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**Impact of Environmental
Chemicals on Lung Development**

Mark D. Miller and Melanie A. Marty

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Title: Impact of Environmental Chemicals on Lung Development

Mark D. Miller^{1,2}, Melanie A. Marty¹

Institution / Affiliation:

1 Office of Environmental Health Hazard Assessment, California Environmental Protection Agency, Oakland, California, USA

2 Pediatric Environmental Health Specialty Unit, University of California San Francisco, San Francisco, California, USA

Corresponding Author:

Mark Miller MD, MPH

Office of Environmental Health Hazard Assessment

1515 Clay Street 16th floor, Oakland, Ca 94612

mmiller@oehha.ca.gov

510 622-3159

510 622-3210 fax

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Abbreviations:

AECs - alveolar epithelial cells

AFP – alpha fetoprotein

AhR - arylhydrocarbon receptor

BMP - bone morphogenetic protein

CDH - congenital diaphragmatic hernia

DEHP - di(2-ethylhexyl) phthalate

ECM - extracellular matrix

EGFR – epidermal growth factor receptor

ER- α - estrogen receptor alpha

FEF₂₅₋₇₅ - forced expiratory flow between 25% and 75% of forced vital capacity

FEV₁ - forced expiratory volume in 1 second

FGF - fibroblast growth factor

FVC - forced vital capacity

GD - gestational day

HDMA - house dust mite antigen

IUGR - intrauterine growth retardation

L-myc – lung associated myc oncogene

Nkx2.1 - transcription factor active in early lung, also called thyroid transcription factor 1
or Ttf1

PBDE - polybrominated diphenyl ether

PCBs - polychlorinated biphenyls

PPAR - peroxisome proliferator activated receptor

RA - retinoic acid

RALDH2 - retinal dehydrogenase2

Shh - sonic hedgehog

Spry2 - sprouty

TCDD - 2,3,7,8-tetrachlorodibenzo-p-dioxin

VEGF - vascular endothelial growth factors

Wnt – wingless signaling gene family

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Abstract

Background: Disruption of fundamental biologic processes and associated signaling events may result in clinically significant alterations in lung development.

Objectives: We review evidence on the impact of environmental chemicals on lung development and key signaling events in lung morphogenesis, and the relevance of potential outcomes to public health and regulatory science.

Data Sources: We evaluated the peer reviewed literature on developmental lung biology and toxicology, mechanistic studies, and supporting epidemiology.

Data Synthesis: Lung function in infancy predicts pulmonary function throughout life. *In utero* and early postnatal exposures influence both childhood and adult lung structure and function, and may predispose to chronic obstructive lung disease and other disorders. The nutritional and endogenous chemical environment affects development of the lung resulting in altered function in the adult. Studies now suggest that similar adverse impacts may occur in animals and humans following exposure to environmentally relevant doses of certain xenobiotics during critical windows in early life. Potential mechanisms include interference with highly conserved factors in developmental processes such as gene regulation, molecular signaling, and growth factors involved in branching morphogenesis and alveolarization.

Conclusions: Assessment of environmental chemical impacts on the lung requires studies that evaluate specific alterations in structure or function, endpoints not regularly assessed in standard toxicity tests. Identifying effects on important signaling events may inform developmental toxicology study protocols. Such knowledge may enable policies promoting true primary prevention of lung diseases. Evidence of relevant signaling disruption in the absence of adequate developmental toxicology data should influence the size of the uncertainty factors used in risk assessments.

Introduction

Over the past twenty years, acknowledgement of the unique vulnerabilities of children to the disruption of normal growth and development caused by environmental exposures has grown. The health impacts of many chemicals have been shown to differ based on the developmental window of susceptibility (e.g., periods of rapid cell proliferation or differentiation) when exposure occurs. While the neurologic system has been most widely studied in this regard, there is a growing body of knowledge about the potential impacts of environmental exposures on lung growth and function. Respiratory disease has a large public health impact. An estimated 24 million U.S. adults have chronic obstructive pulmonary disease, 23 million have asthma, and chronic lower respiratory diseases rank as the fourth leading cause of death in the U.S. (Kung et al. 2008; National Heart, Lung, and Blood Institute 2007). This paper will first present an overview of essential processes in lung development, followed by examples of xenobiotics, including important environmental contaminants, that can disrupt these processes.

Epidemiologic confirmation of the importance of the impact of early life exposures, as originally described by Barker (the Barker hypothesis), has developed into a burgeoning field of study: The fetal origins of adult disease. Barker's work demonstrating that poorer fetal nutrition and lower birth weight is associated with cardiovascular disease in adults has since been confirmed in multiple longitudinal studies around the world (Barker 2007). This finding was based on the observation that populations living in regions with poor fetal nutrition had higher risk of adult cardiovascular disease. Yet, paradoxically, geographical areas most associated with fetal or neonatal mortality and low birth weight

(e.g., from poor nutrition), were not those at greatest risk for known post-natal risk factors for cardiovascular disease (e.g., high income, increased fat in diet, etc.). The premise for the early origins of adult disease is that during early life, “programming” in response to poor fetal nutrition results in changes in organ structure, metabolism and function that are permanent. For example, lower birth weight has been associated with increased likelihood of having adult lipid profiles linked to cardiovascular risk as well as hypertension and impaired glucose regulation (Kajantie et al. 2007; Gluckman et al. 2008).

Though Barker’s original ecologic epidemiologic findings also showed a link between low birth weight and adult respiratory health, this was not accorded the same importance since geographic areas that had higher levels of low birth weight and neonatal mortality were also regions with more post-natal risk factors (e.g. infection) for adult respiratory diseases. However, more recent studies provide evidence that *in utero* and early postnatal exposures set the stage for both childhood and later life deficiencies in lung function that are predisposing factors for chronic obstructive lung disease and other disorders (Bush 2008; Shi et al. 2007; Canoy 07). For example, Stern et al (2007) found that functional residual capacity measured in 123 infants at two months of age was associated with lung function measurements including forced expiratory volume in 1 second / forced vital capacity (FEV_1/FVC ratio), forced expiratory flow between 25% and 75% of FVC (FEF_{25-75}), and FEV_1 , up to age 22 years (Figure 1). In a longitudinal study of 243 infants, four week olds with flow limited tidal expansion (inability to increase expiratory flow with increased effort) were 7 times more likely to be diagnosed

with asthma at 2 years of age (Young et al. 1994). These investigators observed a statistically significant increase in wheezing and a trend to reduced FEF₂₅₋₇₅ at eleven years of age compared to those children who as infants had normal tidal expansion. (Turner et al. 2002). Similarly, airway responsiveness to histamine at one month of age was associated with abnormal airway function (decreased FEV₁, and FVC), lower respiratory symptoms, and emergence of asthma by age 6 in a study of 95 children (Palmer et al. 2001). Several sizable studies from various countries have demonstrated that lung function in both asthmatics and non-asthmatics tracks from early childhood through adolescence and up to mid-life, and is set by early life events (Oswald et al. 1997; Phelan et al. 2002; Stern et al. 2007; Haland et al. 2006; Morgan et al. 2005). These studies support the importance of early life programming of respiratory system structure and function and its life long implications.

The endogenous fetal environment: Impacts on fetal respiratory development

Evidence for the potential impact of the early life environment on adult respiratory status stems from examination of the effects of fetal nutrition and gender. Intrauterine growth retardation (IUGR), often defined as low birth weight (<10th percentile) for gestational age, has been identified as a risk factor for impaired lung function in children (Ergaz et al. 2005; Nikolajev et al. 2008; Kotecha et al. 2010). The reduction in nutrients and oxygenation related to IUGR may interfere with structural development of the lung (Lipsett et al. 2006). In an animal model, induced IUGR in late gestation produces alterations in alveolar function, corresponding to rapid development of alveoli during this time period (Maritz et al. 2001). Alterations include a thickened alveolar blood-gas barrier which persists into adulthood. In epidemiologic studies, birth weight has been

demonstrated to be related to reduced lung function in adults (Stein et al. 1997; Canoy et al. 2007; Lawlor et al. 2005) and intrauterine growth retardation has been associated with poorer lung function at 8 to 9 years of age even when accounting for catch up growth (Kotecha et al. 2010).

Endogenous chemicals, including estrogens and androgens are associated with modulation of lung development and function (Carey et al. 2007). Both estrogen and androgen receptors are expressed in the human lung during fetal development, and play a role in sexually dimorphic differentiation. For example, in humans, surfactant production and maturation appears earlier in the female and may be a reason why males are more prone to respiratory distress syndrome (caused by surfactant deficiency) (Fleisher et al. 1985; Perelman et al. 1986). Androgens inhibit surfactant production via alterations in epidermal growth factor and transforming growth factor beta signaling (Dammann et al. 2000). In contrast, estrogen administration can stimulate surfactant production and lung maturation in the fetal rat and rabbit (Khosla et al. 1981). For premature infants, human male singletons or twins are at greater risk for respiratory morbidity and respiratory distress syndrome than female singletons or twins. Shinwell et al. (2007) conducted a population based study of 8,858 very low birth weight premature infants to examine mixed sex premature twins. Female infant respiratory morbidity was comparable to that of males suggesting that a male disadvantage was transferred via an intrauterine paracrine mechanism to the female (Shinwell et al. 2007). Melamed et al. (2009) analyzed 2,704 twin births and found a similar disadvantage to the female in mixed sex twins. Large airway growth lags behind parenchymal growth in human males (relative to females)

resulting in relatively narrower conducting airways (Hoffstein 1986; Becklake and Kauffmann 1999).

These examples show that the nutritional and endogenous chemical environments affect development of the lung, and that these effects can be permanent, altering function in the adult. This paper will examine the basis for concern that similar adverse impacts on lung development may occur following exposure to xenobiotics during critical windows in early life. Implications for regulation of environmental chemicals are also discussed.

Overview of Lung Development

The biology of lung development briefly summarized here is reviewed in many books and papers (e.g. Pinkerton and Joad, 2000; *The Lung: Development, Aging, and the Environment*, Harding et al. Eds. 2004).

Stages of Lung Development

In humans, primary lung buds develop during the fourth week of gestation from the endoderm of the foregut. After early embryonic development, stages in prenatal lung development include pseudoglandular, canalicular, saccular, and alveolar. These are represented along with associated developmental features below in Figure 2 (Kajekar 2007). Only a portion of maturational events are required prenatally for successful survival with a majority occurring postnatally during alveolarization (Pinkerton and Joad 2000).

The **embryonic** period is characterized by initial outpouching of primary bronchi from the primitive gut, which elongate into the mesenchyme and divide into two main bronchi. During the **pseudoglandular** phase (6-16 weeks of gestation in human) branching continues, and mesenchyme differentiates into cartilage, smooth muscle, and connective tissue around the epithelial tubules. By the end of this time, major conducting airways to the terminal bronchioles are developed. Respiratory bronchioles, which end in thin walled dilatations (terminal sacs or primitive alveoli), develop in the **canalicular** period along with a rich vascular supply. During the **saccular** phase, first contact between the air space and proliferating pulmonary capillaries takes place. Epithelial cells differentiate into type I (thin) and type II (cuboidal). During **alveolarization** the primitive alveoli develop secondary septa that form the walls of the true alveoli resulting in a dramatic increase in surface area.

Cell types lining the conducting airway (trachea to mid-sized bronchioles) include ciliated cells, goblet cells (which produce mucous), and basal cells (stem cells for other cell types). The respiratory bronchioles include alveoli periodically along their surface. When the alveoli begin to dominate the surface area, they are termed alveolar ducts.

Type I alveolar epithelial cells (AECs) are predominant in the alveolar wall and associated with gas exchange. The type II AECs, which become mature during the alveolar stage, are the source of pulmonary surfactant. Premature infants lack sufficient numbers of differentiated type II AECs often resulting in respiratory distress syndrome from inadequate production of surfactants. Alveolarization continues from late gestation

(about 30 weeks) through at least the first two postnatal years. Growth of the lung continues through late adolescence (Gauderman et al. 2004).

There are over 40 morphologically differentiated cell types in the mature lung (Warburton et al. 1998). The development of each of these differentiated cell types is influenced by the spatial and temporal distribution of a variety of signaling molecules and their receptors which regulate normal morphological structure and function.

Branching morphogenesis

Repeatedly branched structures develop when there is a need to maximize the contact between a structure and the surrounding environment. This organizational strategy packs a large area of contact into a small space (Davies 2006), and is a highly conserved process for organ growth in many structures including the lung, kidney, salivary glands, prostate, and breast. Branching morphogenesis is a recurring, iterative patterning event of bud growth, bud elongation, and subdivision of terminal units (Cardoso 2004). In the lung, branching is utilized to generate the bronchial tree including secretory glands, blood vessels, and interalveolar septa.

Mesenchyme develops from the mesoderm and gives rise to the lung's connective tissue, endothelial cell precursors, smooth muscle that surrounds the airways and blood vessels, the lymphatic system, and the pleura. The lung develops in proximal to distal fashion but also in a specified right/left asymmetrical manner. These events develop in a cascade, each building on earlier activity and reiterated over several generations of branching to form the respiratory tree. Though many details of the regulation of these events are still

unknown, numerous animal studies have illuminated many of the signaling pathways and transcription factors that direct lung development. Physical factors such as stretch and fluid volume also influence lung development.

Perhaps more important from a toxicological perspective, reciprocal exchange of signaling factors between epithelial and mesenchymal cells are essential to normal development (reviewed in Cardoso and Lu 2006). For example, members of the fibroblast growth factor family (FGF), soluble factors produced in the mesenchymal cells that signal tyrosine kinase receptors (Fgfr1-4) in the epithelial cells, are essential for bud initiation. Studies in organ culture show that the epithelium will migrate and proliferate towards fibroblast growth factor 10 (Fgf10) (Park et al. 1998). Correct development of lung branching requires temporal and spatial control over Fgf10 levels (Mariani 2007; Bellusci et al. 1997). In rodents, deletion of *Fgf10* or lack of retinoic acid, a crucial cofactor for Fgfs, results in lung agenesis (Cardoso and Lu 2006; Desai et al. 2004; Sekine et al. 1999). Inhibitory control over Fgf10 involves highly diffusible factors secreted by epithelium of the developing lung bud, sprouty (*Spry2*) and sonic hedgehog (*Shh*) (Mason et al. 2006; Warburton et al. 2005). The Fgf10 in the distal mesenchyme diffuses into the epithelial bud and binds to the receptor, Fgfr2b, to induce budding and initiate branching (Bellusci et al. 1997). Fgfr2 activity induces *Spry2* which inhibits FGF signaling and inhibits bud growth (Mailleux et al 2001). *Shh* is highly expressed in the bud's distal epithelium (Cardoso 2004). It is believed that *Shh* from the bud progressively downregulates FGF activity as the bud grows towards the Fgf10 expressing mesenchyme (Lebeche et al. 1999). In mice, an over expression of *Spry2* or inhibition of

Shh results in impairment of branching (Mailleux et al. 2001; Bellusci et al. 1997; Lebeche et al. 1999; Cardoso 2004).

Vascular endothelial growth factors (VEGF), necessary for development of the lung vasculature, are expressed in both branching tubular airways and vascular mesenchymal cells. The coordinated development of the epithelial and endothelial compartments depends on a VEGF gradient being present from its production at the tips of growing airway tubules (Voelkel et al. 2006). In a number of murine models, overexpression of *VEGF* results in dysmorphogenesis and underexpression of *VEGF* or neutralization of VEGF results in poor septal formation and emphysematous changes (Gerber et al. 1999; Voelkel et al. 2006). These are only examples of the many signaling pathways that must be expressed in a precise temporal and spatial pattern for normal lung growth and development (Maeda et al. 2007).

The trachea develops from an outpouching of the foregut, and requires formation of a separation between the two tissues. Mice deficient in Shh or retinoic acid (RA), or which are *Nkx2.1*-null, develop an incomplete separation of the foregut and trachea known as a tracheoesophageal fistula (Minoo et al. 1999; Litingtung et al. 1998). This is a relatively common congenital anomaly in humans and has been associated with downregulation of the FGF pathways and aberrant Shh signaling (Spilde et al. 2003; Crisera et al. 2000).

Alveolarization

As reviewed by McGowan and Snyder (2004), during the saccular phase the terminal portions of the airways give rise to alveolar ducts and sacs with thick walls of connective tissue and limited ability for gas exchange. The primary septa (the walls of the terminal sacs) have a central core of fibroblasts and connective tissue surrounded on each side by capillaries and epithelial cells. During alveolarization, ridges develop in the primary septa and become the secondary septa.

In humans, a period of rapid alveolar development lasts from about 36 weeks gestation to about 2 years. From several months until 3 years of age, a second phase of alveolarization occurs characterized by microvascular remodeling, thinning and lengthening of the secondary septae, loss of mesenchymal cells, and change from a dual capillary bed to a single one. From birth until maturity there is a twenty fold increase in gas exchange surface area (Burri 2006). Recent evidence in non-human primates suggests that alveoli increase in number through young adulthood (Hyde et al. 2007). If confirmed, it would expand the sensitive time period during which disruption of molecular mechanisms of alveolar development is of special concern from the first few years of life until termination of longitudinal growth.

Cellular Differentiation and Repair

With upwards of forty distinct cell phenotypes represented in the mature lung, undisturbed cellular differentiation is important to future lung function. In general, differentiation occurs in a proximal to distal sequence with the tracheal epithelial cells differentiating first. Thus, identical critical processes involving cell signaling in

development are occurring at different times depending on the location proximal to distal in the respiratory tree.

Cross-talk between endothelial cells and epithelial cells is essential for differentiation of these cell types. Vascular endothelial growth factor-A (VEGF-A) expressed by the lung epithelium is essential to pulmonary capillary development. In mice, deficient capillary development by selective inactivation of the *Vegf-A* gene in the respiratory epithelium results in disruption of primary septae formation during alveolarization (Yamamoto et al. 2007; Gerber et al. 1999). Yamamoto et al. (2007) also demonstrated that Hepatocyte growth factor (*Hgf*) expression in the developing endothelium is essential for normal epithelial development.

The transcription regulatory protein GATA-6, expressed in lung epithelial cells interacts with thyroid transcription factor (TTF1). In mouse models, inhibition of GATA-6 during fetal life inhibited terminal differentiation of respiratory epithelial cells (Liu et al. 2002; Yang et al. 2002). Elevated levels of GATA-6 postnatally (when levels normally are decreased) resulted in alterations in alveolar septation causing increased lung volumes and air space enlargement that persisted into adulthood (Liu et al. 2003). Airway resistance and airway and tissue elastance were significantly decreased. This provides an example of morphogenesis dependent on the precise timing of expression of GATA-6. The impact of environmental chemicals on GATA-6 expression has not received much study.

Clara cells are non-ciliated bronchiolar cells and are the principal epithelial cells in the distal airway whose products include Clara cell secretory protein and some components of surfactant. Clara cells are important in the metabolism of airborne toxicants due to their high CYP450 content, and are particularly sensitive to injury by xenobiotics, in part as a result of their ability to transform them to reactive intermediates (Massaro et al. 1994). Clara cells are considered multipotent progenitor cells that regenerate airway epithelium after oxidant injury (Evans et al. 1976).

Xenobiotic disruption of lung development

Chemical exposures that impact expression of important growth regulators can result in more severe effects or even lethality when the exposure occurs during susceptible periods of lung development. Table 1 summarizes these cellular and subcellular impacts reviewed in this paper along with associated alterations in lung structure or function (and possible clinical implications) for individual chemicals discussed in this paper. Potential windows of susceptibility for exposure can be inferred by comparing the information in Table 1 with the timeline of key developmental periods in humans shown in Figure 2. Below we describe examples of xenobiotics altering lung development by disrupting essential processes such as branching morphogenesis, alveolarization, and cellular differentiation and repair.

The Congenital Diaphragmatic Hernia model and Nitrofen

Congenital diaphragmatic hernia (CDH) is a serious condition of newborns with an incidence of about 1 in 3,000 live births in the U.S. Many die *in utero*; morbidity and

mortality in the first days after birth are high (Hartman et al. 2004). While it is associated with various genetic syndromes, recent understanding of CDH's etiology has been changing. Originally it was believed to be due to malformation in a portion of the diaphragm, which allowed compression of the developing lung by abdominal contents entering the chest cavity resulting in the subsequent characteristic hypoplastic lung. More recently, evidence has supported a "dual hit" theory which postulates that the original injury occurs early in lung development before and not connected to aberrant development of the diaphragm (Keijzer et al. 2000). According to this explanation, the already hypoplastic developing lung is then further inhibited as a result of the mechanical compression on the ipsilateral side resulting from herniation of the intestines into the thoracic cavity.

Exposure to nitrofen (2,4-dichlorophenyl-*p*-nitrophenyl-ether) – a banned pesticide – has been used as a model for CDH in rodents. Using this model, it was shown that the initial event in experimentally induced CDH is the development of hypoplastic lungs which occurred early in development prior to the closure of the diaphragm and herniation (Leinwand et al. 2002). Therefore, compression was not the initial cause of hypoplasia. Hypoplasia has also been observed after nitrofen exposure in animals without CDH (Cilley et al. 1997). Lungs of nitrofen exposed pups had 30% fewer terminal bronchioles than controls and were developmentally immature (Leinwand et al. 2002). Similar observations in human infants with CDH include hypoplastic lungs, fewer alveoli, thickened alveolar walls, increased pulmonary interstitial tissue and less airspace as well as fewer bronchiole and vascular branches (Gallot et al. 2005).

The list of effects induced by nitrofen on various signaling pathways related to branching morphogenesis and lung development has been rapidly increasing. Wnt growth factor signaling has been shown to have a role in regulation of proliferation, differentiation, and lineage specification during embryonic development. In the lung, *Wnt7* inactivation results in decreased branching and subsequent hypoplasia as well as decreases in smooth muscle (Pongracz and Stockley 2006; Shu et al. 2002; Wang et al. 2005). Wnt signaling is an upstream regulator of bone morphogenetic protein 4 (BMP4) and FGF, both important in lung development. *Wnt7* null mice die at birth from severe lung hypoplasia. In mice treated with nitrofen, *GATA 6* (upstream activator of *Wnt7b*) as well as *Wnt7b*, *Wnt2*, and *BMP4* were down-regulated (Takayasu et al. 2007a). *GATA 6*, a zinc finger transcription protein, is an important regulator of distal epithelial cell differentiation as well as proximal airway development (Yang et al. 2002). Prenatal retinoic acid partially mitigated the actions of prenatal nitrofen exposure in nitrofen-induced CDH rats (Montedonico et al. 2008, 2006).

Vitamin A deficient diets have been linked to CDH in animal studies (Andersen 1941) and a small human epidemiologic study found lower retinol (the active metabolite of vitamin A) levels in newborns with CDH than controls (Major et al. 1998). Knockout mice deficient in retinoic acid nuclear receptors had an increased incidence of a spectrum of pulmonary agenesis, hypoplasia, and CDH (Mendelsohn et al. 1994). Nitrofen has been shown to disturb the retinoic acid signaling pathway at an early stage of lung development (Nakazawa et al. 2007a). The incidence of CDH was dramatically reduced

when retinoic acid was given along with nitrofen during pregnancy in rats (Babiuk et al. 2004). In rat lung explants, retinoic acid significantly increased the growth, number of lung buds and lung area of nitrofen-induced hypoplastic lungs but had no effect on controls (Montedonico et al. 2006). One possible mechanism of nitrofen's action on retinol may be to interfere with its cellular uptake during lung morphogenesis (Nakazawa et al. 2007b). Previous studies have suggested activity by inhibiting retinal dehydrogenase2 (RALDH2), a key enzyme for generation of retinoic acid from retinal. Four chemicals shown to be able to precipitate development of CDH in animal models (nitrofen, bisdiamine, 4-diphenyl carboxylic acid, and SB210661 all have been found to inhibit RALDH2 (Mey et al. 2003).

One of the key processes in later gestation is the differentiation of a portion of alveolar type II cells to alveolar type I cells. That process was impaired in the nitrofen-induced CDH lung (Takayasu et al. 2007b). Though it appears that the immediate cause of decreased differentiation into type I cells was mechanical compression, this can be considered a secondary effect of the chemical exposure and was not observed in the absence of nitrofen exposure.

Thyroid disrupting chemicals and branching morphogenesis

Thyroid hormone is important for normal lung development (Van Tuyl et al. 2004). For example, alveolar septation, a largely postnatal structural manifestation was impaired in hypothyroid mouse pups (Van Tuyl et al. 2004). The ratio of surfactant protein mRNA

expression to that of corresponding proteins was influenced by both prenatal and postnatal thyroid hormone deficiency. A reduced ratio is indicative of an immature lung.

Nitrofen is a diphenyl ether and like related chemicals has an anti-thyroid activity (Brandsma et al. 1994), inhibiting T3 receptor binding (Figure 3). Little research has been published on the effect of other environmental chemicals with anti-thyroid activity on lung development. Dioxins, polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDE's) are structurally similar to nitrofen and have known anti-thyroid activity. A significant increase in the incidence of "bronchitis" was noted in a Taiwanese cohort of children exposed to PCBs prenatally (Rogan et al. 1988) which may involve PCB impairment of immune function and/or lung development. An epidemiologic study in the Netherlands found prenatal/lactational exposure to dioxins to be related to a significant reduction in lung function in 41 healthy children between 7 and 12 years of age (Ten Tusscher et al. 2001). Gestational exposure in rats to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) resulted in upregulation of arylhydrocarbon receptor signaling (AhR) in the developing lung, and delayed lung development as evidenced by decreased total lung airspace and increased septal area (Kransler et al. 2009). These hypoplastic changes in lung morphology were associated with functional differences in respiratory mechanics. The study suggests that AhR activation adversely impacts lung development. Upregulation of AhR activity results in decreases in thyroid hormone due to increased metabolism. Thus, decreased thyroid hormone may play a role in these findings.

We identified no other studies examining developmental exposure to thyroid disrupting environmental chemicals and lung function. Lung function studies are a sensitive, minimally invasive method of measuring impact on lung development. Measurements of lung function in experimental animal pups would provide additional useful information on adverse lung impacts in developmental toxicity studies. In addition, more epidemiologic studies evaluating lung function in people exposed pre- and post-natally to thyroid hormone disrupting chemicals are needed.

Examples of disruption of branching morphogenesis by environmental chemicals in other organ systems

Branching morphogenesis is an essential developmental process for many organs that utilizes common signaling pathways. One of the organs that exhibits branching morphogenesis is the salivary gland. TCDD exposure of cultured murine submandibular glands reduced epidermal growth factor signaling. This aryl hydrocarbon associated effect inhibited branching morphogenesis and resulted in smaller glands with enlarged buds (Kiukkonen et al. 2006). This effect was seen in cells cultured at GD 13 but not later in development indicating a specific window of susceptibility. Development of the prostate in the mouse is also inhibited by exposure to TCDD during pregnancy and lactation. Exposure on GD 13 to a single dose resulted in reduced organ weight related to an inhibition of development of prostatic epithelial buds (Ko et al. 2002). Inhibition of prostatic bud development by TCDD is not androgen dependent (Lin et al. 2004).

TCDD exposure during a critical window of gestation produced disruption of branching morphogenesis in the rat mammary gland resulting in smaller glands, limited branching,

decreased numbers of terminal end buds, and a lack of substantial alveolar lobule development independent of hormonal effects (Fenton et al. 2002; Vorderstrasse et al. 2004). This effect was dependent on the time of exposure - it was observed after exposure on GD 15 but not GD 20, and persisted at least until postnatal day 45 (post pubertal) (Fenton 2002).

Retinoids are involved in embryonic kidney patterning and development including branching morphogenesis and are important in kidney development. The Ret receptor modulates ureteric bud branching morphogenesis (Gilbert 2002). Nitrofen exposure resulted in hypoplastic kidneys in rats (Montedonico et al. 2007).

Exposure of rats *in utero* to the herbicide atrazine was associated with altered branching morphogenesis in the mammary gland, though little detail is known of the exact mechanism of action (Rayner et al. 2005). Similarly, the mammary glands of offspring exposed GD 17-19 displayed delay in development of mature gland structures and less epithelial branching. Importantly, the offspring of atrazine treated dams were unable to provide adequate nutritional support for the F₂ offspring resulting in decreased pup weight gain.

Clearly, the impact of exposure during critical windows of development to environmental chemicals that disrupt branching morphogenesis has been demonstrated in various organ systems dependent on this process. Chemicals that inhibit this process in one organ should be investigated for similar impact on lung development.

Nicotine and Alveolarization

The glycolytic pathway is very active during the alveolar phase of lung development and provides energy and precursors to the lung. Maternal exposure to nicotine in the rat resulted in sustained or permanent suppression of glycolysis and glycogenolysis in the lung tissue of the pup (Maritz 1986, 1988), due to reduced synthesis of phosphorylase and phosphofructokinase (the rate limiting step in glycolysis) in nicotine exposed animals (Kordom et al. 2002; Kordom 2004). As a result of inhibited glycolysis, $\text{Na}^+\text{-K}^+$ ATPase is inhibited, which may result in swelling and bleb formation of the alveolar type1 cells (reviewed in Maritz 2008).

In rats, maternal dosing with nicotine during pregnancy and lactation, at doses that did not alter fetal growth, resulted in a significant decrease in number of alveoli and increase in the alveolar volume at maturity in nicotine exposed pups relative to controls (Maritz 2002; Maritz and Windvogel 2003). The increase in alveolar volume in nicotine-exposed pups was attributed to slower alveolar septal formation, flattening of the alveoli as they aged, and disappearance of alveolar walls leading to larger alveoli. The histopathology of the nicotine-exposed animals resembled early emphysema (Maritz and Windvogel 2003). Routine pathological examination of these lungs would likely fail to demonstrate these relatively subtle changes. Such irreversible changes could result in dysfunction (emphysema) in later life.

Xenobiotics and Cell Differentiation and Repair

In the rabbit, Clara cells are actively differentiating postnatally, reaching maturity at 4 weeks. Despite lower levels of CYP450 activity in immature neonatal Clara cells, they were susceptible to injury at lower doses of 4-ipomeanol than adult cells (Plopper et al. 1994). Smiley-Jewel et al. (2000), treated rabbits with ipomeanol during three postnatal time periods that represent early, intermediate, and final stages of Clara cell maturation. In rabbits treated with a single dose of 4-ipomeanol during early or middle periods of differentiation (up to 9 days of age), bronchiolar cell differentiation and repair were inhibited. The period of greatest susceptibility was during mid-differentiation at 7 days of age. On examination at 6 weeks, those rabbits treated at day 7 had more squamous cells, fewer ciliated cells, a reduced expression of Clara cell markers, and an undifferentiated cuboidal cell ultrastructure compared to those treated at adulthood. In those treated at later stages of development (21 days in this study), repair was completed during this time period and the bronchiolar epithelium was indistinguishable from controls at 6 weeks. Thus, the extent of repair was related to the stage of cellular differentiation at the time of injury but independent of the degree of injury (Smiley-Jewell et al. 2000; Smiley-Jewell et al. 1998). Exposure during the critical window of early development disproportionately inhibited differentiation and repair of bronchiolar Clara cells resulting in persistent, possibly permanent effects.

Similarly, naphthalene, another bioactivated Clara cell toxicant, has been found to induce injury at much lower doses in immature mice (Fanucchi et al. 1997). 1-Nitronaphthalene, an atmospherically formed nitroaromatic, more severely impacts immature than mature Clara cells in both mice and rats (Fanucchi et al. 2004).

These findings suggest that Clara cell injury by bio-activated chemicals in the developing animal is not necessarily predicted by the level of CYP450 enzyme activity. There is no reason to believe this is restricted to mice, rats, and rabbits. Susceptibility of the neonate to injury cannot be predicted by the findings in adults. Early life injury may be a precursor to dysfunction later in life.

Wide-spread environmental chemicals that disrupt lung development

Ozone

Lung development occurs not only prenatally but through childhood and lung function grows at least through adolescence. Therefore, exposure to environmental toxicants may impact underlying structural and functional aspects of the lung during a wide window. This may increase susceptibility to asthma as well as other diseases.

An elegant series of studies conducted at University of California, Davis evaluated the impact of ozone and allergens on lung development in rhesus monkeys. These studies integrated early life exposures through multiple windows of susceptibility to observe functional and structural changes relevant to human lung development and lung disease. Tran et al. (2004a) describe conducting airway growth in monkeys with airways doubling in length and increasing by 33% in diameter between 1 and 6 months of age. Postnatal exposure to ozone, alone or in combination with house dust mite antigen (HDMA), resulted in changes in bronchiolar growth patterns, inhibiting growth in diameter and promoting growth in length (see Figure 3) as well as reducing the number of conducting airway branches (by as many as six generations) (Fanucchi et al. 2006; Plopper et al.

2007). These changes appeared to be permanent since they persisted after six months of recovery.

In monkeys, the smooth muscle bundles grow along with the conducting airways and have characteristic alterations depending on location and age (Tran et al. 2004a). Muscle bundles progress with age from a primarily perpendicular orientation relative to the long axis of the airway in early development to one with a large percentage of bundles greater than 30° from perpendicular. This process is disrupted by exposure to HDMA or ozone in rhesus monkeys (Tran et al. 2004b). The smooth muscle bundles were thickened and their orientation relative to the airway in the respiratory bronchioles was altered by exposure to ozone such that a much higher percentage of the bundles are $<15^{\circ}$ perpendicular to the long axis of the bronchiole, an alteration that may increase airway hyper-reactivity (Tran et al. 2004b; Plopper et al. 2007). The changes noted above from ozone exposure were not associated with changes in lung volume or function as measured by routine testing. The effect of the structural changes found after exposure to HDMA and ozone is consistent with alterations in airflow and resistance found in asthmatics (Plopper et al. 2007; Tran et al. 2004b; James and Carroll 2000). Epidemiologic evidence suggests exposure to ozone is associated with asthma induction in children (McConnell et al. 2002) and reduced lung function growth (Tager et al. 2005; Galizia and Kinney 1999).

Chronic cyclic O₃ exposure (0.5 ppm, 8 h/d, 5 days on followed by 9 days of filtered air for 11 cycles) of infant rhesus monkeys induced biochemical and functional alterations

(depleted proteoglycan and Fgf-2, altered Fgfr-1), and thinning of the basement membrane zone (Fanucchi et al., 2006). This tissue binds and releases growth factors, is involved in cell-cell communication, and functions as a barrier. The alteration of Fgf-2 signaling, important for regulating processes in the developing lung, may be associated with the reported O₃ -induced abnormal development of alveolar and bronchiolar regions in animal models.

Arsenic

Arsenic is a known human carcinogen, causing lung cancer after inhalation and oral exposure. Long-term arsenic exposure in drinking water has also been associated with chronic nonmalignant respiratory disease and decreased lung function (Guah Mazumder 2007). In men exposed in West Bengal, India, chronic ingestion of arsenic was a stronger determinant of lung function than smoking, and decreases in FEV₁, FVC, and FEF₂₅₋₇₅ indicates restrictive pathophysiology involving the small airways (von Ehrenstein et al. 2005).

Several studies have demonstrated transplacental carcinogenesis in animal models. *In utero* arsenic exposure in C3H mice results in tumorigenesis in a number of organs including the lung (Waalkes et al., 2003). These investigators reported increased lung tumors in adult females, not males. In humans, exposure to arsenic via drinking water *in utero* and postnatally was associated with much larger increases in lung cancer and bronchiectasis in a population in Chile than later life exposures (Smith et al., 2006). Thus, arsenic exposure early in life is associated with both chronic obstructive lung

disease and lung cancer, and data from animal models indicate that this may result from aberrant signaling during lung development.

Gestational arsenic exposure increased expression of estrogen receptor alpha (*ER- α*) and genes related to estrogen signaling in the female fetal mouse lung after environmentally relevant exposures via drinking water (Shen et al. 2007). This correlated to intense overexpression of *ER- α* protein in lung tumors in adult females exposed gestationally. These authors also observed activation of a number of genes associated with lung cancer development, including *EGFR*, *AFP*, and *L-myc* following gestational exposure.

The extracellular matrix (ECM) in the lung performs various functions including providing structural support for cells, regulating intercellular communication, and storing growth factors locally. ECM fibers include collagen (structural support) and elastin (elasticity). Matrix genes were dysregulated by chronic arsenic exposure in adult mice (10 or 50 ppb) including downregulation of the gene for collagen, elastin, and fibronectin and upregulation of the matrix degrading enzyme MMP-9 (Hays et al. 2008; Lantz and Hays 2006). In vitro exposure of human epithelial cells to arsenic also caused upregulation of MMP-9 and restricted wound repair (Olsen et al. 2008). Collagen knockout mice have increased smooth muscle around the airways (Dekkers et al. 2007; Parameswaran 2006). Similarly, exposure of mice to environmentally relevant levels of arsenic *in utero* and early post-natally resulted in decreased total collagen in the airway adventitia and increased smooth muscle surrounding the airway at 28 days of age (Lantz et al. 2009). These mice exhibited airway hyperresponsiveness as increased

bronchoconstriction in response to methacholine challenge. The structural alterations were not present in those exposed as adults to arsenic. Since these irreversible findings were identified by immunohistochemistry and methacholine challenge they would not likely be identified by standard toxicology studies.

Arsenic can disrupt the highly complex signaling between embryonic lung tissues of mesenchymal and endodermal origin and can permanently alter lung structure and function when exposure occurs at key developmental windows. Petrick et al (2009) exposed pregnant rats starting at GD1 to 500 ppb arsenic via drinking water and evaluated gene expression in the day 18 fetal lung. Arsenic exposed pups had smaller lung weights than controls. Expression of key genes in pathways involved in lung development was altered by arsenic exposure, including the β -catenin pathway which is required for proper cell migration during branching morphogenesis. Deletion of the *β -catenin* gene in epithelial cells resulted in a lack of peripheral lung structures (Mucenski et al., 2003). Thus, altered signaling by arsenic exposure results in poor airway structure. Malformed airways are characteristic of bronchiectasis, which is observed in humans exposed to arsenic via drinking water

Di(2-ethylhexyl) phthalate

The phthalate ester plasticizers interact with the nuclear hormone receptor superfamily Peroxisome Proliferator Activated Receptor (PPAR), and are thereby capable of modulating gene transcription in a number of tissues.

There are significant levels of PPAR γ in human lung (Chang and Szabo 2000), and di(2-

ethylhexyl) phthalate (DEHP) metabolites can bind to these receptors. Epithelial cell PPAR γ has been shown through the use of transgenic mice to be directly involved in lung maturation, probably through altering airway cell differentiation and resultant phenotype (Simon et al. 2006). Lungs of mice whose conducting airways are deficient in PPAR γ develop abnormally and have enlarged airspaces, and altered lung mechanics, possibly due to altered epithelial-mesenchymal interactions during development.

Rats exposed during the last week of pregnancy and first two days postnatally via oral administration of 1000 mg/kg-day DEHP to the dams had abnormal lung histology (Magliozzi et al. 2003). Focal thickening of primary septa was evident, and there were more dilated air spaces in the parenchyma in treated pups, resulting in a decrease in gas exchange surface. Type II pneumocytes, which are a major source of surfactant, were enlarged and more numerous in treated relative to control pups. The authors note that lung parenchymal changes were similar to that seen in children with chronic lung disease and in animal models of bronchopulmonary dysplasia. The histopathology suggests impaired alveolar maturation and not tissue degeneration. Magliozzi et al. also note that although type II pneumocytes were more numerous and larger in the DEHP treated pups, the peroxisomes in the alveolar type II pneumocytes of treated pups appeared unaffected, and suggest that DEHP action in the fetal lung is not dependent on interaction with the PPAR α . The effect might be due to interaction with PPAR γ to alter surfactant production, secretion or reabsorption by type II cells. It has been shown that PPAR γ ligands can downregulate surfactant protein B expression in alveolar type II cells (Yang et al, 2003). DEHP may affect alveolar development by disrupting the timing of

epithelial and mesenchymal cell proliferation (Rosicarelli and Stefanni 2009). DEHP treatment of the dams was associated with both an impaired secretion pattern and altered proliferation rate of septal myofibroblasts. A nested case control study of 198 children with allergic symptoms and 202 controls identified a statistically significant association between DEHP levels in household dust and doctor diagnosed asthma (Bornehag et al. 2004). The interactions of the ubiquitous phthalate ester plasticizers such as DEHP with PPAR γ during lung development need further study.

Conclusion

The lung is susceptible to many influences during early development including endogenous hormones, pharmaceuticals, and environmental chemicals. Chemical exposure during developmental windows may produce lifelong structural and functional alterations; some may become apparent only later in life (e.g., as lung function naturally declines with age). Susceptible maturational events occur throughout prenatal development, postnatally, and up through adolescence. Evidence is accumulating that clinically significant disruption of lung development may be caused by some xenobiotics at environmentally relevant doses (e.g., arsenic, ozone). Nonetheless, there is a paucity of literature evaluating the impact of early life exposure to environmental chemicals on lung structure and function.

Many fundamental biologic processes (e.g. branching morphogenesis) and associated signaling events involved in development of multiple organs are highly conserved. A variety of transcription factors and morpho-regulatory molecules essential to these

processes are susceptible to interference during critical developmental stages. Examples presented in this paper highlight the potential of a chemical to impact development in multiple organs that utilize the same fundamental patterning and developmental building blocks. Although local tissue differences may alter the impact of signaling disruption, a chemical which is identified as having the potential to disrupt fundamental processes in one organ (e.g., dioxin disruption of branching morphogenesis in the prostate) should be evaluated appropriately for similar impacts in the lung. In addition, chemicals structurally similar (e.g., PCBs) to those known to affect the developing lung (e.g., nitrofen) should be evaluated with appropriate studies for their impact on lung development.

Studies to determine the potential toxicity of xenobiotics resulting from early life exposures should incorporate knowledge of early signaling events into experimental protocols. Evidence from animal studies indicates that many lung alterations induced by environmental chemicals require functional and/or highly specific studies targeted at identifying alterations of structure or function. Many of the functionally significant impacts of early life exposures on lung development discussed in this paper would not have been identified with standard toxicologic study protocols.

Risk assessment practice should utilize data on disruption of basic developmental processes to inform the size of applied uncertainty factors. When there is evidence that a chemical can disrupt relevant signaling pathways, but there are inadequate developmental toxicity data, uncertainty related to this data gap should be reflected in the assessment.

For example, the risk assessor could increase the size of the intraspecies uncertainty factor used in noncancer risk assessment to account for increased sensitivity of early life stages.

US EPA's Strategic Plan for Evaluating the Toxicity of Chemicals (U.S. EPA 2009) and the National Academy of Sciences report on Toxicity Testing in the 21st Century (National Research Council 2007) envision a transformation in the approach to toxicity testing that focuses on "toxicity pathways". Toxicity pathways are defined in the NAS report as "cellular response pathways that, when sufficiently perturbed in an intact animal, are expected to result in adverse health effects". These cellular pathways include key signaling pathways in development. The EPA document notes that "an inventory of toxicity pathways and their involvement in a variety of toxicological responses needs to be created". We suggest that the key signaling pathways in branching morphogenesis (e.g. a highly conserved fundamental developmental process that utilizes similar signaling pathways across multiple organs) represent important toxicity pathways. Evidence that a chemical interferes with an important signaling event should inform decisions on relevant endpoints for studies of developmental toxicity, and help identify chemical groupings for which a cumulative evaluation may be appropriate because they impact the same toxicity pathway. Ultimately, knowledge of the impacts of xenobiotics on lung development can be utilized to develop policies promoting true primary prevention of chronic obstructive pulmonary disease, asthma, and other lung diseases.

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Table 1 Cellular, structural, and functional impacts on lung development of xenobiotics.

Chemical	Cellular and subcellular level impacts	Structural or functional impact	Possible clinical implications	References
Nitrofen 2,4-dichloro- <i>p</i> -nitrophenyl-ether	Downregulates GATA 6 Wnt7, BMP4, FGF, retinal dehydrogenase2; decreases retinoic acid synthesis; inhibits T3 receptor binding	Decreased branching, altered smooth muscle, alteration in surfactant and alveolar septation	Pulmonary hypoplasia, immature lung	Pongracz and Stockley 2006; Shu et al. 2002; Wang et al. 2005; Takayasu et al. 2007; Montedonico et al. 2008; Major et al. 1998; Mendelsohn et al. 1994; Brandsma et al. 1994; Leinwand et al. 2002
TCDD	Arylhydrocarbon receptor, thyroid hormone	Delayed lung development, decreased total lung space, increased septal area	Chronic bronchitis*, decreased functional capacity, COPD?	Kransler et al. 2009; Ten Tuscher et al. 2001; Rogan et al. 1988*
Nicotine	Suppresses glycolysis and glycogenolysis; reduces synthesis of phosphorylase and phosphofructokinase; inhibits Na ⁺ K ⁺ ATPase	Slower septal formation, bleb formation, decreased number alveoli, increased alveolar volume	Decreased functional capacity; emphysematous changes	Maritz 1988, 1986 2002; Kordom et al. 2002; Kordom 2004; Maritz and Windvogel 2003
4-ipomeanol, naphthalene, 1-nitronaphthalene	Inhibits bronchiolar cell differentiation and repair	Injury/loss of Clara cells	Increased susceptibility to inhaled toxicants, alteration in surfactant	Plopper et al. 1994; Smiley-Jewel et al. 1998 2000; Fanucchi et al. 1997 2004
Ozone	Depletes proteoglycan and Fgf-2, alters FGF receptor-1, thinning basement membrane zone	Altered bronchiolar growth (longer/decreased diameter), fewer branches, alteration in orientation bronchiolar smooth muscle	Increased airway hyper-reactivity*, emphysema? decrease in lung function growth*, asthma induction*	Tager et al. 2005*; Tran et al. 2004; Fanucchi et al. 2006 Plopper et al. 2007; McConnell et al. 2002*, Galizia and Kinney 1999*

*Includes evidence in humans

Table 1 cont.

Chemical	Cellular and subcellular level impacts	Structural or functional impact	Possible clinical implications	References
Arsenic	Increases estrogen receptor alpha expression; dysregulates matrix genes, β -catenin up regulates EGFR, L-myc and AFP-	Altered branching and cell migration, decreased elasticity, structural support	Bronchiectasis*, airway hyperreactivity; lung cancer*	Von Ehrenstein et al. 2005*; Guah Mazumder 2007*; Petrick et al. 2009; Waalkes et al. 2003; Smith et al. 2006*; Shen et al. 2007; Hays et al 2008; Lantz et al. 2009
Di(2-ethylhexyl) Phthalate	Binds to PPAR γ altering airway cell differentiation surfactant protein production	Thickened primary septa, fewer/more dilated airspaces, increased type II pneumocytes	Bronchopulmonary dysplasia, altered lung mechanics; altered surfactant regulation; asthma*	Chang and Szabo 2000; Yang et al. 2003; Maggliozi et al. 2003; Rosicarelli and Stefanini 2009; Bornehag et al. 2004*

*Includes evidence in humans

Figure Legends

Figure 1

Predicted mean values for lung function in males at ages 11, 16, and 22 years by length-adjusted infant lung function measured as maximal expiratory flows at functional residual capacity ($V_{max\text{fre}}$)

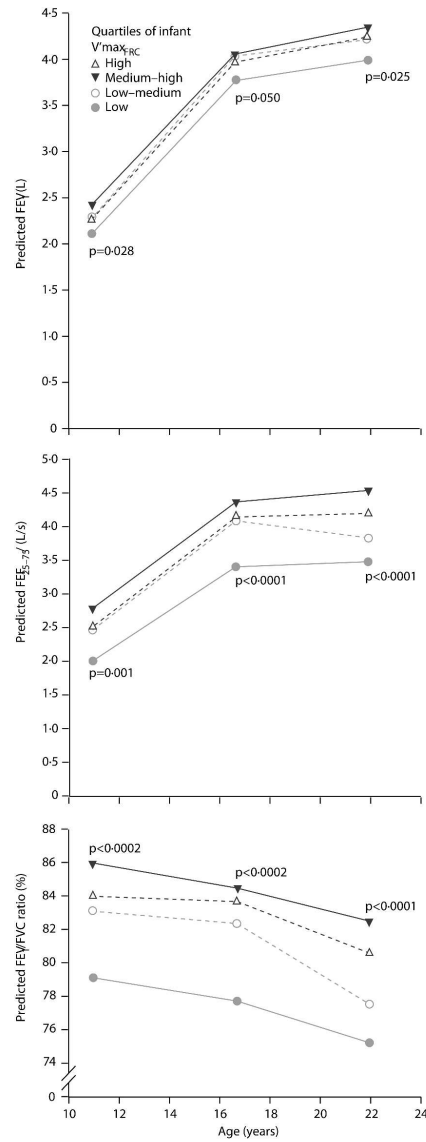
14 % of variance in lung function of young adults was related to airway function at 2 months. Predicted values were standardized to the mean height and weight for male participants at ages 11, 16, and 22 years. (Reprinted from *The Lancet* 370: Stern et al. Poor airway function in early infancy and lung function by 22 years: a non-selective longitudinal cohort study. Pg 761, copyright 2007 with permission from Elsevier)

Figure 2

Principal stages of lung development in humans. Diagrammatic representations of the time-line and developmental organization of trachea, primary bronchi, intrapulmonary bronchi and acinus in the mammalian respiratory system. (Reprinted from *Pharmacology and Therapeutics* (114), R. Kajekar, Environmental factors and developmental outcomes in the lung. 129-145, 2007 with permission of Elsevier)

Figure 3

Diagrammatic comparison of differences in the size of one generation of distal bronchiole in the left cranial lobe of infant rhesus monkeys (180 days of age) following 11 cycles of exposure to filtered air (FA), house dust mite allergen (HDMA), ozone (O₃) or both (HDMA+O₃). The airway measured is the bronchiole proximal to the terminal bronchiole in the axial airway path of the caudal segment of the left cranial lobe of each animal. Relative scaling for length (l) and diameter (d) is based on setting the value for 30-day-old animals (when exposure began) equal to "1". (From Plopper et al., *Toxicologic Pathology* 35 (1), figure 5, pg 101, copyright 2007. Reprinted by permission of SAGE publications.)

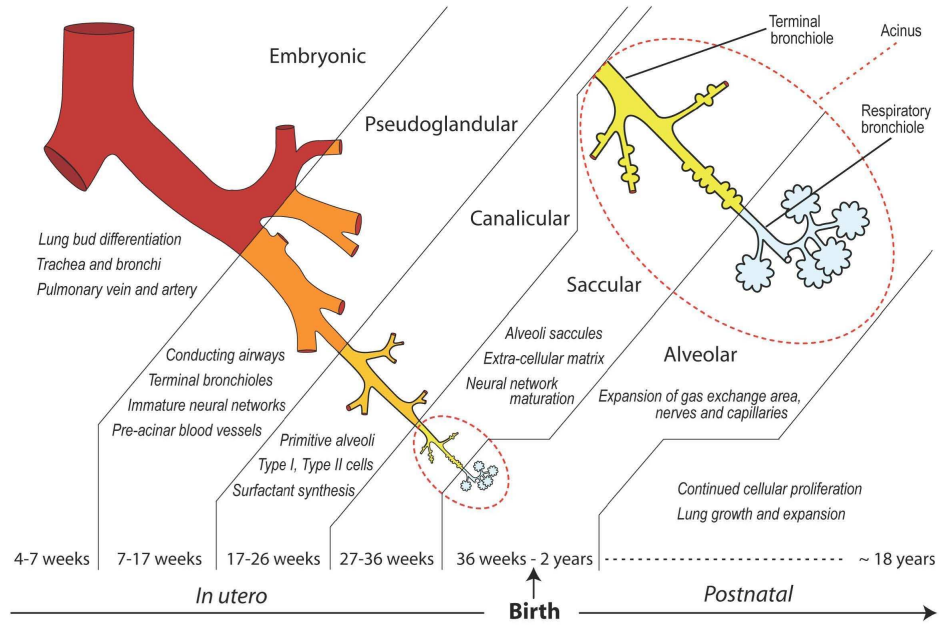


Predicted mean values for lung function in males at ages 11, 16, and 22 years by length-adjusted infant lung function measured as maximal expiratory flows at functional residual capacity (V_{max_frc})

14 % of variance in lung function of young adults was related to airway function at 2 months. Predicted values were standardized to the mean height and weight for male participants at ages 11, 16, and 22 years. An interaction term between survey (age 11, 16, and 22) and quartiles of infant V_{max_frc} was included in the random-effects models. (Reprinted from The Lancet 370: Stern et al.

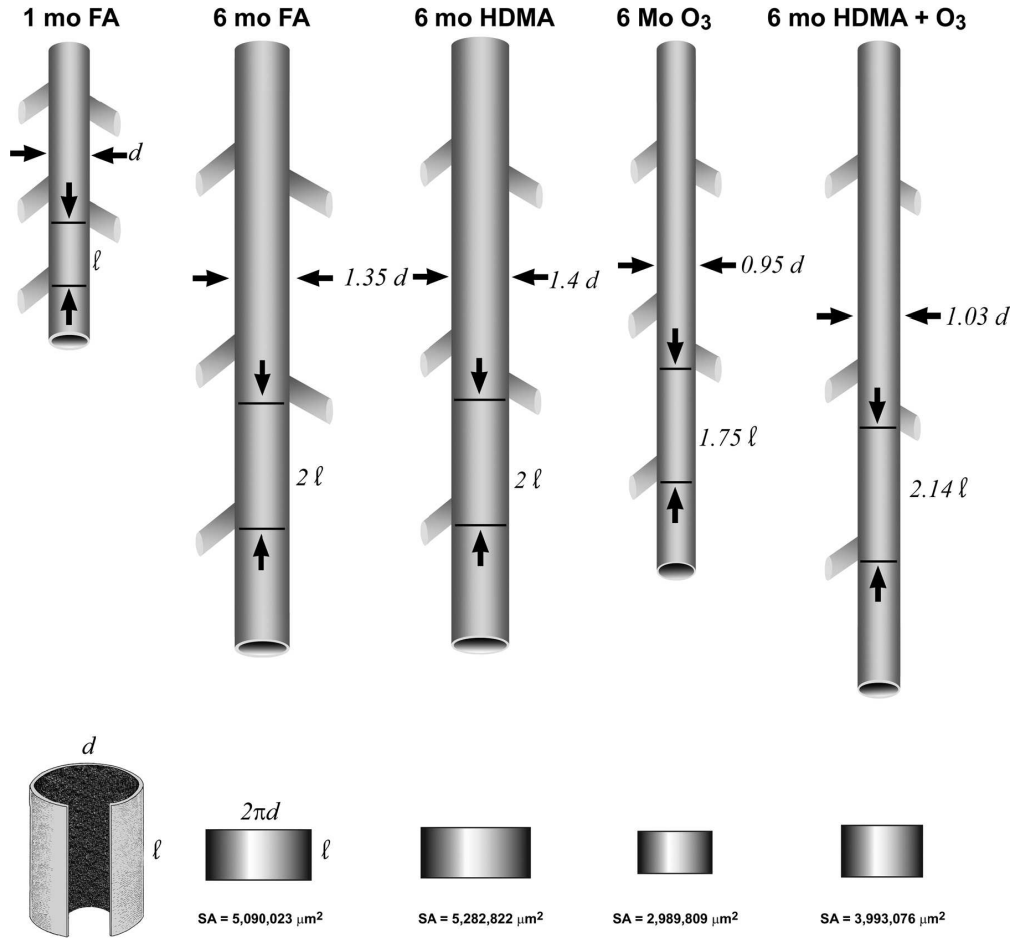
Poor airway function in early infancy and lung function by 22 years: a non-selective longitudinal cohort study. Pg 761, copyright 2007 with permission from Elsevier)

90x208mm (600 x 600 DPI)



Principal stages of lung development in humans. Diagrammatic representations of the time-line and developmental organization of trachea, primary bronchi, intrapulmonary bronchi and acinus in the mammalian respiratory system. (Reprinted from Pharmacology and Therapeutics (114), R. Kajekar, Environmental factors and developmental outcomes in the lung. 129-145, 2007 with permission of Elsevier)

279x216mm (227 x 227 DPI)



153x142mm (299 x 299 DPI)