatal bronchopulmonary dysplasia predicts abnormal pulmonary HRCT scans in long-term survivors of extreme preterm birth. Thorax. 2009;64(5):405.

45. Simpson SJ, Logie KM, O'Dea CA, Banton GL, Murray C, Wilson AC, et al. Altered lung structure and function in mid-childhood survivors of very preterm birth. Thorax. 2017.
46. Howling SJ, Northway WH, Hansell DM, Moss RB, Ward S, Müller NL. Pulmonary sequelae of bronchopulmonary dysplasia survivors: high-resolution CT findings. AJR American journal of roentgenology. 2000;174(5):1323.
47. Wong PM, Lees AN, Louw J, Lee FY, French N, Gain K, et al. Emphysema in young adult survivors of moderate-to-severe bronchopulmonary dysplasia. The European respiratory journal. 2008;32(2):321.
48. Liu N, Cummings OW, Lagstein A, Hage CA, Chan KM, Zhang C. Lung transplantation for bronchopulmonary dysplasia in adults: a clinical and pathologic study of 3 cases. The American journal of surgical pathology. 2020;44(4):509-15.
49. Narayanan M, Beardsmore CS, Owers-Bradley J, Dogaru CM, Mada M, Ball I, et al. Catch-up alveolarization in ex-preterm children. evidence from 3He magnetic resonance. American journal of respiratory and critical care medicine. 2013;187(10):1104-9.
50. Jobe AH, Bancalari E. An All-Inclusive Perspective on Bronchopulmonary Dysplasia. The Journal of Pediatrics. 2021.
51. Pramana IA, Latzin P, Schlapbach LJ, Hafen G, Kuehni CE, Nelle M, et al. Respiratory symptoms in preterm infants: burden of disease in the first year of life.(Clinical report). 2011;16(197).
52. Greenough A, Alexander J, Burgess S, Chetcuti P, Cox S, Lenney W, et al. Home oxygen status and rehospitalisation and primary care requirements of infants with chronic lung disease. Archives of disease in childhood. 2002;86(1):40-3.
53. Been JV, Lugtenberg MJ, Smets E, van Schayck CP, Kramer BW, Mommers M, et al. Preterm birth and childhood wheezing disorders: a systematic review and meta-analysis. PLoS Med. 2014;11(1):e1001596.
54. Simpson SJ, Hall GL, Wilson AC. Lung function following very preterm birth in the era of 'new' bronchopulmonary dysplasia. Respirology. 2015;20(4):535-40.
55. Fawke J, Lum S, Kirkby J, Hennessy E, Marlow N, Rowell V, et al. Lung function and respiratory symptoms at 11 years in children born extremely preterm: the EPICure study. American journal of respiratory and critical care medicine. 2010;182(2):237-45.
56. Korhonen P, Laitinen J, Hyoudynmaa E, Tammela O. Respiratory outcome in school-aged, very-low-birth-weight children in the surfactant era. Acta Pædiatrica. 2004;93(3):316-21.
57. Thunqvist P, Gustafsson PM, Schultz ES, Bellander T, Berggren-Broström E, Norman M, et al. Lung Function at 8 and 16 Years After Moderate-to-Late Preterm Birth: A Prospective Cohort Study. Pediatrics. 2016;137(4).
58. Landry J, Tremblay G, Li P, Wong C, Benedetti A, Taivassalo T. Lung Function and Bronchial Hyperresponsiveness in Adults Born Prematurely A Cohort Study. Annals of the American Thoracic Society. 2016;13(1):17-24.
59. Gough A, Linden M, Spence D, Patterson CC, Halliday HL, McGarvey LPA. Impaired lung function and health status in adult survivors of bronchopulmonary dysplasia. The European respiratory journal. 2014;43(3):808.
60. van Katwyk S, Augustine S, Thébaud B, Thavorn K. Lifetime patient outcomes and healthcare utilization for Bronchopulmonary dysplasia (BPD) and extreme preterm infants: a microsimulation study. BMC pediatrics. 2020;20(1):1-10.
61. Hennessy EM, Bracewell M, Wood N, Wolke D, Costeloe K, Gibson A, et al. Respiratory health in pre-school and school age children following extremely preterm birth. Archives of disease in childhood. 2008;93(12):1037-43.
62. Simpson SJ, Turkovic L, Wilson A, Verheggen M, Logie KM, Pillow JJ, et al. Lung function trajectories throughout childhood in survivors of very preterm birth: A longitudinal cohort study. The Lancet Child and Adolescent Health. 2018.
63. Kotecha SJ, Watkins WJ, Paranjothy S, Dunstan FD, Henderson AJ, Kotecha S. Effect of late preterm birth on longitudinal lung spirometry in school age children and adolescents. Thorax. 2012;67(1):54.
64. Filippone M, Sartor M, Zacchello F, Baraldi E. Flow limitation in infants with bronchopulmonary dysplasia and respiratory function at school age. The Lancet. 2003;361(9359):753-4.

65. Doyle LW, Adams A-M, Robertson C, Ranganathan S, Davis NM, Lee KJ, et al. Increasing airway obstruction from 8 to 18 years in extremely preterm/low-birthweight survivors born in the surfactant era. Thorax. 2017;72(8):712.
66. Vollsaeter M, Roksund OD, Eide GE, Markestad T, Halvorsen T. Lung function after preterm birth: development from mid-childhood to adulthood.(Report). Thorax. 2013;68(8):767.
67. Vollsæter M, Clemm H, Satrell E, Eide G, Røksund O, Markestad T, et al. Adult Respiratory Outcomes of Extreme Preterm Birth: A Regional Cohort Study. Annals of the American Thoracic Society. 2015;12(3):313-22.
68. Fakhoury KF, Sellers C, Smith EOB, Rama JA, Fan LL. Serial measurements of lung function in a cohort of young children with bronchopulmonary dysplasia. Pediatrics. 2010;125(6):e1441.
69. Lum S, Kirkby J, Welsh L, Marlow N, Hennessy E, Stocks J. Nature and severity of lung function abnormalities in extremely pre-term children at 11 years of age. European Respiratory Journal. 2011;37(5):1199-207.
70. Filbrun AG, Popova AP, Linn MJ, McIntosh NA, Hershenson MB. Longitudinal measures of lung function in infants with bronchopulmonary dysplasia. Pediatric pulmonology. 2011;46(4):369.
71. Greenough A, Dimitriou G, Bhat RY, Broughton S, Hannam S, Rafferty GF, et al. Lung volumes in infants who had mild to moderate bronchopulmonary dysplasia. European journal of pediatrics. 2005;164(9):583-6.
72. Hjalmarson O, Sandberg KL. Lung function at term reflects severity of bronchopulmonary dysplasia. The Journal of pediatrics. 2005;146(1):86-90.
73. Vrijlandt EJLE, Boezen HM, Gerritsen J, Stremmelaar EF, Duiverman EJ. Respiratory Health in Prematurely Born Preschool Children with and without Bronchopulmonary Dysplasia. The Journal of Pediatrics. 2007;150(3):256-61.
74. Thunqvist P, Gustafsson P, Norman M, Wickman M, Hallberg J. Lung function at 6 and 18 months after preterm birth in relation to severity of bronchopulmonary dysplasia. Pediatric pulmonology. 2015;50(10):978-86.
75. Udomittipong K, Sly PD, Patterson HJ, Gangell CL, Stick SM, Hall GL. Forced oscillations in the clinical setting in young children with neonatal lung disease. The European respiratory journal. 2008;31(6):1292.
76. Hülskamp G, Pillow JJ, Dinger J, Stocks J. Lung function tests in neonates and infants with chronic lung disease of infancy: functional residual capacity. Pediatric pulmonology. 2006;41(1):1-22.
77. Latzin P, Roth S, Thamrin C, Hutten GJ, Pramana I, Kuehni CE, et al. Lung volume, breathing pattern and ventilation inhomogeneity in preterm and term infants. PloS one. 2009;4(2):e4635.
78. Yammine S, Schmidt A, Sutter O, Fouzas S, Singer F, Frey U, et al. Functional evidence for continued alveolarisation in former preterms at school age? The European respiratory journal. 2016;47(1):147.
79. Satrell E, Røksund O, Thorsen E, Halvorsen T. Pulmonary gas transfer in children and adolescents born extremely preterm. The European respiratory journal. 2013;42(6):1536.
80. Vom Hove M, Prenzel F, Uhlig HH, Robel-Tillig E. Pulmonary Outcome in Former Preterm, Very Low Birth Weight Children with Bronchopulmonary Dysplasia: A Case-Control Follow-Up at School Age. The Journal of Pediatrics. 2014;164(1):40-5.e4.
81. Filippone M, Bonetto G, Cherubin E, Carraro S, Baraldi E. Childhood course of lung function in survivors of bronchopulmonary dysplasia. Jama. 2009;302(13):1418-20.
82. Narang I, Rosenthal M, Cremonesini D, Silverman M, Bush A. Longitudinal Evaluation of Airway Function 21 Years after Preterm Birth. American Journal of Respiratory and Critical Care Medicine. 2008;178(1):74-80.
83. Chambers DC. Lung Function in Ex-preterm Adults. American journal of respiratory and critical care medicine. 2009;179(6):517-.
84. Gibson AM, Reddington C, McBride L, Callanan C, Robertson C, Doyle LW. Lung function in adult survivors of very low birth weight, with and without bronchopulmonary dysplasia. Pediatric Pulmonology. 2015;50(10):987-94.
85. Saarenpaa H-K, Tikanmaki M, Sipola-Leppanen M, Hovi P, Wehkalampi K, Siltanen M, et al. Lung function in very low birth weight adults.(Report). 2015;136(4).
86. Martinez FD. Early-life origins of chronic obstructive pulmonary disease. New England Journal of Medicine. 2016;375(9):871-8.

87. Doyle LW, Andersson S, Bush A, Cheong JL, Clemm H, Evensen KAI, et al. Expiratory airflow in late adolescence and early adulthood in individuals born very preterm or with very low birthweight compared with controls born at term or with normal birthweight: a meta-analysis of individual participant data. The Lancet respiratory medicine. 2019;7(8):677-86.
88. Crump C. Preterm birth and mortality in adulthood: a systematic review. Journal of Perinatology. 2020;40(6):833-43.
89. Crump C, Sundquist J, Winkleby MA, Sundquist K. Gestational age at birth and mortality from infancy into mid-adulthood: a national cohort study. The Lancet Child & Adolescent Health. 2019;3(6):408-17.
90. Lavoie PM, Dubé M-P. Genetics of bronchopulmonary dysplasia in the age of genomics. Current opinion in pediatrics. 2010;22(2):134.
91. Siezen CL, Bont L, Hodemaekers HM, Ermers MJ, Doornbos G, Van't Slot R, et al. Genetic susceptibility to respiratory syncytial virus bronchiolitis in preterm children is associated with airway remodeling genes and innate immune genes. The Pediatric infectious disease journal. 2009;28(4):333-5.
92. Melville JM, Moss TJ. The immune consequences of preterm birth. Frontiers in neuroscience. 2013;7.
93. Collado MC, Cernada M, Neu J, Pérez-Martínez G, Gormaz M, Vento M. Factors influencing gastrointestinal tract and microbiota immune interaction in preterm infants. Pediatric research. 2015;77(6):726-31.
94. Gentle SJ, Lal CV. Predicting BPD: lessons learned from the airway microbiome of preterm infants. Frontiers in pediatrics. 2020;7:564.
95. Rofael SA, McHugh TD, Troughton R, Beckmann J, Spratt D, Marlow N, et al. Airway microbiome in adult survivors of extremely preterm birth: the EPICure study. European Respiratory Journal. 2019;53(1).
96. Matías V, San Feliciano L, Fernández JE, Lapeña S, Garrido E, Ardura J, et al. Host and environmental factors influencing respiratory secretion of pro-wheezing biomarkers in preterm children. Pediatric Allergy and Immunology. 2012;23(5):441-7.
97. Korhonen PH, Suursalmi PH, Kopeli T, Nieminen R, Lehtimaki L, Luukkaala T, et al. Inflammatory activity at school age in very low birth weight bronchopulmonary dysplasia survivors. Pediatr Pulmonol. 2015;50(7):683-90.
98. Teig N, Allali M, Rieger C, Hamelmann E. Inflammatory markers in induced sputum of school children born before 32 completed weeks of gestation. J Pediatr. 2012;161(6):1085-90.
99. Halvorsen T, Skadberg BT, Eide GE, Roksund O, Aksnes L, Oymar K. Characteristics of asthma and airway hyper-responsiveness after premature birth. Pediatr Allergy Immunol. 2005;16(6):487-94.
100. MacNee W, Tuder RM. New paradigms in the pathogenesis of chronic obstructive pulmonary disease I. Proceedings of the American Thoracic Society. 2009;6(6):527-31.
101. Filippone M, Bonetto G, Corradi M, Frigo AC, Baraldi E. Evidence of unexpected oxidative stress in airways of adolescents born very pre-term. The European respiratory journal. 2012;40(5):1253-9.
102. Carraro S, Giordano G, Stocchero M, Baraldi E. Exhaled breath condensate: metabolomics. Eur Respir Mon. 2010;49:231-6.
103. Piersigilli F, Bhandari V. Metabolomics of bronchopulmonary dysplasia. Clinica Chimica Acta. 2020;500:109-14.
104. Piersigilli F, Bhandari V. Biomarkers in neonatology: the new "omics" of bronchopulmonary dysplasia. The Journal of Maternal-Fetal & Neonatal Medicine. 2016;29(11):1758-64.
105. Piersigilli F, Lam TT, Vernocchi P, Quagliariello A, Putignani L, Aghai ZH, et al. Identification of new biomarkers of bronchopulmonary dysplasia using metabolomics. Metabolomics. 2019;15(2):20.
106. Pintus MC, Lussu M, Dessì A, Pintus R, Noto A, Masile V, et al. Urinary 1H-NMR metabolomics in the first week of life can anticipate bpd diagnosis. Oxidative medicine and cellular longevity. 2018;2018.
107. Fanos V, Cristina Pintus M, Lussu M, Atzori L, Noto A, Stronati M, et al. Urinary metabolomics of bronchopulmonary dysplasia (BPD): preliminary data at birth suggest it is a congenital disease. The Journal of Maternal-Fetal & Neonatal Medicine. 2014;27(sup2):39-45.

108. Carraro S, Giordano G, Pirillo P, Maretti M, Reniero F, Cogo PE, et al. Airway metabolic anomalies in adolescents with bronchopulmonary dysplasia: new insights from the metabolomic approach. J Pediatr. 2015;166(2):234-9 e1.
109. Evans CR, Karnovsky A, Kovach MA, Standiford TJ, Burant CF, Stringer KA. Untargeted LC–MS metabolomics of bronchoalveolar lavage fluid differentiates acute respiratory distress syndrome from health. Journal of proteome research. 2014;13(2):640-9.
110. Adamko DJ, Nair P, Mayers I, Tsuyuki RT, Regush S, Rowe BH. Metabolomic profiling of asthma and chronic obstructive pulmonary disease: a pilot study differentiating diseases. Journal of Allergy and Clinical Immunology. 2015;136(3):571-80. e3.
111. Reinke SN, Gallart-Ayala H, Gómez C, Checa A, Fauland A, Naz S, et al. Metabolomics analysis identifies different metabotypes of asthma severity. European Respiratory Journal. 2017;49(3).
112. Kelly RS, Dahlin A, McGeachie MJ, Qiu W, Sordillo J, Wan ES, et al. Asthma metabolomics and the potential for integrative omics in research and the clinic. Chest. 2017;151(2):262-77.
113. Mattarucchi E, Baraldi E, Guillou C. Metabolomics applied to urine samples in childhood asthma; differentiation between asthma phenotypes and identification of relevant metabolites. Biomedical Chromatography. 2012;26(1):89-94.
114. Urs R, Stoecklin B, Pillow JJ, Hartmann B, Hall GL, Simpson SJ. Collecting exhaled breath condensate from non-ventilated preterm-born infants: a modified method. Pediatric Research. 2021:1-3.
115. Singer F, Kieninger E, Abbas C, Yammine S, Fuchs O, Proietti E, et al. Practicability of nitrogen multiple-breath washout measurements in a pediatric cystic fibrosis outpatient setting. Pediatric pulmonology. 2013;48(8):739-46.
116. Quine D, Wong CM, Boyle EM, Jones JG, Stenson BJ. Non-invasive measurement of reduced ventilation: perfusion ratio and shunt in infants with bronchopulmonary dysplasia: a physiological definition of the disease. Archives of Disease in Childhood-Fetal and Neonatal Edition. 2006;91(6):F409-F14.
117. Asher M, Keil U, Anderson H, Beasley R, Crane J, Martinez F, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. European respiratory journal. 1995;8(3):483-91.
118. Menzies-Gow A, Mansur AH, Brightling CE. Clinical utility of fractional exhaled nitric oxide in severe asthma management. European Respiratory Journal. 2020;55(3).
119. Dweik RA, Boggs PB, Erzurum SC, Irvin CG, Leigh MW, Lundberg JO, et al. An official ATS clinical practice guideline: interpretation of exhaled nitric oxide levels (FENO) for clinical applications. American journal of respiratory and critical care medicine. 2011;184(5):602-15.
120. King GG, Bates J, Berger KI, Calverley P, de Melo PL, Dellacà RL, et al. Technical standards for respiratory oscillometry. European Respiratory Journal. 2020;55(2).
121. Calogero C, Simpson SJ, Lombardi E, Parri N, Cuomo B, Palumbo M, et al. Respiratory impedance and bronchodilator responsiveness in healthy children aged 2–13 years. Pediatric pulmonology. 2013;48(7):707-15.
122. Oostveen E, Boda K, van der Grinten CP, James AL, Young S, Nieland H, et al. Respiratory impedance in healthy subjects: baseline values and bronchodilator response. European Respiratory Journal. 2013;42(6):1513-23.
123. Czövek D, Shackleton C, Hantos Z, Taylor K, Kumar A, Chacko A, et al. Tidal changes in respiratory resistance are sensitive indicators of airway obstruction in children. Thorax. 2016;71(10):907-15.
124. Graham BL, Steenbruggen I, Miller MR, Barjaktarevic IZ, Cooper BG, Hall GL, et al. Standardization of spirometry 2019 update. An official American thoracic Society and European respiratory Society technical statement. American journal of respiratory and critical care medicine. 2019;200(8):e70-e88.
125. Stanojevic S, Cole T, Baur X, Hall G, Culver B, Enright P. Multi-ethnic reference values for spirometry for the 3-95 year age range: The global lung function 2012 equations. The European respiratory journal. 2012;40(6):1324-43.
126. Pellegrino R, Viegi G, Brusasco V, Crapo R, Burgos F, Casaburi R, et al. Interpretative strategies for lung function tests. European respiratory journal. 2005;26(5):948-68.

127. Broadhurst D, Goodacre R, Reinke SN, Kuligowski J, Wilson ID, Lewis MR, et al. Guidelines and considerations for the use of system suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies. Metabolomics. 2018;14(6):1-17.
128. Mendez KM, Pritchard L, Reinke SN, Broadhurst DI. Toward collaborative open data science in metabolomics using Jupyter Notebooks and cloud computing. Metabolomics. 2019;15(10):1-16.
129. Kononikhin A, Starodubtseva N, Chagovets V, Ryndin A, Burov A, Popov I, et al. Exhaled breath condensate analysis from intubated newborns by nano-HPLC coupled to high resolution MS. Journal of Chromatography B. 2017;1047:97-105.
130. Rosso MI, Roark S, Taylor E, Ping X, Ward JM, Roche K, et al. Exhaled breath condensate in intubated neonates-a window into the lung's glutathione status. Respiratory research. 2014;15(1):1-9.
131. Horváth I, Barnes PJ, Loukides S, Sterk PJ, Högman M, Olin A-C, et al. A European Respiratory Society technical standard: exhaled biomarkers in lung disease. European Respiratory Journal. 2017;49(4):1600965.
132. Muller W, Morini F, Eaton S, Peters M, Jaffe A. Safety and feasibility of exhaled breath condensate collection in ventilated infants and children. European Respiratory Journal. 2006;28(3):479-85.
133. Moeller A, Franklin P, Hall GL, Horak Jr F, Wildhaber JH, Stick SM. Measuring exhaled breath condensates in infants. Pediatric pulmonology. 2006;41(2):184-7.
134. Frey U, Stocks J, Coates A, Sly PD, Bates J. Specifications for equipment used for infant pulmonary function testing. The European respiratory journal. 2000;16:731-40.
135. Schulzke S, Deeptha K, Sinhal S, Baldwin D, Pillow J. Nasal versus face mask for multiple-breath washout technique in preterm infants. Pediatric pulmonology. 2008;43(9):858-65.
136. Horváth I, Hunt J, Barnes PJ. Exhaled breath condensate: methodological recommendations and unresolved questions. European Respiratory Journal. 2005;26(3):523-48.
137. Carpagnano GE, Barnes PJ, Geddes DM, Hodson ME, Kharitonov SA. Increased leukotriene B4 and interleukin-6 in exhaled breath condensate in cystic fibrosis. American journal of respiratory and critical care medicine. 2003;167(8):1109-12.
138. Patel K, Davis SD, Johnson R, Esther Jr CR. Exhaled breath condensate purines correlate with lung function in infants and preschoolers. Pediatric pulmonology. 2013;48(2):182-7.
139. Yen E, Weinberger B, Laumbach R, Ohman-Strickland P, Vetrano A, Gow A, et al. Exhaled breath condensate nitrite in premature infants with bronchopulmonary dysplasia. Journal of neonatal-perinatal medicine. 2018;11(4):399-407.
140. Goldoni M, Caglieri A, Andreoli R, Poli D, Manini P, Vettori MV, et al. Influence of condensation temperature on selected exhaled breath parameters. BMC Pulmonary Medicine. 2005;5(1):1-9.
141. Ferraro V, Carraro S, Bozzetto S, Zanconato S, Baraldi E. Exhaled biomarkers in childhood asthma: old and new approaches. Asthma research and practice. 2018;4(1):1-7.
142. Course CW, Kotecha S, Kotecha SJ. Fractional exhaled nitric oxide in preterm-born subjects: A systematic review and meta-analysis. Pediatric pulmonology. 2019;54(5):595-601.
143. Magnussen C, Ojeda FM, Rzayeva N, Zeller T, Sinning CR, Pfeiffer N, et al. FEV1 and FVC predict all-cause mortality independent of cardiac function—Results from the population-based Gutenberg Health Study. International journal of cardiology. 2017;234:64-8.
144. Shackleton C. Improving the clinical utility of the forced oscillation technique in preschool-aged children. 2018.
145. Quanjer PH, Stanojevic S, Cole TJ, Baur X, Hall GL, Culver BH, et al. Multi-ethnic reference values for spirometry for the 3-95-yr age range: the global lung function 2012 equations. The European respiratory journal. 2012;40(6):1324-43.
146. Gough A, Spence D, Linden M, Halliday HL, McGarvey LPA. General and Respiratory Health Outcomes in Adult Survivors of Bronchopulmonary Dysplasia: A Systematic Review: A Systematic Review. Chest. 2012;141(6):1554-67.
147. Simpson SJ, Champion Z, Hall GL, French N, Reynolds V. Upper airway pathology contributes to respiratory symptoms in children born very preterm. The Journal of Pediatrics. 2019;213:46-51.
148. Abu-Hasan M, Tannous B, Weinberger M. Exercise-induced dyspnea in children and adolescents: if not asthma then what? Annals of allergy, asthma & immunology. 2005;94(3):366-71.

149. Reynolds V, Meldrum S, Simmer K, Vijayasekaran S, French N. Laryngeal pathology at school age following very preterm birth. International Journal of Pediatric Otorhinolaryngology. 2015;79(3):398-404.
150. O'Dea CA, Logie K, Wilson AC, Pillow JJ, Murray C, Banton G, et al. Lung abnormalities do not influence aerobic capacity in school children born preterm. European Journal of Applied Physiology. 2021;121(2):489-98.
151. Duke JW, Lovering AT. Respiratory and cardiopulmonary limitations to aerobic exercise capacity in adults born preterm. Journal of Applied Physiology. 2020;129(4):718-24.
152. Welsh L, Kirkby J, Lum S, Odendaal D, Marlow N, Derrick G, et al. The EPICure study: maximal exercise and physical activity in school children born extremely preterm. Thorax. 2010;65(2):165-72.
153. MacLean J, DeHaan K, Fuhr D, Hariharan S, Kamstra B, Hendson L, et al. Altered breathing mechanics and ventilatory response during exercise in children born extremely preterm. Thorax. 2016;71(11):1012-9.
154. Smith LJ, van Asperen PP, McKay KO, Selvadurai H, Fitzgerald DA. Reduced exercise capacity in children born very preterm. Pediatrics. 2008;122(2):e287-e93.
155. Clemm H, Røksund O, Thorsen E, Eide GE, Markestad T, Halvorsen T. Aerobic capacity and exercise performance in young people born extremely preterm. Pediatrics. 2012;129(1):e97-e105.
156. Kriemler S, Keller H, Saigal S, Bar-Or O. Aerobic and lung performance in premature children with and without chronic lung disease of prematurity. Clinical journal of sport medicine. 2005;15(5):349-55.
157. Lombardi E, Fainardi V, Calogero C, Puglia M, Voller F, Cuttini M, et al. Lung function in a cohort of 5-year-old children born very preterm. Pediatric pulmonology. 2018;53(12):1633-9.
158. Roberts D, Brown J, Medley N, Dalziel SR. Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. Cochrane database of systematic reviews. 2017(3).
159. Yang J, Kingsford RA, Horwood J, Epton MJ, Swanney MP, Stanton J, et al. Lung function of adults born at very low birth weight. Pediatrics. 2020;145(2).
160. Halvorsen T, Torunn Skadberg B, Egil Eide G, Drange Røksund O, Markestad T. Better care of immature infants; has it influenced long-term pulmonary outcome? Acta Paediatrica. 2006;95(5):547-54.
161. Cheong JL, Doyle LW. Long-term pulmonary outcome of preterm infants. The Newborn Lung. 2019:161-70.
162. Jobe AH, Kemp M, Schmidt A, Takahashi T, Newnham J, Milad M. Antenatal corticosteroids: a reappraisal of the drug formulation and dose. Pediatric Research. 2021;89(2):318-25.
163. Jobe AH, Goldenberg RL. Antenatal corticosteroids: an assessment of anticipated benefits and potential risks. American journal of obstetrics and gynecology. 2018;219(1):62-74.
164. Kemp MW, Schmidt AF, Jobe AH, editors. Optimizing antenatal corticosteroid therapy. Seminars in Fetal and Neonatal Medicine; 2019: Elsevier.
165. Malmberg L, Mieskonen S, Pelkonen A, Kari A, Sovijarvi A, Turpeinen M. Lung function measured by the oscillometric method in prematurely born children with chronic lung disease. European Respiratory Journal. 2000;16(4):598-603.
166. Verheggen M, Wilson AC, Pillow JJ, Stick SM, Hall GL. Respiratory function and symptoms in young preterm children in the contemporary era. Pediatr Pulmonol. 2016;51(12):1347-55.
167. Gray DM, Czovek D, McMillan L, Turkovic L, Stadler JA, Vanker A, et al. Intra-breath measures of respiratory mechanics in healthy African infants detect risk of respiratory illness in early life. European Respiratory Journal. 2019;53(2).
168. Lorx A, Czövek D, Gingl Z, Makan G, Radics B, Bartusek D, et al. Airway dynamics in COPD patients by within-breath impedance tracking: effects of continuous positive airway pressure. European Respiratory Journal. 2017;49(2).
169. Riedel T, Kyburz M, Latzin P, Thamrin C, Frey U. Regional and overall ventilation inhomogeneities in preterm and term-born infants. Intensive care medicine. 2009;35(1):144-51.
170. Schulzke SM, Hall GL, Nathan EA, Simmer K, Nolan G, Pillow JJ. Lung volume and ventilation inhomogeneity in preterm infants at 15-18 months corrected age. The Journal of pediatrics. 2010;156(4):542-9. e2.

171. Kotecha SJ, Edwards MO, Watkins WJ, Lowe J, Henderson AJ, Kotecha S. Effect of bronchodilators on forced expiratory volume in 1 s in preterm-born participants aged 5 and over: a systematic review. Neonatology. 2015;107(3):231-40.
172. Thunqvist P, Tufvesson E, Bjermer L, Winberg A, Fellman V, Domellöf M, et al. Lung function after extremely preterm birth—A population-based cohort study (EXPRESS). Pediatric pulmonology. 2018;53(1):64-72.
173. Bui DS, Lodge CJ, Burgess JA, Lowe AJ, Perret J, Bui MQ, et al. Childhood predictors of lung function trajectories and future COPD risk: a prospective cohort study from the first to the sixth decade of life. The lancet Respiratory medicine. 2018;6(7):535-44.
174. Gibbons JT, Wilson AC, Simpson SJ. Predicting lung health trajectories for survivors of preterm birth. Frontiers in Pediatrics. 2020;8:318.
175. Robroeks C, Van De Kant K, Jöbsis Q, Hendriks H, Van Gent R, Wouters E, et al. Exhaled nitric oxide and biomarkers in exhaled breath condensate indicate the presence, severity and control of childhood asthma. Clinical & Experimental Allergy. 2007;37(9):1303-11.
176. Borrill Z, Roy K, Singh D. Exhaled breath condensate biomarkers in COPD. European Respiratory Journal. 2008;32(2):472-86.
177. Groneck P, Gotze-Speer B, Oppermann M, Eiffert H, Speer CP. Association of pulmonary inflammation and increased microvascular permeability during the development of bronchopulmonary dysplasia: a sequential analysis of inflammatory mediators in respiratory fluids of high-risk preterm neonates. Pediatrics. 1994;93(5):712-8.
178. Huang A-X, Lu L-W, Liu W-J, Huang M. Plasma inflammatory cytokine IL-4, IL-8, IL-10, and TNF-α levels correlate with pulmonary function in patients with asthma-chronic obstructive pulmonary disease (COPD) overlap syndrome. Medical science monitor: international medical journal of experimental and clinical research. 2016;22:2800.
179. Chakrabarti S, Patel KD. Matrix metalloproteinase-2 (MMP-2) and MMP-9 in pulmonary pathology. Experimental lung research. 2005;31(6):599-621.
180. Rosias PP, Dompeling E, Hendriks HJ, Heijnens JW, Donckerwolcke RA, Jöbsis Q. Exhaled breath condensate in children: pearls and pitfalls. Pediatric allergy and immunology. 2004;15(1):4-19.
181. Davis MD, Montpetit A, Hunt J. Exhaled breath condensate—an overview. Immunology and allergy clinics of North America. 2012;32(3):363.
182. Adamko DJ, Bear M, Regush S, Robinson JL. Urine metabolomic profiling of children with respiratory tract infections in the emergency department: a pilot study. BMC infectious diseases. 2016;16(1):1-11.
183. Rabinovitch N, Zhang L, Gelfand EW. Urine leukotriene E4 levels are associated with decreased pulmonary function in children with persistent airway obstruction. Journal of allergy and clinical immunology. 2006;118(3):635-40.
184. Yokomizo T, Izumi T, Shimizu T. Leukotriene B4: metabolism and signal transduction. Archives of biochemistry and biophysics. 2001;385(2):231-41.
185. van't Erve TJ, Kadiiska MB, London SJ, Mason RP. Classifying oxidative stress by F2-isoprostane levels across human diseases: A meta-analysis. Redox biology. 2017;12:582-99.
186. Kotecha S, Mildner R, Prince L, Vyas J, Currie A, Lawson R, et al. The role of neutrophil apoptosis in the resolution of acute lung injury in newborn infants. Thorax. 2003;58(11):961-7.
187. Oei J, Lui K, Wang H, Henry R. Decreased neutrophil apoptosis in tracheal fluids of preterm infants at risk of chronic lung disease. Archives of Disease in Childhood-Fetal and Neonatal Edition. 2003;88(3):F245-F9.
188. Kallapur S, Jobe A. Contribution of inflammation to lung injury and development. Archives of Disease in Childhood-Fetal and Neonatal Edition. 2006;91(2):F132-F5.
189. Papi A, Bellettato CM, Braccioni F, Romagnoli M, Casolari P, Caramori G, et al. Infections and airway inflammation in chronic obstructive pulmonary disease severe exacerbations. American journal of respiratory and critical care medicine. 2006;173(10):1114-21.
190. Benjamin JT, Plosa EJ, Sucre JM, van der Meer R, Dave S, Gutor S, et al. Neutrophilic inflammation during lung development disrupts elastin assembly and predisposes adult mice to COPD. The Journal of clinical investigation. 2021;131(1).

191. Fischer BM, Pavlisko E, Voynow JA. Pathogenic triad in COPD: oxidative stress, protease–antiprotease imbalance, and inflammation. International journal of chronic obstructive pulmonary disease. 2011;6:413.
192. Esteban-Gorgojo I, Antolín-Amérigo D, Domínguez-Ortega J, Quirce S. Non-eosinophilic asthma: current perspectives. Journal of asthma and allergy. 2018;11:267.
193. Pammi M, Lal CV, Wagner BD, Mourani PM, Lohmann P, Luna RA, et al. Airway microbiome and development of bronchopulmonary dysplasia in preterm infants: a systematic review. The Journal of Pediatrics. 2019;204:126-33. e2.
194. Hunt J. Exhaled breath condensate: an evolving tool for noninvasive evaluation of lung disease. Journal of Allergy and Clinical Immunology. 2002;110(1):28-34.
195. Poggi C, Dani C. Antioxidant strategies and respiratory disease of the preterm newborn: an update. Oxidative medicine and cellular longevity. 2014;2014.
196. Tao JL, Chen YZ, Dai QG, Tian M, Wang SC, Shan JJ, et al. Urine metabolic profiles in paediatric asthma. Respirology. 2019;24(6):572-81.
197. Maskey-Warzęchowska M, Gorska K, Nejman-Gryz P, Paplińska-Goryca M, Grzela T, Krejner A, et al. Matrix metalloproteinase 9 in exhaled breath condensate in patients with stable chronic obstructive pulmonary disease: an observational study. Polish archives of internal medicine. 2018;128(7-8):427-33.
198. Karakoc GB, Yukselen A, Yilmaz M, Altintas DU, Kendirli SG. Exhaled breath condensate MMP-9 level and its relationship wıth asthma severity and interleukin-4/10 levels in children. Annals of Allergy, Asthma & Immunology. 2012;108(5):300-4.
199. Kwiatkowska S, Noweta K, Zieba M, Nowak D, Bialasiewicz P. Enhanced exhalation of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in patients with COPD exacerbation: a prospective study. Respiration. 2012;84(3):231-41.
200. Harada A, Sekido N, Akahoshi T, Wada T, Mukaida N, Matsushima K. Essential involvement of interleukin-8 (IL-8) in acute inflammation. Journal of leukocyte biology. 1994;56(5):559-64.
201. Pease JE, Sabroe I. The role of interleukin-8 and its receptors in inflammatory lung disease. American Journal of Respiratory Medicine. 2002;1(1):19-25.
202. Ko FW, Leung T-F, Wong GW, Ngai J, To KW, Ng S, et al. Measurement of tumor necrosis factor-α, leukotriene B4, and interleukin 8 in the exhaled breath condensate in patients with acute exacerbations of chronic obstructive pulmonary disease. International journal of chronic obstructive pulmonary disease. 2009;4:79.
203. Karrasch S, Linde K, Rücker G, Sommer H, Karsch-Völk M, Kleijnen J, et al. Accuracy of FENO for diagnosing asthma: a systematic review. Thorax. 2017;72(2):109-16.
204. Wood LG, Fitzgerald DA, Gibson PC, Cooper DM, Garg ML. Lipid peroxidation as determined by plasma isoprostanes is related to disease severity in mild asthma. Lipids. 2000;35(9):967-74.
205. Stoecklin B, Simpson SJ, Pillow JJ. Bronchopulmonary dysplasia: rationale for a pathophysiological rather than treatment based approach to diagnosis. Paediatric respiratory reviews. 2019;32:91-7.
206. Nambiar S, Bong How S, Gummer J, Trengove R, Moodley Y. Metabolomics in chronic lung diseases. Respirology. 2020;25(2):139-48.
207. Baraldi E, Giordano G, Stocchero M, Moschino L, Zaramella P, Tran MR, et al. Untargeted metabolomic analysis of amniotic fluid in the prediction of preterm delivery and bronchopulmonary dysplasia. PLoS One. 2016;11(10):e0164211.
208. Villamor-Martinez E, Álvarez-Fuente M, Ghazi AM, Degraeuwe P, Zimmermann LJ, Kramer BW, et al. Association of chorioamnionitis with bronchopulmonary dysplasia among preterm infants: a systematic review, meta-analysis, and metaregression. JAMA network open. 2019;2(11):e1914611-e.
209. Svedenkrans J, Stoecklin B, Jones JG, Doherty DA, Pillow JJ. Physiology and predictors of impaired gas exchange in infants with bronchopulmonary dysplasia. American journal of respiratory and critical care medicine. 2019;200(4):471-80.
210. Fabiano A, Gazzolo D, Zimmermann LJ, Gavilanes AW, Paolillo P, Fanos V, et al. Metabolomic analysis of bronchoalveolar lavage fluid in preterm infants complicated by respiratory distress syndrome: preliminary results. The Journal of Maternal-Fetal & Neonatal Medicine. 2011;24(sup2):55-8.

211. Verder H, Heiring C, Ramanathan R, Scoutaris N, Verder P, Jessen TE, et al. Bronchopulmonary dysplasia predicted at birth by artificial intelligence. Acta Paediatrica. 2020.
212. Burattini I, Bellagamba MP, Biagetti C, Carnielli VP. Amino Acid Intake in Preterm Infants. Protein in Neonatal and Infant Nutrition: Recent Updates. 86: Karger Publishers; 2016. p. 151-60.
213. Contreras MT, Gallardo MJ, Betancourt LR, Rada PV, Ceballos GA, Hernandez LE, et al. Correlation between plasma levels of arginine and citrulline in preterm and full-term neonates: Therapeutical implications. Journal of clinical laboratory analysis. 2017;31(6):e22134.
214. Fike CD, Dikalova A, Kaplowitz MR, Cunningham G, Summar M, Aschner JL. Rescue treatment with L-citrulline inhibits hypoxia-induced pulmonary hypertension in newborn pigs. American journal of respiratory cell and molecular biology. 2015;53(2):255-64.
215. Vadivel A, Aschner JL, Rey-Parra GJ, Magarik J, Zeng H, Summar M, et al. L-citrulline attenuates arrested alveolar growth and pulmonary hypertension in oxygen-induced lung injury in newborn rats. Pediatric research. 2010;68(6):519-25.
216. Smith HA, Canter JA, Christian KG, Drinkwater DC, Scholl FG, Christman BW, et al. Nitric oxide precursors and congenital heart surgery: a randomized controlled trial of oral citrulline. The Journal of thoracic and cardiovascular surgery. 2006;132(1):58-65.
217. Montgomery AM, Bazzy-Asaad A, Asnes JD, Bizzarro MJ, Ehrenkranz RA, Weismann CG. Biochemical screening for pulmonary hypertension in preterm infants with bronchopulmonary dysplasia. Neonatology. 2016;109(3):190-4.
218. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. New England Journal of Medicine. 1993;329(27):2002-12.
219. Oliveira-Paula GH, Lacchini R, Tanus-Santos JE. Clinical and pharmacogenetic impact of endothelial nitric oxide synthase polymorphisms on cardiovascular diseases. Nitric Oxide. 2017;63:39-51.
220. Posod A, Müller S, Komazec IO, Dejaco D, Peglow UP, Griesmaier E, et al. Former very preterm infants show alterations in plasma amino acid profiles at a preschool age. Pediatric research. 2017;81(5):787-94.
221. Pencharz P, House J, Wykes L, Ball R. What are the essential amino acids for the preterm and term infant? Recent Developments in Infant Nutrition: Springer; 1996. p. 278-96.
222. Synderman S, Boyer A, Roitman E, Jr HL, Prose P. The histidine requirement of the infant. Pediatrics. 1963;31:786-801.
223. Huang L, Hogewind-Schoonenboom JE, de Groof F, Twisk JW, Voortman GJ, Dorst K, et al. Lysine requirement of the enterally fed term infant in the first month of life. The American journal of clinical nutrition. 2011;94(6):1496-503.
224. Hao H, Li S, Zhou W, Wang H, Liu M, Shi C, et al. Metabolic products in urine of preterm infants characterized via gas chromatography-mass spectrometry. International journal of clinical and experimental medicine. 2015;8(9):16454.
225. van der Schoor SR, Reeds PJ, Stellaard F, Wattimena J, Sauer P, Büller HA, et al. Lysine kinetics in preterm infants: the importance of enteral feeding. Gut. 2004;53(1):38-43.
226. Carraro S, Giordano G, Pirillo P, Maretti M, Reniero F, Cogo PE, et al. Airway metabolic anomalies in adolescents with bronchopulmonary dysplasia: new insights from the metabolomic approach. The Journal of pediatrics. 2015;166(2):234-9. e1.
227. Lowe J, Watkins WJ, Edwards MO, Spiller OB, Jacqz-Aigrain E, Kotecha SJ, et al. Association between pulmonary ureaplasma colonization and bronchopulmonary dysplasia in preterm infants: updated systematic review and meta-analysis. The Pediatric infectious disease journal. 2014;33(7):697-702.
228. Ntontsi P, Ntzoumanika V, Loukides S, Benaki D, Gkikas E, Mikros E, et al. EBC metabolomics for asthma severity. Journal of breath research. 2020;14(3):036007.
229. McClay JL, Adkins DE, Isern NG, O'Connell TM, Wooten JB, Zedler BK, et al. 1H nuclear magnetic resonance metabolomics analysis identifies novel urinary biomarkers for lung function. Journal of proteome research. 2010;9(6):3083-90.
230. Carrola J, Rocha CM, Barros AS, Gil AM, Goodfellow BJ, Carreira IM, et al. Metabolic signatures of lung cancer in biofluids: NMR-based metabonomics of urine. Journal of proteome research. 2011;10(1):221-30.

231. Duijts L, van Meel ER, Moschino L, Baraldi E, Barnhoorn M, Bramer WM, et al. European Respiratory Society guideline on long-term management of children with bronchopulmonary dysplasia. European respiratory journal. 2020;55(1).
232. Aukland SM, Halvorsen T, Fosse KR, Daltveit AK, Rosendahl K. High-resolution CT of the chest in children and young adults who were born prematurely: findings in a population-based study. American Journal of Roentgenology. 2006;187(4):1012-8.
233. Gibson PG, Yang IA, Upham JW, Reynolds PN, Hodge S, James AL, et al. Effect of azithromycin on asthma exacerbations and quality of life in adults with persistent uncontrolled asthma (AMAZES): a randomised, double-blind, placebo-controlled trial. The Lancet. 2017;390(10095):659-68.
234. Raju TN, Pemberton VL, Saigal S, Blaisdell CJ, Moxey-Mims M, Buist S. Long-term healthcare outcomes of preterm birth: an executive summary of a conference sponsored by the National Institutes of Health. The Journal of pediatrics. 2017;181:309-18. e1.
235. Kamath BD, MacGuire ER, McClure EM, Goldenberg RL, Jobe AH. Neonatal mortality from respiratory distress syndrome: lessons for low-resource countries. Pediatrics. 2011;127(6):1139-46.
236. Doyle LW, Ehrenkranz RA, Halliday HL. Early (< 8 days) postnatal corticosteroids for preventing chronic lung disease in preterm infants. Cochrane Database Syst Rev. 2014(5):CD001146.
237. Bassler D, Plavka R, Shinwell ES, Hallman M, Jarreau PH, Carnielli V, et al. Early Inhaled Budesonide for the Prevention of Bronchopulmonary Dysplasia. N Engl J Med. 2015;373(16):1497-506.
238. Baud O, Maury L, Lebail F, Ramful D, El Moussawi F, Nicaise C, et al. Effect of early low-dose hydrocortisone on survival without bronchopulmonary dysplasia in extremely preterm infants (PREMILOC): a double-blind, placebo-controlled, multicentre, randomised trial. Lancet. 2016;387(10030):1827-36.
239. Committee on F, Newborn. Postnatal corticosteroids to treat or prevent chronic lung disease in preterm infants. Pediatrics. 2002;109(2):330-8.
240. Jobe AH. Postnatal corticosteroids for preterm infants--do what we say, not what we do. N Engl J Med. 2004;350(13):1349-51.
241. Shah VS, Ohlsson A, Halliday HL, Dunn M. Early administration of inhaled corticosteroids for preventing chronic lung disease in very low birth weight preterm neonates. The Cochrane Library. 2017.
242. Bassler D, Shinwell ES, Hallman M, Jarreau P-H, Plavka R, Carnielli V, et al. Long-Term Effects of Inhaled Budesonide for Bronchopulmonary Dysplasia. New England Journal of Medicine. 2018;378(2):148-57.
243. Nair V, Loganathan P, Soraisham AS. Azithromycin and other macrolides for prevention of bronchopulmonary dysplasia: a systematic review and meta-analysis. Neonatology. 2014;106(4):337-47.
244. Madurga A, Mizíková I, Ruiz-Camp J, Morty R. Recent advances in late lung development and the pathogenesis of bronchopulmonary dysplasia. American Journal of Physiology. 2013;305(12):L893.
245. Nixon PA, Washburn LK, Schechter MS, O'Shea TM. Follow-up study of a randomized controlled trial of postnatal dexamethasone therapy in very low birth weight infants: effects on pulmonary outcomes at age 8 to 11 years. The Journal of pediatrics. 2007;150(4):345-50.
246. Chan KN, Silverman M. Increased airway responsiveness in children of low birth weight at school age: effect of topical corticosteroids. Archives of disease in childhood. 1993;69(1):120-4.
247. Pelkonen AS, Hakulinen AL, Hallman M, Turpeinen M. Effect of inhaled budesonide therapy on lung function in schoolchildren born preterm. Respir Med. 2001;95(7):565-70.
248. Yuksel B, Greenough A. Randomised trial of inhaled steroids in preterm infants with respiratory symptoms at follow up. Thorax. 1992;47(11):910-3.
249. Moreno-Macias H, Romieu I. Effects of antioxidant supplements and nutrients on patients with asthma and allergies. Journal of Allergy and Clinical Immunology. 2014;133(5):1237-44.
250. Simpson SJ, Hallberg J. The PELICAN (Prematurity's Effect on the Lungs In Children and Adults Network) ERS Clinical Research Collaboration: understanding the impact of preterm birth on lung health throughout life. Eur Respiratory Soc; 2021.

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Appendix A

AUTHORSHIP ATTRIBUTION STATEMENTS

Peer-reviewed publications arising from this thesis

1. Urs, Rhea, Kotecha, Sailesh, Hall, Graham L. and Simpson, Shannon J. 2018. Persistent and progressive long-term lung disease in survivors of preterm birth. Paediatric Respiratory Reviews 28, pp. 87-94. https://doi.org/10.1016/j.prrv.2018.04.001

AUTHOR CONTRIBUTIONS

R.U. was responsible for literature searching, extracting data from the included articles, and leading discussions regarding interpretation of the data. R.U. was responsible for writing the manuscript, as well as final approval and submission. All co-authors assisted with the conceptualisation and design of the study and were involved in discussions regarding data interpretation. All co-authors were also involved in editing the manuscript and providing final approval before submission.

Co-author signatures:

Professor Sailesh Kotecha	**Professor Graham Hall**	**Dr Shannon Simpson**
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2. Urs, R., Stoecklin, B., Pillow, J.J. *et al*. Collecting exhaled breath condensate from non-ventilated preterm-born infants: a modified method. Pediatr Res (2021). (DOI: 10.1038/s41390-021-01474-x)

AUTHOR CONTRIBUTIONS

R.U. collected and analysed the data, interpreted the data, drafted the initial manuscript, performed literature search, drafted the figures, and approved the final manuscript as submitted. B.S. assisted with collection and analysis of the data, reviewed and revised the manuscript, and approved the final manuscript as submitted. B.H. co-developed the design for the adapted R-Tube and 3D-printed the custom connector, reviewed and revised the manuscript; and approved the final manuscript as submitted. J.J.P. was the principal investigator of the PIFCO study, critically reviewed and revised the manuscript, and approved the final manuscript as submitted. G.H. contributed to the overall study design and reviewed and approved the final manuscript as submitted. S.S. contributed to the overall study design, critically reviewed and revised the manuscript, and approved the final manuscript as submitted.

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Other research outputs within this Thesis

Chapter 5: Impaired respiratory function in survivors of preterm birth using spirometry and oscillometry measures

	Conception and Design	Acquisition of Data and Method	Data Conditioning and Manipulation	Analysis and Statistical Method	Interpretation and Discussion
Rhea Urs		**X**	**X**	**X**	**X**
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Graham Hall	**X**				**X**
Co-Author 2 Acknowledgment: I acknowledge that these represent my contribution to the above research output. Signed:					
Shannon Simpson	**X**				**X**
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Chapter 6: Elevated neutrophilic inflammation and oxidative stress in survivors of preterm birth Impaired respiratory function in survivors of preterm birth using spirometry and oscillometry measures

	Conception and Design	Acquisition of Data and Method	Data Conditioning and Manipulation	Analysis and Statistical Method	Interpretation and Discussion
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Chapter 7: Urinary metabolomics of bronchopulmonary dysplasia in preterm-born neonates at 36 weeks postmenstrual age

	Conception and Design	Acquisition of Data and Method	Data Conditioning and Manipulation	Analysis and Statistical Method	Interpretation and Discussion
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Stacey Reinke	**X**	**X**	**X**	**X**	**X**
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Shannon Simpson	**X**				**X**
Co-Author 7 Acknowledgment: I acknowledge that these represent my contribution to the above research output. Signed:					

Chapter 8: Differences in urinary metabolomic profile between term- and preterm-born children and young-adults

	Conception and Design	Acquisition of Data and Method	Data Conditioning and Manipulation	Analysis and Statistical Method	Interpretation and Discussion
Rhea Urs	**X**	**X**	**X**	**X**	**X**
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David Broadhurst	**X**	**X**	**X**	**X**	
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Appendix B

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Appendix A.2 Copyright notice for Pediatric Research Journal, Springer Nature for Chapter 4 Publication

Collecting exhaled breath condensate from non-ventilated preterm-born infants: a modified method

Author: Rhea Urs *et al.*

Publication: Pediatric Research

Publisher: Springer Nature

Date: Apr 9, 2021

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Appendix C

This is the peer-reviewed but unedited manuscript version of the following article: Urs, Rhea, Kotecha, Sailesh, Hall, Graham L. and Simpson, Shannon J. 2018. Persistent and progressive long-term lung disease in survivors of preterm birth. Paediatric Respiratory Reviews 28, pp. 87-94. 10.1016/j.prrv.2018.04.001. The final, published version is available at https://doi.org/10.1016/j.prrv.2018.04.001

Persistent and progressive long-term lung disease in survivors of preterm birth

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Preterm birth, bronchopulmonary dysplasia, chronic lung disease of prematurity, lung function, pulmonary inflammation

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Educational aims:

The reader will be able to:

- Understand the role of inflammation in the development of chronic lung disease in prematurity
- Understand the known long-term respiratory implications of preterm birth
- Identify possible mechanisms of long-term respiratory disease in survivors of preterm birth
- Identify possible tools to help better characterise and treat chronic lung disease in survivors of preterm birth

Directions for future research:

- Comprehensively characterise respiratory symptoms, lung structure, lung function and lung inflammation beyond the neonatal period in survivors of preterm birth
- Investigate the longitudinal trajectory of respiratory symptoms, lung structure and lung function in individuals who were born preterm
- Investigate targeted treatments for chronic lung disease in those born preterm

List of Abbreviations:

Gestational age (GA), bronchopulmonary dysplasia (BPD), neonatal intensive care unit (NICU), reactive oxygen species (ROS), computed tomography (CT), helium-3 magnetic resonance (3HeMR), inhaled corticosteroids (ICS), forced expiratory volume in 1 second (FEV_1), forced mid-expiratory flow (FEF_{25-75}), residual volume/total lung capacity (RV/TLC), diffusing capacity of the lung for carbon monoxide (DLCO), vascular endothelial growth factor (VEGF), respiratory syncytial virus (RSV), transforming growth factor-beta (TGF-β), chronic obstructive pulmonary disease (COPD)

Summary

Preterm birth accounts for approximately 11 % of births globally, with rates increasing across many countries. Concurrent advances in neonatal care have led to increased survival of infants of lower gestational age (GA). However, infants born <32 weeks GA experience adverse respiratory outcomes, manifesting with increased respiratory symptoms, hospitalisation and health care utilisation into early childhood. The development of bronchopulmonary dysplasia (BPD) – the chronic lung disease of prematurity – further increases the risk of poor respiratory outcomes throughout childhood, into adolescence and adulthood. Indeed, survivors of preterm birth have shown increased respiratory symptoms, altered lung structure, persistent and even declining lung function throughout childhood. The mechanisms behind this persistent and sometimes progressive lung disease are unclear, and the implications place those born preterm at increased risk of respiratory morbidity into adulthood. This review aims to summarise what is known about the long-term pulmonary outcomes of contemporary preterm birth, examine the possible mechanisms of long-term respiratory morbidity in those born preterm and discuss addressing the unknowns and potentials for targeted treatments.

1.1 Preterm birth and chronic lung disease in the NICU

Prematurity is associated with adverse neonatal outcomes, particularly respiratory outcomes as a result of interrupted lung development (1). Very premature infants are particularly vulnerable as they are born during the canalicular (16-26 weeks GA) and saccular (26-36 weeks GA) stages of lung development (2) and consequently forced to exchange gas with an immature lung, with lung development occurring in a relatively hyper-oxic *ex utero* environment compared to the *in utero* milleau (1).

The chronic lung disease of prematurity, bronchopulmonary dysplasia (BPD), was first described over 50 years ago in infants with an average gestational age (GA) of 34 weeks (w) and prolonged exposure to high oxygen concentrations (3). Significant improvements in neonatal critical care – including routine use of surfactant therapy – occurred during the 1990s (4), such that approximately half of babies born at 25 w GA now survive in high-middle income countries (5). Consequently, the clinical and pathological characteristics of prematurity and BPD have changed profoundly. "New" BPD is defined as the requirement for supplemental oxygen for at least 28 days, and is characterised by fewer and larger alveoli, decreased pulmonary vasculature, inflammation and variable smooth muscle hyperplasia (6). Prenatal factors such as in utero inflammation (7), intrauterine growth restriction (8), maternal smoking (9), male sex (10), Caucasian race (11) and genetic factors (12) increase the risk of poor respiratory outcomes and BPD (Figure 1). Lower gestational age (13) and longer mechanical ventilation are associated with supplemental oxygen duration and therefore more severe BPD (13). Postnatal pulmonary inflammation and oxidative stress play a key role in the pathogenesis of BPD (7, 14) and are discussed in more detail in the context of initiating a pathological process that may indeed persist beyond the neonatal intensive care unit (NICU).

1.1.1 Inflammation in the neonatal period

Pro-inflammatory cytokines, adhesion molecules, selectins, and chemokines are found in high levels in infants with BPD and are associated with endothelial interactions leading to decreased vascularisation and simplified alveoli (7). The inflammatory pathway involved in BPD can be initiated in the prenatal period, for example chorioamnionitis (inflammation of the foetal membranes) elicits a pulmonary inflammatory response in the neonate (7). Inflammation is then exacerbated by neonatal events including supplemental oxygen therapy, prolonged mechanical ventilation and pulmonary and systemic infections following preterm birth, which decrease alveolar number, internal lung surface area and in turn, lung function (15-17).

1.1.2 Oxidative stress in the neonatal period

Oxidative stress results from the production of reactive oxygen species (ROS) exceeding the capacity of antioxidant defences (18). Preterm infants are often supplemented with oxygen at high concentrations to ensure adequate tissue oxygenation, which can produce ROS, particularly in the presence of inflammation in the lung (14). Subsequent lung injury is of particular consequence to preterm infants, who have decreased intracellular antioxidant defences, such as reduced levels of superoxide dismutase and catalase, compared to term infants (14). Additionally, many preterm neonates have detectable free iron in their airway surface liquid, which is associated with increased risk of oxidative injury (19) due to the production of toxic hydroxyl radicals (14). Several studies observe associations between high levels of oxidative stress markers – including o-tyrosine, super-oxide proteinases, uric acid, ascorbic acid, surfactant lipid peroxidation and hydroxyl radicals – with prematurity and greater risk of BPD (20, 21).

1.1.3 Interplay between inflammation, oxidative stress and lung injury in prematurity:

During pulmonary inflammation, activated neutrophils release ROS, which functionally deactivate protease inhibitors, leading to excess proteases (22), which may contribute to BPD pathogenesis (7). The release of ROS not only favours tissue damage, but increases capillary permeability, facilitating the passage of cytokines and contributing to a further increase in inflammation (23). In this way, inflammation and oxidative stress occur in a complex simultaneous and circular manner. Inflammation-induced oxidative stress is evident in infants with BPD, whose alveolar macrophages produce larger amounts of hydrogen peroxide than controls (24).

In summary, pulmonary inflammation and oxidative stress play a key role in lung injury and BPD pathogenesis. We hypothesise that consequences of these factors may persist beyond the neonatal period and contribute to the poor long-term respiratory outcomes seen in survivors of preterm birth. Certainly, as the oldest survivors of this "New" BPD reach their 20s, it is becoming clear that survivors of preterm birth are at increased risk of significant and ongoing respiratory disease (25).

2.1 Beyond the NICU

2.1.1 Structural abnormalities

Most of the evidence underpinning our current understanding of the developmental and structural abnormalities of the lung in BPD come from histopathology studies of a few fatal cases in infants; with only one case described beyond 3 years of age (26). These studies describe simplified alveoli, decreased vasculature and variable smooth muscle hyperplasia in BPD, and all suggest a delay in alveolar development in survivors of prematurity. Animal models of 'new' BPD describe decreased alveolar septation and hyperplasia (27), enlarged and simplified alveoli and increased pulmonary fibrosis, which worsen over time (28). These are associated with abnormal lung mechanics (29) and shortened life-span (30). These findings suggest that arrested development and insults to the lung in the neonatal period can result in long-term function-limiting structural alterations.

Indeed, computed tomography (CT) scans from preterm infants and children born in the surfactant era show high rates of structural abnormalities with the presence of bronchial wall thickening (suggesting inflammation or post-inflammatory changes), linear and triangular sub-pleural opacities (likely scarring) and decreased pulmonary attenuation with limited amounts of emphysema detected (31, 32). Similar findings of sub-pleural opacities were found in several cross-sectional studies at school age (33-35), with emphysema, bronchial wall thickening and fibrosis also noted (33, 36). More severe structural lung disease is associated with increased BPD severity and poorer lung function outcomes (31, 32, 34, 35). Mixed patterns of reduced lung attenuation, bronchial wall thickening and inverse bronchopulmonary artery diameter ratios have also been observed in preterm-born adults (37), with one study of young adult survivors of severe "old" BPD reporting an 84 % incidence of emphysema, which correlated with lower FEV_1 values (38). As no longitudinal CT imaging studies have been performed, it remains unknown whether the decreased pulmonary attenuation seen during mid-childhood resolves, persists or progresses to the emphysema observed in young adults.

In contrast to the results noted by chest CT, the helium-3 magnetic resonance (3HeMR) imaging technique, which assesses alveolar dimensions and uniformity, has shown normalisation after preterm birth (39). Despite lower FEV_1 values in preterm-born children, alveolar dimensions were not different to term-born children; possibly suggesting catch-up alveolarisation in preterm-born children (39).

2.1.2 Ongoing respiratory morbidity

Beyond the neonatal period, survivors of prematurity experience persistent respiratory symptoms. In the first years of life, preterm-born infants experience more wheeze, use inhaled medications and are re-hospitalised more frequently than their term-born counterparts (40). Approximately 50 % of those

with BPD are re-hospitalised in the first year (41). At school age, preterm-born children are up to 5 times more at risk of wheezing disorders than their term born counterparts (42), and commonly report respiratory symptoms independent of a neonatal diagnosis of BPD (43). Additionally, preterm children are more likely to have exercise-induced respiratory symptoms, be diagnosed with asthma and are twice as likely to be prescribed inhaler medications, including inhaled corticosteroids (ICS), than term born children (44, 45).

The burden of respiratory disease likely persists beyond childhood with adolescents from a large Swedish cohort (born in the late-preterm period) reporting more wheeze than their term counterparts (46). Conversely, young adults born preterm from a small study by Landry *et al*. reported no differences in respiratory symptoms compared to those born at term (47). Adult survivors of 'old' BPD report increased symptoms, with much higher rates of wheeze and asthma medication use than full-term controls (48).

Although cross-sectional studies generally show increased rates of respiratory symptoms in those born preterm compared to those born at term, longitudinal data describing how these symptoms change over time is lacking. In extremely preterm children, prevalence of respiratory symptoms, hospitalisation and medication use is reported to significantly decrease between 2 and 6 years of age, although high rates of chest deformities at 6 years suggest ongoing respiratory morbidity (49). Simpson *et al.* showed that in children born at less than 32 weeks gestation, rates of wheeze, cough and asthma medication use remained consistent between early and mid-childhood (50). As the oldest survivors of prematurity in the post-surfactant era are only now reaching early adulthood, more adequately powered studies are needed to establish the prevalence and characteristics of respiratory symptoms, and the consequences of 'new' BPD throughout life.

2.1.3 Impaired lung function

Respiratory symptoms are often reported in the presence of lung function abnormalities in preterm children and lung function is further decreased in children with BPD (25). Cross-sectional studies report obstructive lung disease throughout childhood and into adulthood with abnormal spirometry parameters in preterm children – namely lower forced expiratory volumes in 1 second (FEV_1), lower forced mid-expiratory flow (FEF_{25-75}) with normal forced vital capacities (33, 36, 43-47, 51-55). Airway obstruction is partially reversible with bronchodilators in about one third of preterm-born infants (56), with studies reporting between 25-60 % of those with BPD responding to bronchodilators at school age (33, 44, 52, 56). However, these studies have not reported whether regular, long-term use of bronchodilators is associated with improved lung function outcomes in this population.

Some studies report airway obstruction in the presence of modest restriction (57, 58) with lower lung volumes in preterm infants with and without BPD (59, 60) and reduced residual volume to total lung capacity ratio (RV/TLC) in preterm children at school-age (45) compared to those born at term. Preterm born children also exhibit altered respiratory mechanics in comparison to term born controls, which is more pronounced in those with BPD, with increased respiratory system resistance and altered elastic properties of the respiratory system (reactance); indicating peripheral lung disease from infancy to at least school-age (33, 36, 44, 61-63).

There is conflicting evidence of ventilation inhomogeneity in preterm born children using multiple breath washout techniques, with one study reporting elevated inhomogeneity (64) in preterm infants compared to controls, with another detecting no difference (65). In the mid-childhood extremely preterm EPICure cohort, slightly elevated ventilation inhomogeneity (lung clearance index) was reported in preterm children compared to those born at term (57) yet no differences were seen in a West Australian cohort of similar age (43), although this cohort included children up to 32 weeks gestational

age. Another recent study found that although lung clearance index was not different, the alveolar phase III of washout revealed elevated ventilation inhomogeneity in the conducting airways (Scond) but not the acinar airways (Sacin) of extremely preterm-born children at school age, suggesting a functionally normal alveolar compartment with impaired function of the proximal airways in comparison to controls (66).

Assessments of gas exchange (DLCO) also provide conflicting results, with some studies suggesting decreased alveolar-capillary membrane function in preterm-born children throughout childhood and adolescence (44, 45, 47, 67), while others fail to detect a difference (36, 68). Those who reported impaired gas exchange assessed cohorts that were born more premature than those who reported no difference, which may account for the inconsistencies between studies. Taken together, it therefore remains unclear whether the alveolar compartment and pulmonary vasculature of preterm-born children develops normally and whether any altered alveolar or pulmonary vascular development is functionally significant in mid-childhood and beyond.

2.1.4 Longitudinal Lung Function

Few studies have examined longitudinal lung function beyond infancy in survivors of preterm birth, and often in small numbers of participants, of the most extreme neonatal course, prior to routine use of exogenous surfactant and in the absence of all-ages reference equations. Consequently, findings are conflicting.

Across childhood, Filippone *et al.* showed poor lung function in 17 BPD survivors, which tracked (but did not change trajectory compared to controls) through 2, 9 and 15 years of age (69). Similarly, no 'catch-up' in lung function from mid-childhood to adolescence was observed in moderate-late preterm-born children with poor lung function (46). Some studies have documented improvements in FEV_1 z-score with individuals born at 33-34 week gestation improving from 8 to 16 years of age (51). Other studies of preterm children of lower gestational age report significant declines in FEV_1 z-scores from childhood to adolescence or young adulthood (70, 71) and more decline in those with BPD or current smoking status (53). Studies into adulthood report both persistently low (54, 55) and declining (72, 73) lung function in those born preterm in the pre-surfactant era, with one study reporting lung function declines of at least 0.1 z-scores per year during childhood in those with BPD (Figure 2.) (50).

Consequently, it remains to be established whether lung function deficits in those born preterm track through life or actually worsens over time, especially those born in the era of "new" BPD. Regardless, low 'peak' lung function (FEV_1) and unknown age-related rate of FEV_1 decline during childhood may put ex-preterm children at risk of early onset chronic lung disease in adulthood and early mortality (74). Additionally, the preterm population are highly susceptible to known predictors of lung function decline, such as increased risk of respiratory infection during early childhood, increased asthma diagnoses and increased airway hyper-responsiveness (74). Those born preterm may also be subject to other mechanisms of persistent or progressive lung disease that are not influenced by exposures, such as altered growth, genetics, immunity and persistent inflammation and oxidative stress, which are described below.

3.1 Possible mechanisms of persistent disease?

3.1.1 Genetics

Although genetics are known to play a role in the susceptibility of developing BPD (12), it is unknown whether these genetic variances influence the persistence of respiratory morbidity beyond the neonatal period. Because of the multi-factorial nature of the disease, no specific genetic predictors of BPD have been identified, although associations have been made between BPD and altered innate and adaptive

immune responses, surfactant metabolism and potential reductions in growth factors like vascular endothelial growth factor (VEGF) (75). Siezen and colleagues reported increased genetic susceptibility to respiratory syncytial virus (RSV) in preterm-born compared to term-born children, with these differences likely manifested in airway remodelling and innate immunity such as altered interferon and transforming growth factor-beta (TGF-β) function and altered response to inflammation (76). This current evidence suggests that genetics may predispose preterm-born individuals to altered immunity, lung repair and lung growth, which can have long-term implications on respiratory health (Figure 3).

3.1.2 Immunity

Normal 'programming' of the immune system occurs in utero and throughout the first year of life (77). Birth prior to term results in incomplete maternal transfer of antibodies (which occurs during the 3rd trimester) to the preterm infant, which may play a role in the increased susceptibility to infection during the first year of life (77). Prenatal exposures like antenatal glucocorticoids, prenatal infections and inflammation contribute to altered immunity in preterm infants, who have deficiencies in antimicrobial peptides, altered cellular responses to infection (77) and may have a different and unstable microbial colonisation (78). These early life influences may result in altered immune programming and long-term immune deficiencies (77), putting survivors of preterm birth at risk of increased incidence and severity of respiratory infection, a known risk factor for accelerated lung function decline in adulthood (74).

3.1.3 Inflammation & Oxidative Stress

Beyond the neonatal period, data to identify and characterise ongoing inflammation and oxidative stress in survivors of preterm birth is limited. Increased levels of chemokines, growth factors, T-helper cytokines (Th-1, Th-2 and Th-17 cytokines) and immunomodulatory mediators have been detected in nasopharyngeal aspirates from preterm infants at 1 year of age (79). One small study reported that inflammatory markers in exhaled breath condensate did not differ between school-aged healthy and preterm-born children, although as only a few subjects were studied this finding may lack statistical power (80). Other studies report increased neutrophilic inflammation, such as a 16-fold increase in sputum neutrophils and a 3-fold increase in sputum IL-8 in 16 children with "old BPD" (81) and increased levels of urinary leukotriene E4 in children born preterm regardless of BPD diagnosis (82). It is clear that inflammation is a common underlying process that affects and is affected by predictors of respiratory morbidity like environmental exposures, immune responses, growth factors and genetics. As such, it is likely that inflammation persists in the preterm lung throughout childhood, and must be considered in light of persisting and possibly declining lung function throughout life.

Markers of oxidative stress are abundant in other chronic lung disorders and are linked to ongoing airway inflammation and remodelling (83), however there are limited data in survivors of preterm birth beyond infancy. Filippone *et al.* reported that adolescents born preterm with and without a history of BPD, had "unexpected" ongoing oxidative stress with increased levels of 8-isoprostane in exhaled breath condensates in the presence of lower lung function when compared to term-born adolescents (84). The underlying mechanisms of this continuing oxidative stress remain unclear, but the implication that oxidative stress persists through to young adulthood suggests that oxidative damage may play a role in ongoing respiratory morbidity.

Given the integral role of inflammatory and oxidative stress pathways in the pathogenesis of BPD – and their key role in similar respiratory diseases like chronic obstructive pulmonary disease (COPD) – it is an important area for future investigation.

4.1 Emerging tools to characterise lung disease

Risk factors for long-term respiratory morbidity are multifactorial and influenced by prenatal, postnatal and childhood events. To examine the complex, multifactorial mechanisms of lung function decline in this vulnerable group more comprehensive, systems biology approaches are needed. 'Omics methods – which include genomics, epigenomics, microbiomics, transcriptomics, proteomics and metabolomics – have been able to distinguish between neonates who go on to develop BPD and those who do not (85). Beyond infancy, however, only one "'omics" investigation has been reported in survivors of preterm birth - a metabolomics study which clearly delineated between the metabolomic profile of exhaled breath condensate in healthy term adolescents and those with BPD (86). This study suggests differing surfactant lipid profiles, despite the small number of adolescents (all with BPD) exhibiting heterogeneous clinical symptoms and inhaled corticosteroid usage (86). Although more adequately powered studies are needed, 'omics tools provide a promising approach to better understanding the mechanisms underlying ongoing lung disease in survivors of preterm birth. Importantly, these methods may help to identify potential therapeutic targets and capture the metabolic response to pharmaceutical interventions.

4.1.1 Potential treatments and interventions

Prevention of long-term lung disease in preterm children has been focussed to intervention in the NICU, with the aim of decreasing lung injury. Corticosteroids have anti-inflammatory properties, with systemic dexamethasone administration in the NICU becoming popular for the prevention of BPD in the 1990s. The respiratory benefits of dexamethasone treatment include decreased ventilator requirements, earlier extubation, improved lung function, decreased lung inflammation, and ultimately improved survival with reduced BPD incidence (87-89). However, adverse long term neurodevelopmental effects and increased risk of gastro-intestinal perforation have led to the early termination of some clinical trials and cautious use of postnatal systemic corticosteroids during the neonatal period after preterm birth (90). Nevertheless, systemic corticosteroids continue to be used especially to aid extubation of chronically ventilator-dependent preterm-born infants (91). Inhaled corticosteroids (ICS) administered in the neonatal period have not shown effectiveness in preventing BPD (92), and although one recent trial showed no association with adverse neurodevelopmental outcome, rates of mortality were higher in neonates who received ICS (93). Azithromycin, which has been shown to reduce disease severity in other inflammatory lung diseases, may be effective in both the short- and long-term in preterm infants but further studies are needed to determine its efficacy in reducing lung injury in preterm infants (94). Other potential anti-inflammatory therapies that have shown improvements in respiratory outcomes in animal models include curcumin and mesenchymal stromal cell therapy, but these are yet to be assessed in humans (95).

The benefits of anti-inflammatory treatment in survivors of preterm birth after the neonatal period have been less well studied: in part because the inflammatory contribution to lung disease in survivors of preterm birth remains poorly described. A follow-up at 8-11 years of very preterm children randomised to receive postnatal dexamethasone or placebo in the newborn period demonstrated long-lasting effects of corticosteroid administration, with fewer children exhibiting abnormal spirometry measures in the treated group (40 %; N=35) than the placebo group (68 %; N=28) (96). Smaller studies of no more than 18 participants each have shown inconsistent results, with no improvements in lung function or symptoms observed in children aged 7-13 years after ICS; however, a decrease in the variability of the peak expiratory flow was observed (97, 98), suggesting a reduction in bronchial reactivity. One study reported significant improvements in symptoms, lung function and reduced bronchodilator usage in ex-preterm infants aged around 10 months who used ICS (99). Although there is evidence that ICS may be of some benefit in the preterm population, its clinical use in this population is not clear and larger studies are needed to answer this question. Interventions targeted towards neutrophilic inflammation or those

that promote lung growth may be more appropriate considering the phenotype of chronic lung disease after preterm birth.

5.1 Conclusion

Survivors of preterm birth face a lifetime of respiratory morbidity, with impaired lung structure and function which may worsen over time. It is becoming increasingly clear that not only do prenatal and neonatal events increase the risk of impaired pulmonary function in preterm-born individuals, but exposures through childhood – such as poor growth, infection, inflammation and oxidative stress - may play a larger role than previously thought. Adequately powered systems biology approaches are needed to discern predictors and targets for preventing chronic lung disease after prematurity, due to its complex and multi-factorial nature.

References

1. Smith LJ, McKay KO, van Asperen PP, Selvadurai H, Fitzgerald DA. Normal development of the lung and premature birth. Paediatric respiratory reviews. 2010;11(3):135-42.

2. Kotecha S. Lung growth: implications for the newborn infant. Archives of Disease in Childhood-Fetal and Neonatal Edition. 2000;82(1):F69-F74.

3. Northway WH, Jr., Rosan RC, Porter DY. Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. N Engl J Med. 1967;276(7):357-68.

4. Kamath BD, Macguire ER, McClure EM, Goldenberg RL, Jobe AH. Neonatal mortality from respiratory distress syndrome: lessons for low-resource countries. Pediatrics. 2011;127(6):1139-46.

5. Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller AB, Narwal R, et al. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. Lancet. 2012;379(9832):2162-72.

6. Jobe AH. The new bronchopulmonary dysplasia. Current opinion in pediatrics. 2011;23(2):167.

7. Speer CP. Inflammation and bronchopulmonary dysplasia: a continuing story. Semin Fetal Neonatal Med. 2006;11(5):354-62.

8. Lal MK, Manktelow BN, Draper ES, Field DJ. Chronic lung disease of prematurity and intrauterine growth retardation: a population-based study. Pediatrics. 2003;111(3):483-7.

9. Hoo A-F, Henschen M, Dezateux C, Costeloe K, Stocks J. Respiratory function among preterm infants whose mothers smoked during pregnancy. American journal of respiratory and critical care medicine. 1998;158(3):700-5.

10. Costeloe KL, Hennessy EM, Haider S, Stacey F, Marlow N, Draper ES. Short term outcomes after extreme preterm birth in England: comparison of two birth cohorts in 1995 and 2006 (the EPICure studies). Bmj. 2012;345:e7976.

11. Avery ME, Tooley WH, Keller JB, Hurd SS, Bryan MH, Cotton RB, et al. Is chronic lung disease in low birth weight infants preventable? A survey of eight centers. Pediatrics. 1987;79(1):26-30.

12. Somaschini M, Castiglioni E, Volonteri C, Cursi M, Ferrari M, Carrera P. Genetic predisposing factors to bronchopulmonary dysplasia: preliminary data from a multicentre study. The Journal of Maternal-Fetal & Neonatal Medicine. 2012;25(sup4):119-22.

13. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. Am J Respir Crit Care Med. 2001;163(7):1723-9.

14. Saugstad OD, editor Bronchopulmonary dysplasia—oxidative stress and antioxidants. Seminars in Neonatology; 2003: Elsevier.

15. Schmalisch G, Wilitzki S, Roehr CC, Proquitté H, Bührer C. Differential effects of immaturity and neonatal lung disease on the lung function of very low birth weight infants at 48–52 postconceptional weeks. Pediatric pulmonology. 2013;48(12):1214-23.

16. Hislop AA, Wigglesworth JS, Desai R, Aber V. The effects of preterm delivery and mechanical ventilation on human lung growth. Early Human Development. 1987;15(3):147-64.

17. Schmalisch G, Wilitzki S, Fischer HS, Bührer C. Effect of intubation and mechanical ventilation on exhaled nitric oxide in preterm infants with and without bronchopulmonary dysplasia measured at a median postmenstrual age of 49 weeks. BMC research notes. 2014;7(1):389.

18. Dennery PA. Role of redox in fetal development and neonatal diseases. Antioxidants and Redox Signaling. 2004;6(1):147-53.

19. Gerber C, Bruchelt G, Stegmann H, Schweinsberg F, Speer CP. Presence of bleomycin-detectable free iron in the alveolar system of preterm infants. Biochemical and biophysical research communications. 1999;257(1):218-22.

20. Schock BC, Sweet DG, Halliday HL, Young IS, Ennis M. Oxidative stress in lavage fluid of preterm infants at risk of chronic lung disease. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2001;281(6):L1386-L91.

21. Clerch LB, Wright AE, Coalson JJ. Lung manganese superoxide dismutase protein expression increases in the baboon model of bronchopulmonary dysplasia and is regulated at a posttranscriptional level. Pediatric research. 1996;39(2):253-8.

22. Speer C. Pulmonary inflammation and bronchopulmonary dysplasia. Journal of Perinatology. 2006;26(S1):S57.

23. Perrone S, Tataranno ML, Buonocore G. Oxidative stress and bronchopulmonary dysplasia. Journal of clinical neonatology. 2012;1(3):109.

24. Speer CP, Groneck P, editors. Oxygen radicals, cytokines, adhesion molecules and lung injury in neonates. Seminars in Neonatology; 1998: Elsevier.

25. Kotecha SJ, Edwards MO, Watkins WJ, Henderson AJ, Paranjothy S, Dunstan FD, et al. Effect of preterm birth on later FEV1: a systematic review and meta-analysis.(forced expiratory volume)(Report). Thorax. 2013;68(8):760.

26. Husain AN, Siddiqui NH, Stocker JT. Pathology of arrested acinar development in postsurfactant bronchopulmonary dysplasia. Human Pathology. 1998;29(7):710-7.

27. Coalson JJ, Winter VT, Siler-Khodr T, Yoder BA. Neonatal chronic lung disease in extremely immature baboons. American journal of respiratory and critical care medicine. 1999;160(4):1333-46.

28. Warner BB, Stuart LA, Papes RA, Wispé JR. Functional and pathological effects of prolonged hyperoxia in neonatal mice. American Journal of Physiology-Lung Cellular and Molecular Physiology. 1998;275(1):L110-L7.

29. Yee M, Chess PR, McGrath-Morrow SA, Wang Z, Gelein R, Zhou R, et al. Neonatal oxygen adversely affects lung function in adult mice without altering surfactant composition or activity. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2009;297(4):L641-L9.

30. Yee M, White RJ, Awad HA, Bates WA, McGrath-Morrow SA, O'Reilly MA. Neonatal hyperoxia causes pulmonary vascular disease and shortens life span in aging mice. The American journal of pathology. 2011;178(6):2601-10.

31. Tonson La Tour A, Spadola L, Sayegh Y, Combescure C, Pfister R, Barazzone Argiroffo C, et al. Chest CT in bronchopulmonary dysplasia: Clinical and radiological correlations. Pediatric Pulmonology. 2013;48(7):693-8.

32. Mahut B, De Blic J, Emond S, Benoist M-R, Jarreau P-H, Lacaze-Masmonteil T, et al. Chest computed tomography findings in bronchopulmonary dysplasia and correlation with lung function. Archives of Disease in Childhood - Fetal and Neonatal Edition. 2007;92(6):F459.

33. Broström EB, Thunqvist P, Adenfelt G, Borling E, Katz-Salamon M. Obstructive lung disease in children with mild to severe BPD. Respir Med. 2010;104(3):362-70.

34. Aukland SM, Halvorsen T, Fosse KR, Daltveit AK, Rosendahl K. High-resolution CT of the chest in children and young adults who were born prematurely: findings in a population-based study. AJR American journal of roentgenology. 2006;187(4):1012.

35. Aukland SM, Rosendahl K, Owens CM, Fosse KR, Eide GE, Halvorsen T. Neonatal bronchopulmonary dysplasia predicts abnormal pulmonary HRCT scans in long-term survivors of extreme preterm birth. Thorax. 2009;64(5):405.

36. Simpson SJ, Logie KM, O'Dea CA, Banton GL, Murray C, Wilson AC, et al. Altered lung structure and function in mid-childhood survivors of very preterm birth. Thorax. 2017.

37. Howling SJ, Northway WH, Hansell DM, Moss RB, Ward S, Müller NL. Pulmonary sequelae of bronchopulmonary dysplasia survivors: high-resolution CT findings. AJR American journal of roentgenology. 2000;174(5):1323.

38. Wong PM, Lees AN, Louw J, Lee FY, French N, Gain K, et al. Emphysema in young adult survivors of moderate-to-severe bronchopulmonary dysplasia. The European respiratory journal. 2008;32(2):321.

39. Narayanan M, Beardsmore CS, Owers-Bradley J, Dogaru CM, Mada M, Ball I, et al. Catch-up alveolarization in ex-preterm children. evidence from 3He magnetic resonance. American journal of respiratory and critical care medicine. 2013;187(10):1104-9.

40. Pramana IA, Latzin P, Schlapbach LJ, Hafen G, Kuehni CE, Nelle M, et al. Respiratory symptoms in preterm infants: burden of disease in the first year of life.(Clinical report). 2011;16(197).

41. Greenough A, Alexander J, Burgess S, Chetcuti P, Cox S, Lenney W, et al. Home oxygen status and rehospitalisation and primary care requirements of infants with chronic lung disease. Archives of disease in childhood. 2002;86(1):40-3.

42. Been JV, Lugtenberg MJ, Smets E, van Schayck CP, Kramer BW, Mommers M, et al. Preterm birth and childhood wheezing disorders: a systematic review and meta-analysis. PLoS Med. 2014;11(1):e1001596.

43. Simpson SJ, Hall GL, Wilson AC. Lung function following very preterm birth in the era of 'new' bronchopulmonary dysplasia. Respirology. 2015;20(4):535-40.

44. Fawke J, Lum S, Kirkby J, Hennessy E, Marlow N, Rowell V, et al. Lung function and respiratory symptoms at 11 years in children born extremely preterm: the EPICure study. American journal of respiratory and critical care medicine. 2010;182(2):237-45.

45. Korhonen P, Laitinen J, Hyoudynmaa E, Tammela O. Respiratory outcome in school-aged, very-low-birth-weight children in the surfactant era. Acta Pædiatrica. 2004;93(3):316-21.

46. Thunqvist P, Gustafsson PM, Schultz ES, Bellander T, Berggren-Broström E, Norman M, et al. Lung Function at 8 and 16 Years After Moderate-to-Late Preterm Birth: A Prospective Cohort Study. Pediatrics. 2016;137(4).

47. Landry J, Tremblay G, Li P, Wong C, Benedetti A, Taivassalo T. Lung Function and Bronchial Hyperresponsiveness in Adults Born Prematurely A Cohort Study. Annals of the American Thoracic Society. 2016;13(1):17-24.

48. Gough A, Linden M, Spence D, Patterson CC, Halliday HL, McGarvey LPA. Impaired lung function and health status in adult survivors of bronchopulmonary dysplasia. The European respiratory journal. 2014;43(3):808.

49. Hennessy EM, Bracewell M, Wood N, Wolke D, Costeloe K, Gibson A, et al. Respiratory health in pre-school and school age children following extremely preterm birth. Archives of disease in childhood. 2008;93(12):1037-43.

50. Simpson SJ, Turkovic L, Wilson A, Verheggen M, Logie KM, Pillow JJ, et al. Lung function trajectories throughout childhood in survivors of very preterm birth: A longitudinal cohort study. The Lancet Child and Adolescent Health. 2018.

51. Kotecha SJ, Watkins WJ, Paranjothy S, Dunstan FD, Henderson AJ, Kotecha S. Effect of late preterm birth on longitudinal lung spirometry in school age children and adolescents. Thorax. 2012;67(1):54.

52. Filippone M, Sartor M, Zacchello F, Baraldi E. Flow limitation in infants with bronchopulmonary dysplasia and respiratory function at school age. The Lancet. 2003;361(9359):753-4.

53. Doyle LW, Adams A-M, Robertson C, Ranganathan S, Davis NM, Lee KJ, et al. Increasing airway obstruction from 8 to 18 years in extremely preterm/low-birthweight survivors born in the surfactant era. Thorax. 2017;72(8):712.

54. Vollsaeter M, Roksund OD, Eide GE, Markestad T, Halvorsen T. Lung function after preterm birth: development from mid-childhood to adulthood.(Report). Thorax. 2013;68(8):767.

55. Vollsæter M, Clemm H, Satrell E, Eide G, Røksund O, Markestad T, et al. Adult Respiratory Outcomes of Extreme Preterm Birth: A Regional Cohort Study. Annals of the American Thoracic Society. 2015;12(3):313-22.

56. Fakhoury KF, Sellers C, Smith EOB, Rama JA, Fan LL. Serial measurements of lung function in a cohort of young children with bronchopulmonary dysplasia. Pediatrics. 2010;125(6):e1441.

57. Lum S, Kirkby J, Welsh L, Marlow N, Hennessy E, Stocks J. Nature and severity of lung function abnormalities in extremely pre-term children at 11 years of age. European Respiratory Journal. 2011;37(5):1199-207.

58. Filbrun AG, Popova AP, Linn MJ, McIntosh NA, Hershenson MB. Longitudinal measures of lung function in infants with bronchopulmonary dysplasia. Pediatric pulmonology. 2011;46(4):369.

59. Greenough A, Dimitriou G, Bhat RY, Broughton S, Hannam S, Rafferty GF, et al. Lung volumes in infants who had mild to moderate bronchopulmonary dysplasia. European journal of pediatrics. 2005;164(9):583-6.

60. Hjalmarson O, Sandberg KL. Lung function at term reflects severity of bronchopulmonary dysplasia. The Journal of pediatrics. 2005;146(1):86-90.

61. Vrijlandt EJLE, Boezen HM, Gerritsen J, Stremmelaar EF, Duiverman EJ. Respiratory Health in Prematurely Born Preschool Children with and without Bronchopulmonary Dysplasia. The Journal of Pediatrics. 2007;150(3):256-61.

62. Thunqvist P, Gustafsson P, Norman M, Wickman M, Hallberg J. Lung function at 6 and 18 months after preterm birth in relation to severity of bronchopulmonary dysplasia. Pediatric pulmonology. 2015;50(10):978-86.

63. Udomittipong K, Sly PD, Patterson HJ, Gangell CL, Stick SM, Hall GL. Forced oscillations in the clinical setting in young children with neonatal lung disease. The European respiratory journal. 2008;31(6):1292.

64. Hülskamp G, Pillow JJ, Dinger J, Stocks J. Lung function tests in neonates and infants with chronic lung disease of infancy: functional residual capacity. Pediatric pulmonology. 2006;41(1):1-22.

65. Latzin P, Roth S, Thamrin C, Hutten GJ, Pramana I, Kuehni CE, et al. Lung volume, breathing pattern and ventilation inhomogeneity in preterm and term infants. PloS one. 2009;4(2):e4635.

66. Yammine S, Schmidt A, Sutter O, Fouzas S, Singer F, Frey U, et al. Functional evidence for continued alveolarisation in former preterms at school age? The European respiratory journal. 2016;47(1):147.

67. Satrell E, Røksund O, Thorsen E, Halvorsen T. Pulmonary gas transfer in children and adolescents born extremely preterm. The European respiratory journal. 2013;42(6):1536.

68. Vom Hove M, Prenzel F, Uhlig HH, Robel-Tillig E. Pulmonary Outcome in Former Preterm, Very Low Birth Weight Children with Bronchopulmonary Dysplasia: A Case-Control Follow-Up at School Age. The Journal of Pediatrics. 2014;164(1):40-5.e4.

69. Filippone M, Bonetto G, Cherubin E, Carraro S, Baraldi E. Childhood course of lung function in survivors of bronchopulmonary dysplasia. Jama. 2009;302(13):1418-20.

70. Narang I, Rosenthal M, Cremonesini D, Silverman M, Bush A. Longitudinal Evaluation of Airway Function 21 Years after Preterm Birth. American Journal of Respiratory and Critical Care Medicine. 2008;178(1):74-80.

71. Chambers DC. Lung Function in Ex-preterm Adults. American journal of respiratory and critical care medicine. 2009;179(6):517-.

72. Gibson AM, Reddington C, McBride L, Callanan C, Robertson C, Doyle LW. Lung function in adult survivors of very low birth weight, with and without bronchopulmonary dysplasia. Pediatric Pulmonology. 2015;50(10):987-94.

73. Saarenpaa H-K, Tikanmaki M, Sipola-Leppanen M, Hovi P, Wehkalampi K, Siltanen M, et al. Lung function in very low birth weight adults.(Report). 2015;136(4).

74. Martinez FD. Early-life origins of chronic obstructive pulmonary disease. New England Journal of Medicine. 2016;375(9):871-8.

75. Lavoie PM, Dubé M-P. Genetics of bronchopulmonary dysplasia in the age of genomics. Current opinion in pediatrics. 2010;22(2):134.

76. Siezen CL, Bont L, Hodemaekers HM, Ermers MJ, Doornbos G, Van't Slot R, et al. Genetic susceptibility to respiratory syncytial virus bronchiolitis in preterm children is associated with airway remodeling genes and innate immune genes. The Pediatric infectious disease journal. 2009;28(4):333-5.

77. Melville JM, Moss TJ. The immune consequences of preterm birth. Frontiers in neuroscience. 2013;7.

78. Collado MC, Cernada M, Neu J, Pérez-Martínez G, Gormaz M, Vento M. Factors influencing gastrointestinal tract and microbiota immune interaction in preterm infants. Pediatric research. 2015;77(6):726-31.

79. Matías V, San Feliciano L, Fernández JE, Lapeña S, Garrido E, Ardura J, et al. Host and environmental factors influencing respiratory secretion of pro-wheezing biomarkers in preterm children. Pediatric Allergy and Immunology. 2012;23(5):441-7.

80. Korhonen PH, Suursalmi PH, Kopeli T, Nieminen R, Lehtimaki L, Luukkaala T, et al. Inflammatory activity at school age in very low birth weight bronchopulmonary dysplasia survivors. Pediatr Pulmonol. 2015;50(7):683-90.

81. Teig N, Allali M, Rieger C, Hamelmann E. Inflammatory markers in induced sputum of school children born before 32 completed weeks of gestation. J Pediatr. 2012;161(6):1085-90.

82. Halvorsen T, Skadberg BT, Eide GE, Roksund O, Aksnes L, Oymar K. Characteristics of asthma and airway hyper-responsiveness after premature birth. Pediatr Allergy Immunol. 2005;16(6):487-94.

83. MacNee W, Tuder RM. New paradigms in the pathogenesis of chronic obstructive pulmonary disease I. Proceedings of the American Thoracic Society. 2009;6(6):527-31.

84. Filippone M, Bonetto G, Corradi M, Frigo AC, Baraldi E. Evidence of unexpected oxidative stress in airways of adolescents born very pre-term. The European respiratory journal. 2012;40(5):1253-9.

85. Piersigilli F, Bhandari V. Biomarkers in neonatology: the new "omics" of bronchopulmonary dysplasia. The Journal of Maternal-Fetal & Neonatal Medicine. 2016;29(11):1758-64.

86. Carraro S, Giordano G, Pirillo P, Maretti M, Reniero F, Cogo PE, et al. Airway metabolic anomalies in adolescents with bronchopulmonary dysplasia: new insights from the metabolomic approach. J Pediatr. 2015;166(2):234-9 e1.

87. Doyle LW, Ehrenkranz RA, Halliday HL. Early (< 8 days) postnatal corticosteroids for preventing chronic lung disease in preterm infants. Cochrane Database Syst Rev. 2014(5):CD001146.

88. Bassler D, Plavka R, Shinwell ES, Hallman M, Jarreau PH, Carnielli V, et al. Early Inhaled Budesonide for the Prevention of Bronchopulmonary Dysplasia. N Engl J Med. 2015;373(16):1497-506.

89. Baud O, Maury L, Lebail F, Ramful D, El Moussawi F, Nicaise C, et al. Effect of early low-dose hydrocortisone on survival without bronchopulmonary dysplasia in extremely preterm infants (PREMILOC): a double-blind, placebo-controlled, multicentre, randomised trial. Lancet. 2016;387(10030):1827-36.

90. Committee on F, Newborn. Postnatal corticosteroids to treat or prevent chronic lung disease in preterm infants. Pediatrics. 2002;109(2):330-8.

91. Jobe AH. Postnatal corticosteroids for preterm infants--do what we say, not what we do. N Engl J Med. 2004;350(13):1349-51.

92. Shah VS, Ohlsson A, Halliday HL, Dunn M. Early administration of inhaled corticosteroids for preventing chronic lung disease in very low birth weight preterm neonates. The Cochrane Library. 2017.

93. Bassler D, Shinwell ES, Hallman M, Jarreau P-H, Plavka R, Carnielli V, et al. Long-Term Effects of Inhaled Budesonide for Bronchopulmonary Dysplasia. New England Journal of Medicine. 2018;378(2):148-57.

94. Nair V, Loganathan P, Soraisham AS. Azithromycin and other macrolides for prevention of bronchopulmonary dysplasia: a systematic review and meta-analysis. Neonatology. 2014;106(4):337-47.

95. Madurga A, Mizíková I, Ruiz-Camp J, Morty R. Recent advances in late lung development and the pathogenesis of bronchopulmonary dysplasia. American Journal of Physiology. 2013;305(12):L893.

96. Nixon PA, Washburn LK, Schechter MS, O'Shea TM. Follow-up study of a randomized controlled trial of postnatal dexamethasone therapy in very low birth weight infants: effects on pulmonary outcomes at age 8 to 11 years. The Journal of pediatrics. 2007;150(4):345-50.

97. Chan KN, Silverman M. Increased airway responsiveness in children of low birth weight at school age: effect of topical corticosteroids. Archives of disease in childhood. 1993;69(1):120-4.

98. Pelkonen AS, Hakulinen AL, Hallman M, Turpeinen M. Effect of inhaled budesonide therapy on lung function in schoolchildren born preterm. Respir Med. 2001;95(7):565-70.

99. Yuksel B, Greenough A. Randomised trial of inhaled steroids in preterm infants with respiratory symptoms at follow up. Thorax. 1992;47(11):910-3.

Legends

Figure 1: Pathophysiology and risk factors for bronchopulmonary dysplasia (BPD).
Development of BPD is complex and multifactorial. Inflammation and oxidative stress are associated with many of the risk factors and have been implicated in the pathophysiology of BPD, although the precise mechanisms are not entirely understood. BPD is characterised by altered lung development, with simplified alveoli, potential airway remodelling and a decrease in the growth and maturation of the pulmonary vasculature.

Figure 2: Longitudinal change in forced expiratory volume in 1 second (FEV_1) over time in children born preterm in the post surfactant era with and without bronchopulmonary dysplasia (BPD).

1. Simpson *et al.* report declining lung function trajectories in children born preterm, modelled from 347 lung function visits of children aged 4 to 12 years. FEV_1 declined by a rate of at least 0.1 z-scores per year in children with BPD over the course of the study.
2. Doyle *et al.* show increased impairments in airflow from 8 to 18 years of age in survivors of extreme premature birth, which was more pronounced in those with BPD and those smoking at 18 years.
3. Vollsaeter *et al.* reported low FEV_1 in extremely preterm children which tracked between 10 and 18 years of age for those with and without BPD.

Upper and lower limits of normal are represented by dotted lines.
*Indicates significant lung function decline over time relative to a representative population of term-born controls. These studies have shown lung function tracks at z-scores of zero for healthy term-born children.
Note: Confidence intervals are not displayed due to inconsistent reporting of results between studies.

Figure 3: Proposed risk factors for long-term respiratory morbidity after preterm birth.
Survivors of preterm birth are exposed to known risk factors for respiratory morbidity, in addition to genetic factors, altered immunity, impaired lung growth, inflammation and oxidative stress.

Figure 1

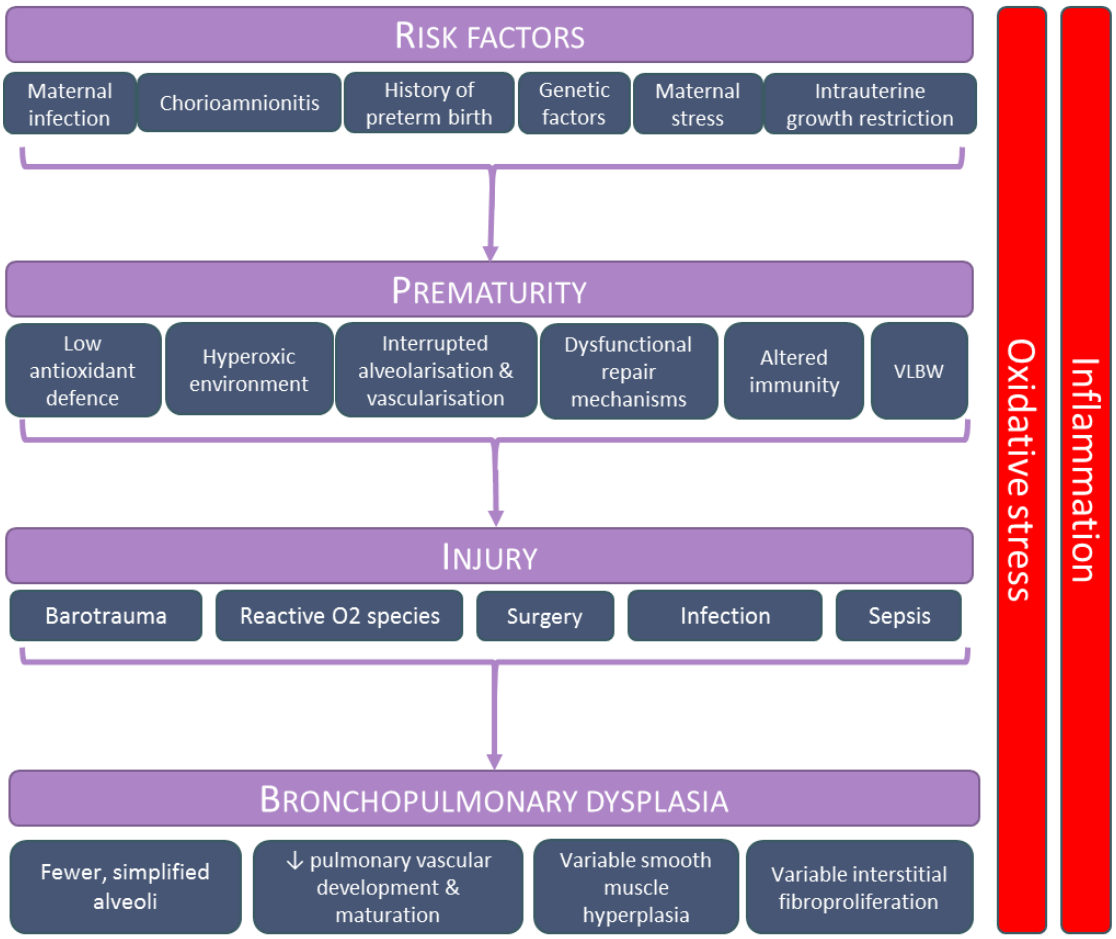

RISK FACTORS
Maternal infection
Chorioamnionitis
History of preterm birth
Genetic factors
Maternal stress
Intrauterine growth restriction
PREMATURITY
Low antioxidant defence
Hyperoxic environment
Interrupted alveolarisation & vascularisation
Dysfunctional repair mechanisms
Altered immunity
VLBW
INJURY
Barotrauma
Reactive O2 species
Surgery
Infection
Sepsis
BRONCHOPULMONARY DYSPLASIA
Fewer, simplified alveoli
↓ pulmonary vascular development & maturation
Variable smooth muscle hyperplasia
Variable interstitial fibroproliferation
Oxidative stress
Inflammation

Figure 2

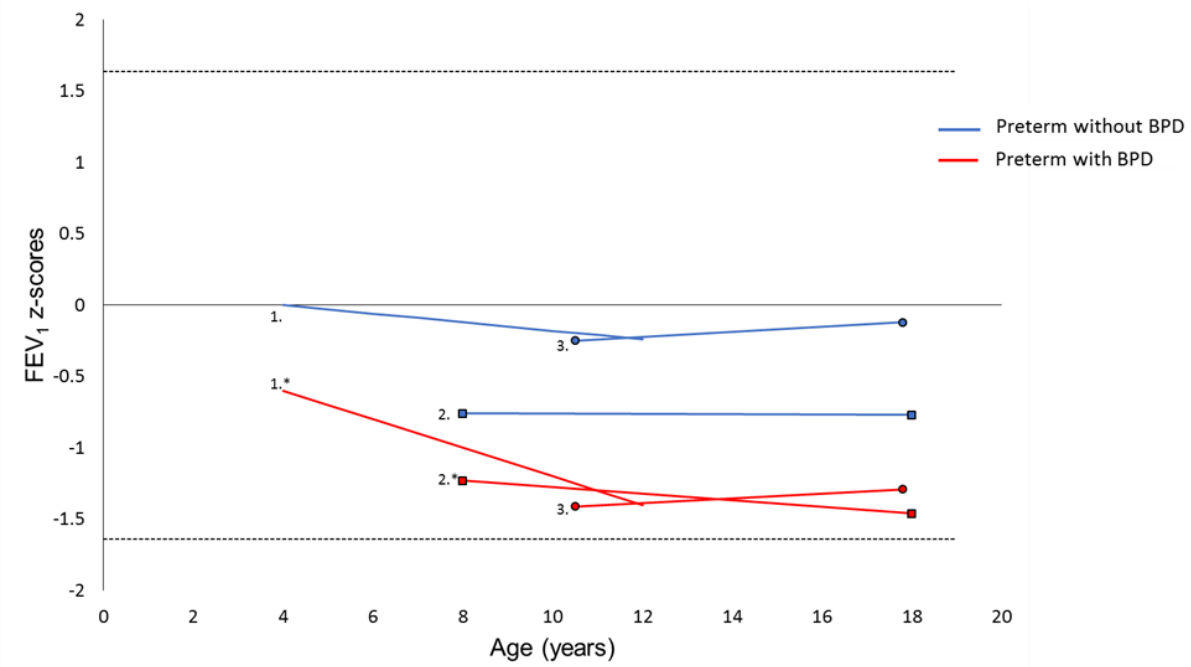
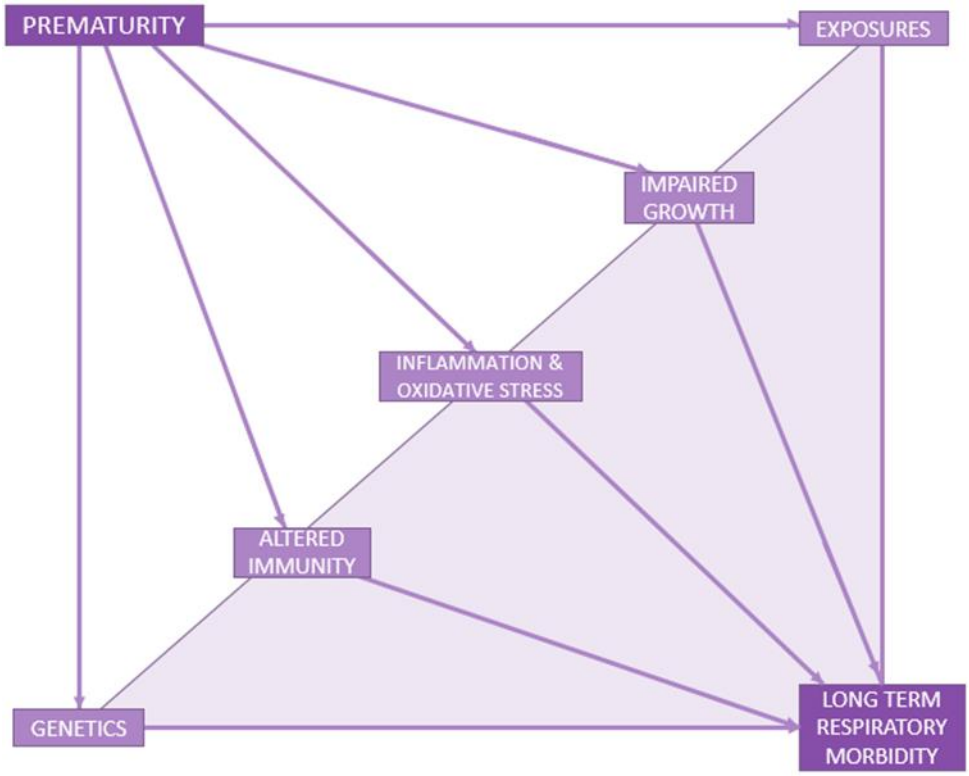

Preterm without BPD
Preterm with BPD
FEV1 z-scores
Age (years)
1.
3.
1.*
2.
2.*
3.

Figure 3


PREMATURITY
EXPOSURES
IMPAIRED GROWTH
INFLAMMATION & OXIDATIVE STRESS
ALTERED IMMUNITY
GENETICS
LONG TERM RESPIRATORY MORBIDITY