Molecular Basis of Pulmonary Disease

Insights from Rare Lung Disorders

Edited by

Francis X. McCormack, MD
Ralph J. Panos, MD
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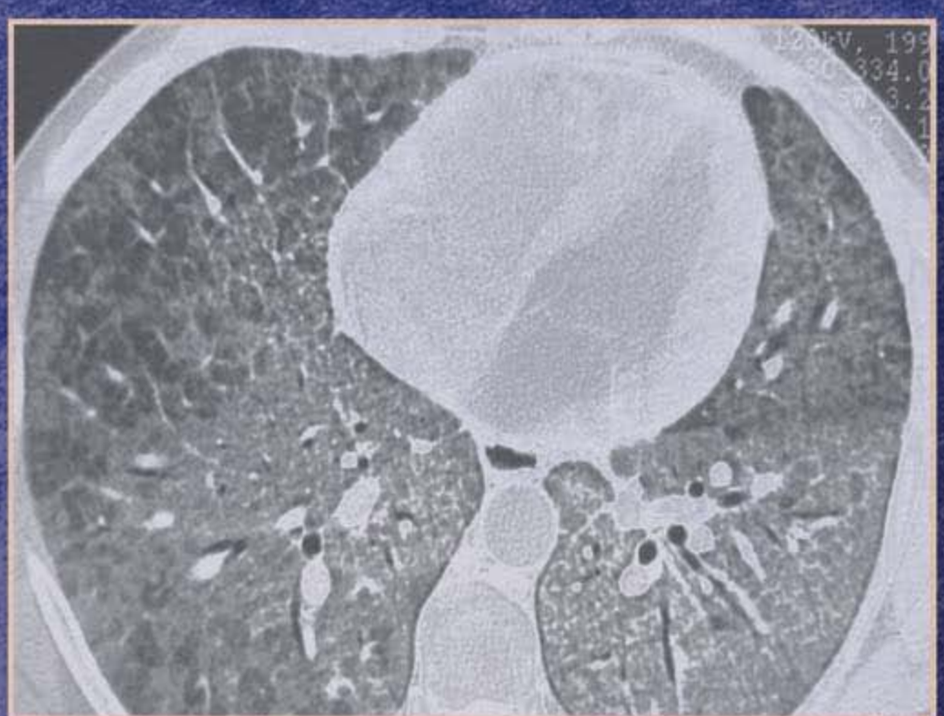

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Molecular Basis of Pulmonary Disease

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Preface

Dr. Sharon Rounds, the editor for this series who invited us to write a book on rare lung diseases, developed the idea after attending the 2004 Lymphangioleiomyomatosis (LAM) Foundation annual research meeting. She was a keynote speaker at that event (during her tenure as the president of the American Thoracic Society) and was witness to the power of patient advocacy and the mission-based scientific effort that had brought this rare disease of women from obscurity to clinical trials with targeted molecular therapies in under a decade. The progress in pulmonary alveolar proteinosis (PAP), pulmonary alveolar microlithiasis (PAM), inherited disorders of surfactant metabolism, and pulmonary arterial hypertension, to name a few, has been no less astounding. Advances have come from the most surprising directions; fruit flies for LAM, genetically engineered mice made for other purposes for PAP, and groundbreaking high-density SNP (single-nucleotide polymorphism) analyses done on a handful of families for PAM. In many cases, insights into biology gained from rare diseases have informed research approaches and treatment strategies for more common diseases; for example, knowledge gained from the study of PAP about the role of GM-CSF in the lung has sparked interest in the use of anti GM-CSF approaches to control both pulmonary and extrapulmonary inflammation in a variety of diseases. The finding that interstitial lung disease develops in families with cytotoxic mutations in surfactant protein C (SP-C), a gene which is expressed only in alveolar type cells, has underscored the importance of the integrity of the alveolar epithelium in the pathogenesis of parenchymal fibrosis. Opportunities to approach lung disease pathogenesis from the vantage point of a primary molecular defect are gifts from nature that are uniquely abundant among the rare lung disorders.

We salute the NIH and the National Center for Research Resources for their vision in facilitating the translation of basic research advances in rare lung diseases into clinical reality through the Rare Lung Disease Consortium, a network of 13 US and international sites that is currently conducting clinical trials and studies in LAM, alpha one antitrypsin deficiency, pediatric interstitial lung disease, and PAP. It has been a rare privilege to work on such fascinating diseases with such capable investigators from all over the world over the past 6 years.

The format for this volume is unique. Most chapters have been authored by a clinician and a basic scientist who are expert in the disease topic and underlying molecular defect, respectively. Their charge was to focus on the genetic basis and molecular pathogenesis of disease, animal models, clinical features, diagnostic approach, conventional management and treatment, and future therapeutic targets and directions. The intent was not to provide a broad overview, but rather to shed light on the molecular mechanisms that evoke the clinical presentation and engender treatment strategies for each disease. We hope that this approach will prove useful for pulmonary clinicians and scientists alike.

We thank our wives, Holly, Jean, and Vicky, for their support and indulgence with late night emails and work-filled weekends, Dr. Rounds for the invitation to write the book, and all of the authors who contributed.

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A Clinical Approach to Rare Lung Diseases

Ralph J. Panos

When you hear hoofbeats behind you, don't expect to see a zebra.
Theodore E. Woodward, MD, University of Maryland, Circa 1950 (1)

Abstract The National Institutes of Health Office of Rare Diseases (ORD) defines a rare or orphan disease as a disorder with a prevalence of fewer than 200,000 affected individuals within the United States whereas in Europe, rare diseases are defined as those disorders that affect 1 or fewer individuals per 2,000 persons. Several consortia exist for the compilation of rare lung disorders: the British orphan lung disease (BOLD) registry, the British pediatric orphan lung disease (BPOLD) registry, the French Groupe d'Etudes et de Recherche sur les Maladies Orphelines Pulmonaires (GERM"O"P") database, and the Rare Lung Disease Consortium (RLDC) in the United States. The National Organization for Rare Diseases (www.raredisease.org) is a nongovernmental federation of organizations to assist individuals with rare diseases that seeks to expand recognition and treatment of individuals with these rare illnesses. This chapter presents an approach to pulmonary medicine that aims to go beyond the usual respiratory disorders to examine the evaluation and understanding of rare lung diseases that have provided extraordinary insights into not only lung function in health and disease but also human biology in general. The respiratory history, physical examination, chest imaging, and related studies are reviewed. The emphasis of this chapter is the formulation of a differential diagnosis that encompasses rare noninfectious, nonmalignant lung diseases of adults and is based on the presence or absence of associated signs and symptoms.

Keywords: rare lung disease, respiratory history, respiratory physical examination, chest imaging

Introduction

In medicine, "zebra" is a common idiom for a rare disease or condition that may be conspicuously noticeable among the herd of common disorders or, more frequently,

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hidden amidst their thundering hooves. When confronted with hoof beats – a patient's constellation of symptoms, signs, and other studies – most physicians consider the simplest and most common diagnosis as the likely cause. This principle of parsimony is based on methodological reductionism and was developed by William of Ockham, a 14th century English logician and Franciscan friar. Ockham's razor, *Entia non sunt multiplicanda praeter necessitatem* (entities should not be multiplied beyond necessity), is a central premise in medical diagnosis *(1)*. In the current medical environment of history and physical examination templates, the physician is frequently presented with a delimited database that constrains the development of a comprehensive differential diagnosis – not only are zebras excluded but the hoofbeats of the herd of horses have been muffled. The time to search for zebras in the busy, frenetic, clinical environment is a luxury that few pulmonologists enjoy. Thus, in many ways, a clinical approach to rare lung diseases is an oxymoron. The concept that common things happen commonly is inculcated into our medical being from medical school onward and reinforced by regimented, templated patient assessments guided by required, bulleted, billing-based guidelines that limit and restrict the formation of an unbiased and comprehensive database from which an expansive differential diagnosis is developed – one that includes the zebras.

The vast spectrum of medical diagnoses is constantly expanding with the recognition and publication of approximately five new disorders each week *(2)*. In the United States, approximately 25 million people are afflicted with over 6,000 rare diseases *(3)*. The National Institutes of Health Office of Rare Diseases (ORD) defines a rare or orphan disease as a disorder with a prevalence of fewer than 200,000 affected individuals within the United States. The ORD maintains a web-based, searchable list of over 7,000 rare diseases with links to various information sources. The National Organization for Rare Diseases (www.rarediseases.org) is a nongovernmental federation of organizations to assist individuals with rare diseases that seeks to expand recognition and treatment of individuals with these rare illnesses. In Europe, rare diseases are defined as those disorders that affect 1 or fewer individuals per 2,000 persons. Orphanet is a European database of nearly 6,000 rare disorders (www.orphan.net). In addition to these general collections of rare diseases, there are several databases limited to rare lung disorders: the British orphan lung disease (BOLD) register was established in 2000 for adult rare lung diseases in the United Kingdom (www.brit-thoracic.org.uk/ClinicalInformation/RareLungDiseasesBOLD/tabid/110/Default.aspx); the British pediatric orphan lung disease (BPOLD) is a registry of nine rare pediatric lung disorders in the United Kingdom (www.bpold.co.uk); and the Groupe d'Etudes et de Recherche sur les Maladies Orphelines Pulmonaires (GERM"O"P") has established a database of patients with rare lung diseases in France (http://germop.univ-lyon1.fr/). In the United States, the Rare Lung Disease Consortium (RLDC) (www.rarediseasesnetwork.epi.usf.edu/rldc/index.htm) was founded in 2003 with collaborating centers throughout the United States and Japan. The RLDC has ongoing clinical trials in several rare lung diseases including lymphangioleiomyomatosis, alpha-1 antitrypsin deficiency, and idiopathic pulmonary fibrosis.

This chapter is an introduction to a safari in pulmonary medicine that aims to go beyond the usual pulmonary disorders to examine the evaluation and understanding of rare lung diseases – the zebras – that have provided extraordinary insights into not only lung function in health and disease but also human biology in general. The evaluation of all patients begins with the history and physical examination. For those individuals

with respiratory symptoms, chest imaging and physiologic studies provide further information to discern the underlying process. The role of the clinical history and pulmonary signs and symptoms as well as chest imaging in the evaluation and diagnosis of respiratory disorders has been reviewed in most textbooks of pulmonary medicine and radiology. We will briefly review the respiratory history, physical examination, chest imaging, and related studies. The emphasis of this chapter is the formulation of a differential diagnosis that encompasses rare noninfectious, nonmalignant lung diseases of adults and is based on the presence or absence of associated signs and symptoms. Environmental exposures, pneumoconioses, and drug-induced pulmonary disorders are not discussed. Many processes are limited strictly or principally to the lungs, and for these disorders, the radiographic imaging, physiologic, other laboratory studies, and genetic testing may be essential for the identification of the underlying disease. Table 1.1 presents a listing of rare lung disorders or conditions that are limited principally to the lungs. Other disorders affect the lungs and other organ systems. For these processes, the key to the diagnosis is the recognition of associated constellations of symptoms that affect the lungs as well as another system or systems. Tables 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 1.10, and 1.11 present differential diagnoses of lung disorders based on associated organ involvement.

Diagnostic Evaluation

The diagnostic evaluation of a patient with suspected lung disease requires a logical sequential series of steps to distinguish the myriad potential causes of pulmonary pathology. The initial approach should include a comprehensive history, physical examination, chest X-rays, and pulmonary function testing.

History

Although most clinicians do not initiate their clinical evaluations looking for rare pulmonary processes, a comprehensive, logical, and sequential evaluation is essential in the evaluation of rare or complex pulmonary disorders. The initial and most important step in this assessment is a comprehensive clinical history to determine the pulmonary symptoms and any associated systemic clues to the etiology of the underlying process. The most frequent presenting respiratory symptoms include breathlessness, cough, chest discomfort, and respiratory sounds or noises. Specific qualities of these presenting symptoms such as onset, duration, location, quality, aggravating or alleviating factors, and associated respiratory or systemic manifestations may help establish a specific diagnosis or limit the differential diagnosis. Occasionally, patients subtly adapt their lifestyle, such as decreasing activity level to minimize or alleviate the sensation of breathlessness. The astute clinician must often delve beyond the initial presenting symptoms to determine whether the patient is attempting to compensate for insidiously progressive respiratory processes. Not infrequently, patients are referred for pulmonary evaluations for an abnormal chest imaging or physiologic study. These patients may or may not have respiratory symptoms.

Breathlessness

Dyspnea is a subjective sensation of abnormal, awkward, or uncomfortable breathing that integrates the subjective perception of breathing *(4)*. Terms used by patients to describe dyspnea include breathlessness, heavy breathing, suffocation, chest tightness, air hunger, and choking. Self-limited, expected breathlessness occurs normally. After strenuous exertion most individuals experience mild shortness of breath that is subsequently relieved with rest. In an individual patient, it may be difficult to discern expected from unanticipated breathlessness. Severity of breathlessness may be difficult to assess as the perception of breathlessness may vary between individuals and over time in a single individual.

The chronicity and onset of breathlessness are important variables in discerning the etiology of dyspnea. Breathlessness that occurs with sudden onset is often due to infections, pulmonary embolism, pneumothorax, or bronchospasm. Breathlessness that develops slowly over time is most often associated with progressive pulmonary processes such as interstitial lung disease, pulmonary vascular disease, or obstructive lung disease. Provocative factors such as plants, pets, or odors may suggest bronchospasm or asthma.

Causes of breathlessness include many non-pulmonary processes including cardiac, metabolic, and hematologic disorders *(5)*. In two-thirds of 85 patients who presented to a pulmonary subspecialty clinic, breathlessness was due to asthma, chronic obstructive pulmonary disease, or cardiomyopathy *(6)*. Interestingly, the clinical impression based on the history, physical examination, and chest X-ray was accurate in 81% of patients when the cause of dyspnea was one of these processes but decreased to 33% for less common causes.

Cough is a protective reflex that eliminates secretions and foreign materials from the airways. The cough reflex is initiated by irritant receptors throughout the airways and extra pulmonary sites including the pleura, pericardium, auditory canals, perinasal sinuses, stomach, and diaphragm. These sensory neurons are triggered by inflammatory, mechanical, chemical, and thermal stimuli; the central nervous system cough center is activated; and motor neurons initiate a forceful exhalation.

The presence of a cough for less than 3 weeks suggests an acute process whereas a longer duration defines a chronic cough. Acute cough is more frequently due to infections but occasionally cardiac disease, pulmonary edema, or pulmonary embolism may be the cause. Common etiologies of chronic cough include smoking-related lung disease, postnasal drainage, asthma, and gastroesophageal reflux. Algorithms for the evaluation and management of patients with chronic cough have been established *(7)*.

The etiology of cough can also be determined by the characteristics of the cough especially whether it is productive or dry and hacking in nature. Productive coughs most frequently suggest an infectious etiology. Hemoptysis may be associated with a bleeding diathesis or anatomic pulmonary abnormality that causes disruption of the normal pulmonary vasculature or mucosa, such as neoplasm, vasculitis, or tissue-destroying infection.

Chest Discomfort

Chest discomfort may originate anywhere in the thorax other than within the lung parenchyma which does not contain pain fibers. Potential origins of chest discomfort include the visceral and parietal pleura, diaphragm, chest wall, muscles, skin, and

other thoracic structures especially the heart, pericardium, and mediastinum. Noncardiac chest pain is infrequently diagnostic but may help to localize an anatomic abnormality that may be visualized with chest imaging.

Respiratory Sounds or Noises

Sounds that may be heard by patients without a stethoscope include snoring, wheezing, and stridor. Snoring is usually a coarse low-pitched sound that occurs during sleep and is strongly suggestive of obstructive sleep apnea or diminished upper airway airflow during sleep. Wheezing is a high-pitched musical sound that is more frequently heard during expiration than inspiration. It usually indicates obstructive airway disease including asthma and chronic obstructive pulmonary disease. Localized wheezes suggest endobronchial obstruction. Stridor is a loud, harsh sound that may occur either during inspiration or expiration. Inspiratory stridor suggests an extrathoracic cause whereas expiratory stridor suggests an intrathoracic etiology. Obstruction of airflow due to intrabronchial lesions, edema of the upper airway, or dynamic airway collapse may cause stridor.

Medical History

The past medical history is an important source of information about systemic processes that may also involve the lung. Associated previous or concurrent systemic medical conditions may also help formulate the differential diagnosis. Some processes intermittently involve different systems or are in evolution and require serial observation.

Family/Social History

The family history and social history may elicit genetic factors or other triggers that might cause the development of lung disease. The family history is an important source of information about familial processes that may affect the lungs. These diseases include cystic fibrosis, alpha-1 antitrypsin deficiency, hereditary telangiectasia, pulmonary fibrosis, and surfactant protein mutations (discussed in detail in Chapters 6, 7, 9, 11, and 16).

Occupational/Environmental History

Particular emphasis should be placed on the patient's occupational and environmental exposures and, occasionally, the spouse's occupational history *(8)*. Obtaining a chronologic listing of all positions held by a patient generates a comprehensive employment resume. The occupational history elicits not just the job title but the actual duties and tasks as well as a comprehensive list of all vapors, gases, dust, or fumes in the work environment. Occasionally a spouse may be exposed to particles such as asbestos fibers that are transported from the job place to the home on the partner's work clothes. The home environment including pets, mold, mildew, down bedding or chemical, fumes, or dusts generated while performing hobbies may also be the source of exposures that may induce various pulmonary disorders.

Review of Systems

A comprehensive systemic review is also extremely useful in complex lung diseases because it may identify associated manifestations that may not be recognized by either the patient or referring physicians. It is often these associated non-pulmonary signs or symptoms that provide the essential clue to the diagnosis of a rare or unusual pulmonary disease. The development of comprehensive differential diagnoses of lung processes based on the presence or absence of associated symptoms is reviewed in the latter portion of this chapter (Tables 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 1.10, and 1.11).

Physical Examination

A thorough physical examination complements the comprehensive history. The examination of patients with respiratory symptoms usually focuses on the chest findings but a comprehensive physical examination is important to determine the presence of a systemic process. The physical examination begins with the vital signs which should include the respiratory rate and oxygen saturation. The four principal parts of the chest examination are inspection, palpation, percussion, and auscultation.

Inspection

Respiratory pattern and rate are assessed initially. Respiratory distress can be identified through the use of accessory muscles, body position during breathing, and use of intercostal muscles. Abnormal respiratory patterns include tachypnea (rapid shallow breathing), hyperpnea (rapid deep breathing), bradypnea (slow breathing), and Cheyne–Stokes respirations (rhythmic breaths in a crescendo–decrescendo pattern that may include apneic episodes). Biot's breathing (ataxic breathing) is an uncommon variant of Cheyne–Stokes respirations in which apneic events are irregularly interspersed among breaths of nonvarying depth and may be associated with meningitis. During inspection, the shape and contour of the thorax is assessed for abnormalities of the thoracic wall such as kyphosis, scoliosis, or pectus excavatum.

Palpation and Percussion

Excursion of the chest wall is determined by feeling the expansion of the chest during inspiration. Asymmetry may suggest an abnormality of the underlying chest wall, pleura, or lung. Palpation can also determine the presence of chest wall masses, lesions, or other abnormalities such as a flail chest. Pneumothorax, pleural effusion, or mediastinal mass may cause lateral deviation of the trachea. Vibratory palpation or tactile fremitus is increased with pulmonary consolidation due to pneumonia or atelectasis but is reduced with pleural effusions or pneumothorax. Percussion is dulled by the loss of aerated pulmonary parenchyma caused by pleural effusion, consolidation, or atelectasis. Hyperresonance or tympany may occur with emphysema, large bullae, or pneumothorax.

Auscultation

Movement of air throughout the tracheobronchial tree produces sounds that range from 60 to 3,000 Hz. Auscultation should be performed in the upper and lower lung zones, anteriorly, posteriorly, and laterally. Breath sounds include tracheal, bronchial, bronchovesicular, and vesicular sounds. Vesicular sounds have a long inspiratory component and a short expiratory phase whereas bronchial sounds have a short inspiratory phase and a long expiratory component. Adventitial sounds include rales or crackles, wheezes, and rhonchi. Crackles are irregular, short, explosive sounds and may be classified as fine or coarse. Fine-end inspiratory crackles are strongly suggestive of interstitial processes, whereas expiratory crackles suggest pulmonary edema or fluid accumulation within the lungs. Wheezes are continuous, musical sounds that may occur during inspiration or expiration but are most common during expiration and suggest obstructive lung disease. Rhonchi are continuous low-pitched sounds that are frequently called dry, coarse rales. Sounds may also emanate from the pleura and include friction rubs which are loud coarse sounds with a raspy quality. These suggest thickening or inflammation of the pleura.

Imaging Studies

Chest imaging studies, especially the chest X-ray and CT scan, are increasingly essential in the evaluation and diagnosis of unusual respiratory conditions. The posterior–anterior and lateral chest roentgenogram is most frequently the initial imaging study in the evaluation of a pulmonary process. Methods for interpretation and generation of differential diagnoses of chest X-ray findings are beyond the scope of this chapter and are the subjects of numerous pulmonary and radiology texts. Fluoroscopy provides dynamic imaging of the thorax and may be used to assess diaphragmatic movement during a sniff test. Other radiographic studies such as the barium esophagram or swallowing study are used to detect functional and anatomic abnormalities within the upper gastrointestinal tract.

Computed tomography is more sensitive than the standard chest X-ray for the detection of differences in tissue density and is used to assess the chest wall, pleura and pleural space, lung parenchyma, and mediastinal structures. High-resolution, thin-section computed tomography (HRCT) imaging using collimation less than 2 mm and high-spatial resolution algorithms that are edge enhancing provides detailed images of the lung parenchyma and has revolutionized the approach to diffuse parenchymal processes *(9)*. Many of the idiopathic interstitial pneumonias have distinct HRCT features that match corresponding histopathologic findings *(9, 10)*. However, because of overlapping findings, HRCT has not completely replaced lung biopsies in the diagnosis of interstitial lung diseases. Multidetector spiral computed tomography with intravenous contrast administration and specialized scanning protocols has replaced pulmonary angiography and ventilation–perfusion scanning in the diagnosis of acute pulmonary emboli. Spiral CT permits three-dimensional reconstruction and display of intrathoracic structures including blood vessels and airways that can be used to perform virtual bronchoscopy with a level of resolution approaching direct videobronchoscopy. Chest CT scanning is increasingly being combined with positron emission tomography (PET, discussed below) for the diagnosis of bronchogenic and metastatic neoplasms within the chest.

Although ultrasound is not useful for imaging the lung parenchyma because sound waves are not transmitted well through the gaseous lung tissue, it is frequently used to assess the pleura and pleural space *(11, 12)*. Ultrasound can also be used to guide thoracenteses and transthoracic needle biopsies *(12, 13)*. Ultrasound is also used to detect and diagnosis congenital lung anomalies antenatally *(14)*. Endobronchial ultrasound (EBUS) is performed using a probe incorporated into the bronchoscope or passed through the working channel *(15)*. The diagnostic yield of EBUS-guided transbronchial aspiration is significantly increased for solitary pulmonary nodules (<2 cm) and hilar and mediastinal lymph nodes compared with conventional bronchoscopy *(15)*. Echocardiography provides functional and anatomic assessment of the heart and great vessels. Doppler echocardiography provides a noninvasive measurement of pulmonary artery pressures for the diagnosis and monitoring of pulmonary hypertension.

Although ventilation perfusion scans have been largely replaced by CT scans using a pulmonary angiogram protocol, nuclear studies are preferred for the diagnosis of pulmonary hypertension due to chronic thromboembolism *(16)*. PET scans utilizing fluorodeoxyglucose are increasingly used to determine whether thoracic lesions are neoplastic *(17)*.

Physiologic Studies

Physiologic studies including spirometry, lung volumes, and diffusing capacity (DLCO) as well as measurement of respiratory muscle strength may be helpful in limiting the differential diagnosis of a complex pulmonary process. Pulmonary function testing determines whether a physiologic abnormality of lung function is present. The major categories of physiologic impairment are obstruction, reduced expiratory flows, and restriction, diminished lung volumes. Obstruction may be caused by asthma, emphysema, or chronic bronchitis. Restriction may be due to interstitial lung disease (ILD), pleural processes, or thoracic wall abnormalities. Lung compliance is normal in thoracic wall processes but reduced in ILD. Increases in DLCO suggest increased intrathoracic blood volume or hemorrhage into the lung parenchyma, whereas reduction in DLCO may be due to decreased surface area for gas exchange caused by interstitial lung disease, loss of lung parenchyma (surgery or emphysema), or pulmonary vascular disease. Provocative studies such as methacholine challenge may be used to incite bronchospasm. Measurement of maximal inspiratory and expiratory pressures provides a global assessment of respiratory muscle strength that may be reduced by neuromuscular disease or thoracic wall abnormalities. Other useful studies include arterial blood gases and oximetry that can be performed in different positions or at rest and with exertion.

Cardiopulmonary exercise testing measures the metabolic, cardiovascular, and pulmonary response to incrementally increasing exercise work load and is frequently used to determine the cause of breathlessness, provide pre-operative assessment of lung function, risk stratification in cardiac disease, and assess disability *(18–20)*.

Polysomnography measures cardiopulmonary responses during the various stages of sleep and is used to diagnose sleep disorders such as obstructive and central sleep apnea, narcolepsy, and parasomnias *(21, 22)*. Sleep disorders associated with other processes such as Cheyne–Stokes respiration in congestive heart failure can also be diagnosed during a sleep study. Specialized studies of sleep such as the multiple sleep latency test

or maintenance of wakefulness test can be used in the diagnosis of narcolepsy and other sleep disorders *(23)*.

Other Studies

Based on the comprehensive history and thorough examination as well as preliminary radiographic and physiologic studies, other laboratory studies may be required to determine the cause of a pulmonary disorder.

Analysis of sputum may suggest an infectious process that is confirmed by culture or immunocytologic staining. Papanicolaou staining may demonstrate neoplastic cells. Induced sputum and exhaled breath markers (exhaled nitric oxide and exhaled breath condensate) are also increasingly being used for the diagnosis and management of pulmonary disorders including obstructive and interstitial diseases *(24–28)*. Pleural fluid obtained by thoracentesis is classified as transudative or exudative based on the protein and LDH levels. Transudative pleural effusions are most commonly due to heart, liver, or renal failure but exudative effusions are caused by many different disorders and require further evaluation. In addition to routine biochemical, microbiologic, and cytologic studies, the presence of lupus erythematosis (LE) cells, reduced complement levels, or elevated rheumatoid factor titers can diagnose a connective tissue disease-associated pleural effusion. Chylous effusions are characterized by a triglyceride level above 100 mg/dl. Either closed or pleuroscopic pleural biopsy may be necessary to establish a histopathologic diagnosis.

Skin testing is performed to determine reactivity to various allergens that might cause atopy, asthma, or allergic rhinitis. Reactivity to *Aspergillus* is a diagnostic criterion for allergic bronchopulmonary aspergillosis (ABPA). Current or prior *Mycobacterium tuberculosis* infection may cause a delayed hypersensitivity reaction to purified protein derivative (PPD). Other skin tests are used to diagnose fungal infections. Cystic fibrosis is diagnosed by sweat chloride measurement.

Serologic testing is used to diagnose connective tissue disorders that may have pulmonary manifestations (see Chapter 19), infections especially caused by fungal pathogens, viral infections including human immunodeficiency or hepatitis viruses that are associated with pulmonary hypertension (see Chapter 3). Elevation of IgE levels may suggest atopy, asthma, ABPA, and reductions in complement or immunoglobulin levels may determine the cause of recurrent respiratory infections or bronchiectasis. Other serologic titers include anti-neutrophil cytoplasmic antibody, PR3, MPO, and antiglomerular basement membrane antibody (see Chapter 13).

As the genetic mutations underlying many pulmonary processes are discovered, increasing numbers of molecular genetic studies are available to diagnose pulmonary processes (see Chapters 6, 9, 11, 15, and 16).

Bronchoscopy permits a direct visual inspection of the upper and lower airway and can be used for obtaining samples from the lower respiratory tract by bronchoalveolar lavage, brushings, and biopsy. Bronchoscopy is most useful for the diagnosis of infections and neoplasms and is usually less informative in diffuse lung diseases other than granulomatous processes. Endobronchial ultrasound improves the yield and safety of transbronchial needle aspiration of mediastinal and hilar adenopathy and nodules and frequently obviates the need for mediastinoscopy *(29)*. Open lung biopsy is often required for the diagnosis of diffuse parenchymal lung disease and is frequently

performed by video-assisted thoracoscopic surgery. Nasal epithelial biopsies and ultrastructural imaging may diagnose ciliary disorders.

Pulmonary Differential Diagnosis of Rare or Unusual Conditions

The most essential aspect of the diagnosis of a rare pulmonary disease or condition is the formulation of a comprehensive differential diagnosis – if a process is not considered, it cannot be diagnosed. The presenting pulmonary symptoms and signs provide the initial clues to the identification of the underlying process. Increasingly, pulmonary differential diagnoses are developed from imaging studies, especially chest X-rays and CT scans. Corroborative studies such as serologies, sputum or pleural fluid analyses, lung biopsy, and, most recently, genetic studies establish a definitive diagnosis.

The remaining chapters in this volume present rare lung diseases that have provided extraordinary insight into the biology of the healthy and diseased lung as well as advanced our understanding of basic human biologic processes.

Table 1.1 Rare pulmonary diseases or conditions limited principally to the lungs (excluding neoplasms, infections, and drug or environmental exposures).

Adult congenital lung disease
Bronchopulmonary
Tracheoesophageal fistula
Tracheobronchomegaly (Mounier–Kuhn syndrome)
Congenital bronchiectasis (Williams-Campbell syndrome)
Lung agenesis–hypoplasia complex
Lung, lobe, or subsegment
Bronchial atresia
Lobar emphysema
Bronchial divisional abnormalities
Cystic adenomatoid malformation
Bronchogenic cyst
Vascular
Absence of main pulmonary artery
Anomalous origin of the left pulmonary artery from the right pulmonary artery
Anomalous pulmonary drainage
Pulmonary venous varix
Arteriovenous malformation
Pulmonary specific
Systemic (hereditary hemorrhagic telangiectasia, Osler–Weber–Rendu disease)
Combined parenchymal–vascular
Hypogenetic lung (Scimitar syndrome)
Bronchopulmonary sequestration
Intralobar
Extralobar
Other
Congenital diaphragmatic hernia
Posterior (Bochdalek)

Table 1.1 (continued)

- Anterior (Morgagni)
- Musculoskeletal

Airway/bronchial processes

Upper airway disorders
- Vocal cord dysfunction
- Saber-sheath trachea
- Tracheobronchopathia osteochondroplastica
- Tracheomalacia
- Tracheal polyps
- Obstructive sleep apnea
- Upper airway resistance syndrome

Bronchial processes
- Respiratory bronchiolitis
- Respiratory bronchiolitis interstitial lung disease
- Peribronchiolar metaplasia–interstitial lung disease
- Proliferative bronchiolitis
  - Bronchiolitis obliterans organizing pneumonia
  - Cryptogenic organizing pneumonia
- Diffuse idiopathic pulmonary neuroendocrine cell hyperplasia

Broncholith

Parenchymal processes

Cellular infiltration or accumulation
- Eosinophils
  - Acute eosinophilic pneumonia
  - Chronic eosinophilic pneumonia
- Macrophages
  - Desquamative interstitial pneumonia
- Lymphocytes
  - Lymphocytic interstitial pneumonia
  - Lymphomatoid granulomatosis
  - Angioimmunoblastic lymphadenopathy
  - Follicular bronchiolitis
  - Familial hemophagocytic lymphohistiocytosis

Erythrocytes
- Idiopathic pulmonary hemosiderosis (capillaritis)

Histiocytes
- Langerhans cell histiocytosis (eosinophilic granuloma)
- Erdheim–Chester disease
- Familial hemophagocytic lymphohistiocytosis

Smooth muscle cells
- Lymphangioleiomyomatosis
- Tuberous sclerosis

Neuroendocrine cells
- Diffuse idiopathic pulmonary neuroendocrine cell hyperplasia

Meningothelial cells
- Pulmonary meningotheliomatosis

Noncellular infiltration or accumulation

Table 1.1 (continued)

- Pulmonary calcification and ossification
- Pulmonary alveolar microlithiasis
- Pulmonary alveolar proteinosis
- Surfactant abnormalities
  - SP-B mutations
  - SP-C mutations
  - ABCA3 mutations

Granulomatous infiltration
- Sarcoidosis
- Necrotizing sarcoid granulomatosis
- Berylliosis
- Hypersensitivity pneumonitis
- Talc granulomatosis
- Wegener's granulomatosis
- Churg–Strauss disease
- Bronchocentric granulomatosis
- Hypocalciuric hypercalcemia and interstitial lung disease

Mixed cellular and noncellular infiltration or accumulation
- Idiopathic pulmonary fibrosis
- Acute interstitial pneumonitis
- Nonspecific interstitial pneumonia (cellular and fibrotic)
- Cryptogenic organizing pneumonia (bronchiolitis obliterans organizing pneumonia)
- Respiratory bronchiolitis interstitial pneumonia
- Peribronchiolar metaplasia–interstitial lung disease
- Hypersensitivity pneumonitis
- Radiation pneumonitis/fibrosis
- Pneumoconiosis
- Inhalational lung injury
- Aspiration
  - Lipoid pneumonia

Vascular processes
- Pulmonary hypertension
- Pulmonary embolism
  - Thrombus
  - Septic
  - Amniotic
  - Neoplastic
  - Air
  - Foreign body
- Pulmonary arteriopathy
- Primary pulmonary arteritis
- Thrombotic pulmonary arteriopathy
- Pulmonary veno-occlusive disease
- Pulmonary capillary hemangiomatosis
- Pulmonary infarction
- Pulmonary artery aneurysm
- Bronchial artery aneurysm

Table 1.1 (continued)

Pleural processes
Effusion
Empyema
Hemothorax
Chylothorax
Urinothorax
Fibrothorax
Rounded atelectasis
Familial pneumothorax

Table 1.2 Cutaneous–pulmonary associations.

Disease	Cutaneous manifestation	Pulmonary manifestation
General		
Atopy	Eczema	Asthma
Yellow nail syndrome	Yellow discolored nails that are thicker than normal, excessive curvature on the long axis Onycholysis	Lymphedema Exudative pleural effusion Recurrent sinusitis Bronchiectasis Recurrent pneumonia
Costello syndrome	Redundant skin Papillomata	Lipoid pneumonia
Alpha-1 antitrypsin deficiency	Necrotizing panniculitis	Emphysema, especially panacinar Obstructive lung disease
Infiltrative/accumulative		
Sarcoid	Erythema nodosum Lupus pernio Erythematous or pigmented papules Annular plaque	Lymphadenopathy Interstitial lung disease
Hermansky–Pudlak syndrome	Oculocutaneous albinism	Interstitial lung disease
Tuberous sclerosis	Hypopigmented macules (ash leaf spots) Facial angiofibromas (adenoma sebaceum) Forehead plague “Shagreen” or leather patch Periungual or ungual fibromas (Koenen tumors) Molluscum fibrosum pendulum Café au lait spots Confetti lesions Poliosis Thumbprint macules	Cystic interstitial lung disease

Table 1.2 (continued)

Disease	Cutaneous manifestation	Pulmonary manifestation
Birt–Hogg–Dube syndrome (Hornstein–Knickenberg syndrome)	Fibrofolliculoma Trichodiscomas	Cystic parenchymal disease Pneumothorax
Neurofibromatosis (von Recklinghausen's disease)	Pigmented macules (cafè au lait spots) Neurofibromas Crowe's sign, axillary freckles Lisch nodules, pigmented iris hamartomas	Interstitial lung disease Bullae Mediastinal and intercostals neurinomas Lateral meningocele Pneumothorax
Dyskeratosis congenita	Hyperpigmentation Nail dystrophy Mucous membrane leukoplakia	Interstitial lung disease
Vascular malformations/vasculitis		
Hereditary hemorrhagic telangiectasia (Osler–Weber–Rendu syndrome)	Telangiectases	Arteriovenous malformations
Ataxia telangiectasia	Oculocutaneous telangiectasia	Sino-pulmonary infections Pulmonary fibrosis Pneumothorax
Wegener's granulomatosis	Palpable purpura Subcutaneous nodules Pyoderma gangrenosum-like lesions Oral ulcers Gingival hyperplasia	Granulomatous vasculitis Cavitating pulmonary nodules Upper respiratory tract inflammatory lesions
Microscopic polyangiitis	Nodules Palpable purpura	Nasopharyngeal lesions Alveolar hemorrhage
Churg–Strauss syndrome	Subcutaneous nodules Palpable purpura Erythematous eruption	Asthma Pulmonary infiltrates (sometimes migratory)
Polyarteritis nodosa	Livedo reticularis Ulcers Tender erythematous nodules Bullous or vesicular eruptions Palpable purpura: leukocytoclastic vasculitis	Bronchial arteritis
Connective tissue diseases		
Ehlers–Danlos syndrome	Skin flaccidity Hyperextensibility of the joints	Panacinar emphysema Bullae Pneumothoraces Bronchiectasis Tracheobronchomegaly
Generalized elastolysis (cutis laxa)	Excessive, redundant skin folds	Panlobular emphysema Bronchiectasis Aortic aneurysms

Table 1.2 (continued)

Disease	Cutaneous manifestation	Pulmonary manifestation
Scleroderma	Raynaud phenomenon Cutaneous sclerosis Calcinosis Sclerodactyly Telangiectasia	Interstitial lung disease Pulmonary hypertension
Systemic lupus erythematosis	Butterfly facial rash Discoid lupus Cutaneous vasculitis Mouth ulcers Photosensitivity Livedo reticularis Palpable purpura	Pleuritis Pleural effusion Interstitial lung disease Lymphocytic interstitial pneumonitis Acute pneumonitis Pulmonary hypertension Pulmonary hemorrhage
Dermatomyositis	Gottron's papules Heliotrope rash Periorbital edema Nail fold inflammation	Interstitial lung disease Respiratory muscle weakness
Behcet's disease	Oral and genital ulcers Papules, pustules, plaques Erythema nodosum-like lesions Thrombophlebitis	Pleurisy Pulmonary artery aneurysm
Relapsing polychondritis	Aphtosis Purpura Urticaria Erythema multiforme Angioedema Livido reticularis Panniculitis Migratory superficial thrombophlebitis	Laryngotracheobronchial collapse/obstruction Respiratory infections

Table 1.3 Ophthalomologic–pulmonary associations.

	Ophthalmologic manifestation	Pulmonary manifestation
General		
Atopy	Conjunctivitis	Asthma
Cystic fibrosis	Dilated, tortuous retinal veins Intraretinal hemorrhage Retinal vein occlusion	Cough Dyspnea Wheezing Sputum production Chronic airflow obstruction Recurrent respiratory infections, especially due to *Haemophilus influenza*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* Bronchiectasis Cystic parenchymal changes

Table 1.3 (continued)

	Ophthalmologic manifestation	**Pulmonary manifestation**
X-Linked retinitis pigmentosa	Retinal degeneration Progressive night blindness Loss of peripheral/central vision	Primary ciliary dyskinesia: Bronchiectasis Respiratory infections
Infiltrative/accumulative processes		
Sarcoidosis	Periorbital cutaneous granulomas Lacrimal gland swelling Conjunctival edema/nodules Conjunctivitis Keratoconjunctivitis sicca Episcleritis Scleritis Xerophthalmia Anterior uveitis Extraocular muscle palsies Chorioretinitis Chorioretinal granulomas Vitreous opacities Preretinal infiltrates (string of pearls) Orbital mass	Dyspnea Cough Chest discomfort Hilar adenopathy Parenchymal interstitial opacifications Nodules
Amyloid	Yellow, waxy deposits on lids/conjunctiva Periorbital ecchymoses Lacrimal gland infiltration and swelling Xerophthalmia Extraocular muscle palsies (frozen globe) Optic nerve compression	Endobronchial lesion: postobstructive atelectasis or pneumonia Parenchymal nodules: single or multiple Interstitial/reticulonodular opacifications Mediastinal/hilar adenopathy Pulmonary hypertension
Erdheim–Chester disease	Peri-/Retro-orbital mass and infiltration	Interstitial lung disease
Hermansky–Pudlak syndrome	Oculocutaneous albinism Reduced visual acuity Nystagmus Strabismus Cataract	Interstitial lung disease
Vasculitis		
Polyarteritis nodosum	Periorbital edema Conjunctival edema Hyperemic conjunctiva Nodular episcleritis Necrotizing sclerokeratitis (ring ulcer) Anterior uveitis	Bronchial arteritis

Table 1.3 (continued)

	Ophthalmologic manifestation	Pulmonary manifestation
Wegener's granulomatosis	Periorbital edema Conjunctival edema Hyperemic conjunctiva Nodular episcleritis Necrotizing sclerokeratitis (ring ulcer) Chemosis Epiphora Anterior uveitis	Hemoptysis Alveolar hemorrhage Parenchymal nodules: multiple or solitary; solid or cavitary Infiltrates Pleural effusion Pleural mass Hilar adenopathy
Churg–Strauss syndrome	Episcleritis Panuveitis	Asthma Pulmonary infiltrates (sometimes migratory)
Connective tissue disorders		
Marfan's syndrome	Refractive errors Ectopia lentis	Emphysematous parenchymal abnormalities Pneumothorax
Systemic lupus erythematosis	Conjunctivitis Keratoconjunctivitis sicca Episcleritis Scleritis Anterior uveitis Sclerosing keratitis (ring ulcer)	Interstitial lung disease Pleurisy Effusion Alveolar hemorrhage Shrinking lung syndrome Pulmonary hypertension Thromboembolism: anticardiolipin antibody
Rheumatoid arthritis	Nodular or necrotizing scleritis Sclerosing keratitis (ring ulcer) Limbal guttering Central corneal ulcers Anterior uveitis	Interstitial lung disease Pleurisy Effusion Rheumatoid nodules Bronchiolitis obliterans organizing pneumonia Follicular bronchiolitis
Dermatomyositis	Lid and periorbital edema Heliotrope discoloration of lids Extraocular muscle palsies	Interstitial lung disease Bronchiolitis obliterans organizing pneumonia Respiratory failure due to respiratory muscle dysfunction
Scleroderma	Lid retraction and xerothalmia due to tightened skin	Interstitial lung disease Pleurisy Effusion Aspiration Pulmonary hypertension
Sjogren's syndrome	Xerothalmia/keratoconjunctivitis sicca	Interstitial lung disease Lymphocytic interstitial pneumonitis Xerotrachea Pseudolymphoma/lymphoma
Behcet's syndrome	Iridocyclitis Hypopyon Vitreitis Retinal vasculitis and occlusion Optic disc hyperemia Macular edema	Pulmonary artery aneurysms Pulmonary embolism Pleural effusion Pulmonary hemorrhage/infarction Pulmonary artery occlusion

Table 1.3 (continued)

	Ophthalmologic manifestation	Pulmonary manifestation
Relapsing polychondritis	Scleritis Episcleritis Conjunctivitis Proptosis Periorbital lid edema Uveitis	Laryngotracheobronchial collapse/obstruction Respiratory infections
Primary biliary cirrhosis (associated with Sjogren's syndrome)	Keratoconjunctivitis sicca/xerothalmia	Lymphocytic interstitial pneumonia Pleural effusion

Table 1.4 Otorhinolaryngeal–pulmonary associations.

Disorder	Otorhinolaryngeal manifestations	Pulmonary manifestations
General		
Cystic fibrosis	Polyposis Dilated nasal base Sinus hypoplasia	Cough Dyspnea Wheezing Sputum production Chronic airflow obstruction Recurrent respiratory infections, especially due to *Haemophilus influenza, Staphylococcus aureus, Pseudomonas aeruginosa* Bronchiectasis Cystic parenchymal changes
Primary ciliary dyskinesia	Recurrent/chronic sinusitis Otitis media	Bronchiectasis Respiratory infections
Panbronchiolitis	Sinusitis	Cough Sputum production/bronchorrhea Bronchiectasis
Yellow nail syndrome	Recurrent sinusitis	Lymphedema Exudative pleural effusion Bronchiectasis Recurrent pneumonia
Infiltrative/accumulative disorders		
Sarcoidosis	Nasal crusting Epistaxis Nasal obstruction Yellow submucosal nodules Septal perforation Saddle nose deformity/supratip depression	Dyspnea Cough Chest discomfort Hilar adenopathy Parenchymal interstitial opacifications Nodules

Table 1.4 (continued)

Disorder	Otorhinolaryngeal manifestations	Pulmonary manifestations
Vascular		
Wegener's granulomatosis	Nasal obstruction /ulcerations/ discharge/crusting/adhesions Epistaxis Septal perforation Saddle nose deformity/supratip depression Otitis media	Hemoptysis Alveolar hemorrhage Parenchymal nodules: multiple or solitary; solid or cavitary Infiltrates Pleural effusion Pleural mass Hilar adenopathy
Churg–Strauss syndrome	Polyposis Allergic rhinitis Nasal crusting Otitis media Sensorineural hearing loss	Asthma Migratory infiltrates
Connective tissue		
Rheumatoid arthritis	Cricoarytenoid arthritis Conductive and sensorineural hearing loss	Interstitial lung disease Pleurisy Effusion Rheumatoid nodules Bronchiolitis obliterans organizing pneumonia Follicular bronchiolitis
Systemic lupus erythematosis	Mucosal ulcerations Septal perforation	Interstitial lung disease Pleurisy Effusion Alveolar hemorrhage Shrinking lung syndrome Pulmonary hypertension Thromboembolism: anticardiolipin antibody
Relapsing polychondritis	Auricular/nasal chondritis Sensorineural hearing loss Saddle nose deformity/supratip depression	Laryngotracheobronchial collapse/obstruction Respiratory infections

Table 1.5 Gastrointestinal–pulmonary associations.

	Disorder	GI manifestation	Pulmonary manifestation
Esophagus	Tracheal–esophageal fistula Achalasia Stricture Primary Acquired Zenker's diverticula Hiatal hernia Gastroesophageal reflux	Dysphagia Reflux Water brash	Pneumonia Recurrent infections Aspiration Hoarseness Cough Wheezing Interstitial lung disease Idiopathic pulmonary fibrosis

Table 1.5 (continued)

	Disorder	GI manifestation	Pulmonary manifestation
Stomach	Sarcoid	Ulcer Obstruction due to infiltration/fibrosis	Dyspnea Cough Chest discomfort Hilar adenopathy Parenchymal interstitial opacifications Nodules
Intestinal	Ulcerative colitis	Abdominal pain Diarrhea Gastrointestinal bleeding Proctitis/colitis Stricture Neoplasm	Vasculitis Interstitial lung disease Bronchiolitis obliterans organizing pneumonia Granulomatous lung disease Bronchitis/bronchiectasis/bronchiolitis Diminished diffusing capacity Pleural effusion
	Crohn's disease	Systemic symptoms Gastrointestinal bleeding Ileitis/colitis Perforation Sinus tract formation	Bronchiectasis Tracheal esophageal disease Lymphocytic alveolitis/pneumonitis
	Whipple's disease	Diarrhea: malabsorption syndrome	Cough Dyspnea Pleuritis Pleural effusion Parenchymal nodules Reticulonodular infiltrates Pulmonary arteriopathy
	Celiac disease	Diarrhea Steatorrhea Malabsorption	Pulmonary hemosiderosis Interstitial lung disease
	Cystic fibrosis	Gastroesophageal reflux Intestinal obstruction Intussusception Constipation Rectal prolapse	Cough Dyspnea Wheezing Sputum production Chronic airflow obstruction Recurrent respiratory infections, especially due to *Haemophilus influenza*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* Bronchiectasis Cystic parenchymal changes
	Polyarteritis nodosa	Abdominal pain Bleeding Ischemia Perforation	Bronchial arteritis

Table 1.5 (continued)

	Disorder	GI manifestation	Pulmonary manifestation
	Churg–Strauss syndrome	Eosinophilic gastroenteritis Abdominal pain Gastrointestinal bleeding Diarrhea	Asthma Migratory infiltrates
	Langerhans histiocytosis	Diarrhea Malabsorption	Cystic, interstitial lung disease
Liver	Cystic fibrosis	Hepatic fatty infiltration Biliary cirrhosis Cholelithiasis	Cough Dyspnea Wheezing Sputum production Chronic airflow obstruction Recurrent respiratory infections, especially due to *Haemophilus influenza*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* Bronchiectasis Cystic parenchymal changes
	Alpha-1-antitrypsin deficiency	Cirrhosis Hepatocellular carcinoma	Emphysema, especially panacinar Obstructive lung disease
	Sarcoid	Hepatomegaly Hepatic nodules Hepatic dysfunction	Dyspnea Cough Chest discomfort Hilar adenopathy Parenchymal interstitial opacifications Nodules
	Hepatopulmonary syndrome	Cirrhosis/chronic hepatic dysfunction	Hypoxemia Pulmonary vascular dilation Pleural effusion (hepatic hydrothorax) Pulmonary hypertension
	Fulminant liver failure	Cirrhosis/chronic hepatic dysfunction	Acute respiratory distress syndrome
	Hereditary hemorrhagic telangiectasis (Osler–Weber–Rendu disease)	Mucosal telangiectases Gastrointestinal bleeding	Arterial–venous malformations Hemoptysis
	Biliary cirrhosis Primary Secondary to: Rheumatoid arthritis Hashimoto's thyroiditis Sjogren's syndrome	Cirrhosis Liver failure	Lymphocytic interstitial pneumonitis Interstitial lung disease Granulomatous lung disease Obstructive airways disease BOOP Pulmonary hypertension

Table 1.5 (continued)

	Disorder	GI manifestation	Pulmonary manifestation
	Scleroderma		Hepatopulmonary syndrome
	Sarcoidosis		Pulmonary hemorrhage
	Primary ciliary dyskinesia	Polycystic liver disease Biliary atresia	Bronchiectasis Respiratory infections
	Langerhans cell histiocytosis	Hepatomegaly Hepatic dysfunction	Cystic, interstitial lung disease
Pancreas	Pancreatitis	Pancreatitis Pancreatic pseudocyst	Atelectasis Pleural effusion Acute respiratory distress syndrome Pancreatic–pleural fistula
	Cystic fibrosis	Pancreatic insufficiency Pancreatitis Endocrine pancreatic insufficiency	Cough Dyspnea Wheezing Sputum production Chronic airflow obstruction Recurrent respiratory infections, especially due to *Haemophilus influenza*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* Bronchiectasis Cystic parenchymal changes
	Sarcoid	Pancreatitis Pancreatic mass	Dyspnea Cough Chest discomfort Hilar adenopathy Parenchymal interstitial opacifications Nodules

Table 1.6 Connective tissue disease–pulmonary associations.

Disorder	Connective tissue manifestations	Pulmonary manifestations
Rheumatoid arthritis	Symmetric erosive arthritis Ligament and tendon laxity	Interstitial lung disease Pleurisy Effusion Rheumatoid nodules Bronchiolitis obliterans organizing pneumonia Follicular bronchiolitis
Systemic lupus erythematosis	Malar or discoid rash Photosensitivity Oral ulcers Nonerosive arthritis Serositis	Interstitial lung disease Pleurisy Effusion Alveolar hemorrhage Shrinking lung syndrome

Table 1.6 (continued)

Disorder	Connective tissue manifestations	Pulmonary manifestations
		Pulmonary hypertension Thromboembolism: anticardiolipin antibody
Scleroderma	Raynaud's phenomenon Skin thickening: reduced joint motility and oral aperture Sclerodactyly Subcutaneous calcinosis Esophageal dysmotility Telangiectasia	Interstitial lung disease Pleurisy Effusion Aspiration Pulmonary hypertension
Polymyositis/ Dermatomyositis	Proximal muscle weakness Arthralgias Heliotrope rash	Interstitial lung disease Bronchiolitis obliterans organizing pneumonia Respiratory failure due to respiratory muscle dysfunction
Sjogren's syndrome	Keratoconjunctivitis sicca Xerostomia Raynaud's phenomenon	Interstitial lung disease Lymphocytic interstitial pneumonitis Xerotrachea Pseudolymphoma/lymphoma
Mixed connective tissue disease or undifferentiated connective tissue disease	Fever Malaise Arthralgias Myalgias Raynaud's phenomenon	Interstitial lung disease Pleurisy Effusion Pulmonary hypertension/vasculitis
Ankylosing spondylitis	Symptomatic sacroiliitis	Apical fibrobullous disease Pneumothorax Restriction due to chest wall deformity
Behcet's disease	Oral and genital ulcers Cutaneous lesions: erythema nodosum-like rash, superficial thrombophlebitis, pustular skin lesions Pathergy Ocular lesions	Pulmonary artery aneurysm
Relapsing polychondritis	Chrondritis of the nose, ears, trachea	Hoarseness Upper airway collapse

Table 1.7 Renal–pulmonary associations.

Disorder	Renal abnormality	Pulmonary abnormality
General		
Goodpasture's syndrome	Rapidly progressive glomerulonephritis Renal failure Hematuria Proteinuria	Hemoptysis Alveolar infiltrates Alveolar hemorrhage Increased diffusing capacity

Table 1.7 (continued)

Disorder	Renal abnormality	Pulmonary abnormality
Primary ciliary dyskinesia	Polycystic renal disease	Recurrent/chronic sinusitis Bronchiectasis Respiratory infections
Infiltrative/accumulative disorders		
Birt–Hogg–Dube syndrome	Renal tumors: chromophobe renal cell carcinoma or hybrid oncocytic tumor	Lung cysts Pneumothorax
Tuberous sclerosis	Polycystic kidney disease Renal tumors: chromophobe renal cell carcinoma or hybrid oncocytic tumor Benign and malignant angiomyolipoma	Smooth muscle cell infiltration of pulmonary parenchyma Multifocal, multinodular pneumocyte hyperplasia Lung cysts Pneumothorax Chylous effusion
Lymphangioleiomyomatosis	Angiomyolipoma	Smooth muscle cell infiltration of pulmonary parenchyma Lung cysts Pneumothorax Chylous effusion
Sarcoid	Granulomatous interstitial nephritis Nephrolithiasis Nephrocalcinosis	Dyspnea Cough Chest discomfort Hilar adenopathy Parenchymal interstitial opacifications Nodules
Vasculitis		
Wegener's granulomatosis	Glomerulonephritis Renal failure	Cough Dyspnea Pleuritis Hemoptysis Pulmonary infiltrates, cavities, effusions
Churg–Strauss syndrome	Focal segmental glomerulonephritis Renal insufficiency/failure Proteinuria Microscopic hematuria Hypertension	Asthma Migratory infiltrates
Polyarteritis nodosa	Renal artery aneurysm Renal hemorrhage Renal failure Hypertension	Bronchial arteritis
Connective tissue diseases		
Scleroderma	Proteinuria Renal insufficiency/failure Hypertension Scleroderma renal crisis	Interstitial lung disease Pleurisy Effusion Aspiration Pulmonary hypertension

Table 1.7 (continued)

Disorder	Renal abnormality	Pulmonary abnormality
Systemic lupus erythematosis	Glomerulonephritis: focal/diffuse Renal insufficiency/failure Proteinuria/nephrotic syndrome	Interstitial lung disease Pleurisy Effusion Alveolar hemorrhage Shrinking lung syndrome Pulmonary hypertension Thromboembolism: anticardiolipin antibody
Rheumatoid arthritis	Glomerulonephritis Rheumatoid vasculitis Hematuria Proteinuria	Interstitial lung disease Pleurisy Effusion Rheumatoid nodules Bronchiolitis obliterans organizing pneumonia Follicular bronchiolitis

Table 1.8 Endocrine/reproductive–pulmonary associations.

Disorder	Endocrine/reproductive abnormality	Pulmonary abnormality
Primary ciliary dyskinesia	Male infertility (50%) Reduced female fertility and increased risk of ectopic pregnancy	Bronchiectasis Respiratory infections
Cystic fibrosis	Male sterility: obstructive azospermia, congenital absence of the vas deferens	Cough Dyspnea Wheezing Sputum production Chronic airflow obstruction Recurrent respiratory infections, especially due to *Haemophilus influenza, Staphylococcus aureus, Pseudomonas aeruginosa* Bronchiectasis Cystic parenchymal changes
Ovarian hyperstimulation syndrome	Induction of superovulation with exogenous gonadotropins	Pleural effusion Restrictive lung disease due to ascites, cystic ovaries
Hypothyroidism	Deficiency of thyroid hormone	Respiratory failure: reduced responsiveness to hypoxemia and hypercapnea, myopathy Obstructive sleep apnea Pleural effusion Upper airway obstruction due to goiter
Hyperthyroidism	Excessive thyroid hormone	Increased ventilation in response to elevated metabolic level, increased responsiveness to hypercapnea and hypoxemia

Table 1.8 (continued)

Disorder	Endocrine/reproductive abnormality	Pulmonary abnormality
		Reduced respiratory muscle strength due to myopathy Upper airway obstruction due to goiter Pulmonary hypertension
Langerhans cell histiocytosis	Diabetes insipidus Thyroid infiltration: diffuse/nodular	Cystic, interstitial lung disease
Sarcoid	Thyroid infiltration: diffuse/nodular	Dyspnea Cough Chest discomfort Hilar adenopathy Parenchymal interstitial opacifications Nodules

Table 1.9 Neurologic–pulmonary associations.

Disorder	Neurologic manifestations	Pulmonary manifestations
General		
Disorders of central ventilatory drive	Ondine's curse Failure of automatic control of ventilation Obesity hypoventilation syndrome (Pickwickian syndrome) Medullary insults: Tumors, infection, infarct, radiation, multiple sclerosis, developmental, abnormalities, seizures, drugs, metabolic derangements Myxedema	Central sleep apnea Central alveolar Hypoventilation: Hypercarbia, hypoxemia Acute/chronic respiratory failure
Neurogenic pulmonary edema		Acute respiratory distress syndrome Pulmonary edema Hypoxemia
Motor neuron diseases	Amyotrophic lateral sclerosis Infections Trauma Multiple sclerosis Neuropathies: Guillain–Barre syndrome Infections Critical illness polyneuropathy Acute ascending motor Paralysis Charcot–Marie–Tooth disease	Acute/chronic respiratory Failure Hypoventilation: Hypercarbia, hypoxemia

Table 1.9 (continued)

Disorder	Neurologic manifestations	Pulmonary manifestations
Neuromuscular junction disruption	Myasthenia gravis Eaton–Lambert syndrome Infection Toxins Drugs	Acute/chronic respiratory Failure Hypoventilation: Hypercarbia, hypoxemia
Myopathies	Muscular dystrophies Primary myopathies Metabolic disorders: Acid maltase deficiency Carnitine Palmitoyltransferase Deficiency Hypokalemic periodic Paralysis Myxedema	Acute/chronic respiratory Failure Hypoventilation: Hypercarbia, hypoxemia
Specific disorders		
Polyarteritis nodosa	Mononeuropathy multiplex: sensory and motor Ischemic stroke Hemorrhage	Bronchial arteritis
Wegener's granulomatosis	Cranial and peripheral neuropathy	Cough Dyspnea Pleuritis Hemoptysis Pulmonary infiltrates, cavities, effusions
Churg–Strauss syndrome	Mononeuritis multiplex	Asthma Migratory infiltrates
Rheumatoid arthritis	Mononeuropathy multiplex: sensory, motor, and sensorimotor	Interstitial lung disease Pleurisy Effusion Rheumatoid nodules Bronchiolitis obliterans organizing pneumonia Follicular bronchiolitis
Langerhans cell histiocytosis	Posterior pituitary infiltration: diabetes insipidus Cerebellar/brainstem infiltration: ataxia, visual field deficits, behavioral/cognitive dysfunction	Cystic, interstitial lung disease
Sarcoid	Cranial/peripheral nerve palsy CNS/meningeal infiltration: endocrine dysfunction, seizure, focal motor deficits, hydrocephalus, aseptic meningitis Spinal cord infiltration: sensory, motor, or sensorimotor deficits Muscle infiltration	Dyspnea Cough Chest discomfort Hilar adenopathy Parenchymal interstitial opacifications Nodules

Table 1.10 Hematologic–pulmonary associations.

Disorder	Hematologic manifestations	Pulmonary manifestations
Hermansky–Pudlak syndrome	Platelet dysfunction	Interstitial lung disease
Sarcoid	Thrombocytopenia Hemolytic anemia Lymphopenia	Dyspnea Cough Chest discomfort Hilar adenopathy Parenchymal interstitial opacifications Nodules
Dyskeratosis congenita	Aplastic anemia	Interstitial lung disease
Sickle cell disease	Hemoglobinopathy	Acute chest syndrome Hypoxemia Infections Parenchymal infarction Pulmonary hypertension
Pulmonary alveolar proteinosis	Granulocyte dysfunction	Intra-alveolar accumulation of surfactant Infections Hypoxemia
Hypocalciuric hypercalcemia and interstitial lung disease	Granulocyte dysfunction	Interstitial lung disease
Autoimmune hemolytic anemia	Anemia	Lymphocytic interstitial pneumonitis Interstitial lung disease Thromboembolism Idiopathic pulmonary hemosiderosis
Dysproteinemias	Hypogammaglobulinemia Monoclonal gammopathy Polyclonal gammopathy	Lymphocytic interstitial pneumonitis
Leukemia	Acute/chronic myelogenous leukemia	Pulmonary alveolar proteinosis

Table 1.11 Metabolic disorders–pulmonary associations.

Disease	Metabolic disorder	Pulmonary manifestations
Gaucher's disease	Autosomal recessive mutations in the glucocerebrosidase gene that produce reduced enzyme activity and the accumulation of glucocerebroside in reticuloendothelial cells	Cough Breathlessness Exercise limitation Interstitial lung disease Pulmonary hypertension

Table 1.11 (continued)

Disease	Metabolic disorder	Pulmonary manifestations
Niemann–Pick disease	A clinically diverse group of at least six inherited disorders of cholesterol and sphingomyelin metabolism. Type A and B Niemann–Pick disease are caused by mutations in the sphingomyelinase gene that reduce enzymatic activity resulting in the accumulation of sphingomyelin within reticuloendothelial cells. Types C1 and C2 Niemann–Pick disease are due to mutations in the *NPC1* and *NPC2* genes, respectively, that encode proteins involved in cholesterol metabolism and cause the accumulation of cholesterol	Breathlessness Cough Interstitial lung disease
Fabry's disease	An X-linked disorder caused by defective lysosomal α-galacotsidase A activity, reduced catabolism of certain glycosphingolipids, and their accumulation within the vasculature and visceral tissues	Breathlessness Wheezing Cough Obstructive pulmonary function studies Air trapping demonstrated on imaging studies
Lysinuric protein intolerance	Autosomal recessive disorder caused by mutations in the solute carrier family 7A member 7 (SLC7A7) gene affecting the y+LAT-1 protein that is a light chain component within the heterodimeric amino acid transporters (HATS) family	Interstitial lung disease
Cerebrotendinous xanthomatosis	A deficiency of hepatic mitochondrial C27-steroid 27-hydoxylase causing increased cholesterol synthesis and the build up of bile acid precursors	Interstitial lung disease

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2

Clinical Trials for Rare Lung Diseases

Jeffrey Krischer

Abstract Clinical trial designs for rare lung diseases must meet the same rigorous standards as do designs for trials for diseases that occur with much more frequency. However, there are many different types of study designs; some of which require only a fraction of the number of subjects required to the randomized controlled trial, which is often considered the gold standard.

Alternate designs can address those issues by the use of external or historical controls or with participants serving as their own control. In the case of external or historical controls, all patients to be recruited on a proposed study would receive the new or experimental therapy and their outcomes would be compared to a population that had already been treated by a standard therapy. If historical data are valid and available, this is a very efficient design because it requires fewer patients to be accrued. The downside of such a design is that the selection of historical controls must be made with extreme caution so as not to bias the study results.

A design that avoids this problem is the use of concurrent controls for which participants can serve as their own control. Such designs are desirable if there is less within patient variability in a treatment response than there is between-patient variability. In such cases, outcome estimates will have less variance and the study design will require less accrual. Examples of these designs include cross-over designs and "N-of-1" designs. A design that is well suited to rare events and rare diseases is the case–control design. In such a design, individuals in whom a certain outcome has been observed (disease severity or particular event) are matched to controls who did not have such an outcome and then the two groups are compared with respect to a particular intervention or exposure. Such designs can be developed from prospective as well as retrospective data collection perspectives.

Examples of prospectively randomized designs include cross-over designs as well as factorial designs. In the former, participants are randomized to a treatment arm for a period at the end of which the outcome is assessed and then "crossed over" to the other treatment. The cross-over design makes the same assumptions as do "N-of-1" trials where participants are randomized to pairs of therapies given in random sequence and a washout period is assumed to eliminate the affect of the treatment after the intervention

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is withdrawn. Factorial designs essentially involve a double randomization in which two questions are asked in the same participant population.

Finally, designs for ranking and selection procedures are often helpful and generally require a smaller sample size than randomized controlled trials. Ranking statistics are often used when information about underlying parametric distributions is unknown. It could be argued that less is learned in such an experimental design and a subsequent experiment is required to measure the actual difference between treatment outcomes.

There are many approaches to the design of a trial and many of them can achieve certain economies in terms of the required number of participants that need to be enrolled. However, the options are not without their drawbacks and require investigators to make a number of assumptions.

Keywords: clinical trials, bias, sample size, randomized control trials, historical controls, cross-over designs, N-of-1 designs, case–control designs, factorial designs

Introduction

The challenges of designing clinical trials for rare diseases have been recognized by many investigators *(1–4)* and the issues apply to much more common disorders as well, in that it is preferable to be able to answer a study question with the fewest number of subjects enrolled, irrespective of the number of subjects available. If two alternative treatments are to be compared in a trial, then a scientific and ethical imperative is to discover which is superior so as to minimize the number of subjects given the inferior treatment. Even in the case of trials designed to establish equivalency between two or more treatments, the imperative is to find the one with the least side effects, least cost, or least inconvenience, while maintaining the same degree of efficacy with the fewest number of subjects exposed to the more toxic therapy.

There are a number of alternative study designs that can be considered in the context of rare diseases (Table 2.1). These same designs are available for more common diseases and, for the most part, clinical trial designs for rare diseases must meet the same rigorous standards as do designs for trials for diseases that occur with much more frequency. They must ask important scientific questions, minimize bias, and have appropriate likelihood of achieving a scientifically acceptable answer. Indeed, there are no designs for rare diseases that are not applicable to any other category of diseases. However, there are many different types of study designs; some of which require only a fraction of the number of subjects required to the randomized controlled trial, which is often considered the gold standard.

To begin, it is helpful to consider that a study is, in its most abstract form, an experiment designed to draw a conclusion about which the scientific community, the population of affected individuals, and the population at large can agree. To the extent possible, a study should be free of bias in that its conduct and results are not affected by factors other than the specific study question. The more evidence that a study is bias-free, the stronger one's conviction about the study results can be.

A randomized controlled trial is considered the gold standard because inherent in its design is the minimization of bias. Thus, the results are often considered as the strongest evidence in testing a hypothesis. However, randomized controlled trials are

Table 2.1 Alternative clinical trial designs.

• Prospective cohort design
• Historical controls design
• Parallel group design
• Case–control design
• Cross-over design
• N-of-1 design
• Factorial design
• Ranking and selection design
• Randomized controlled trials
• Bayesian designs
• Decision analysis-based design
• Randomized withdrawal design
• Early escape design
• Group sequential design
• Adaptive design
• Risk-based allocation design

not easy to do in that many potential participants object to the concept of randomization and many investigators feel that randomization, in of it itself, is unethical *(5)*. Randomization requires that the investigator and the subject consider themselves in the state of equipoise in that they truly feel that the treatment received from either arm of a randomized trial is equivalent unless proven otherwise. This is difficult for participants who want to believe that their treatment will be based upon what is best for them and not the "flip of a coin" and difficult for physicians who also think that they are ethically bound to provide the "best" treatment. Equipoise is made the more difficult since trials are often developed because an investigator feels that an experimental therapy is better and they wish to test that hypothesis in a rigorous fashion. Many subjects object to the trials if they have a likelihood of being assigned a potentially inferior arm (i.e., have a likelihood of not receiving the experimental therapy) or randomized to a placebo.

There are other sources of bias that should also be considered in addition to study design. Bias can result from the conduct of a study as well as its reporting in the literature. In the former, bias can result from the selection of subjects enrolled into a study, allocation to the arms of a study, differences in follow-up, or in ascertainment of study end points. The interpretation of study results from a trial conducted at a single institution might be affected by the types of cases that are referred to that institution for enrollment, if they are not representative of the general population of individuals affected by a certain disorder. For example, methods developed for the identification of rare mucociliary clearance disorders tested at a major referral center might give very different results if they were to be tested in the setting of a primary care practice since the population evaluated at the referral center can be very different. Differences in the study populations could affect the calculations of the sensitivity and specificity or a diagnostic test or its interpretation since the detection of rarer conditions generally requires a high level of specificity as compared to more common conditions to be scientifically and societally acceptable.

Bias that results from subject follow-up or ascertainment of study end points can arise insidiously and be very difficult to control. If a study is designed with historical controls or literature controls then follow-up practices may not be reported or differ in

some unknown way from the contemplated study. Even using concurrent controls may be biased if one treatment group is followed more closely than another leading to earlier recognition of study end points. A difference in the drop-out rates between study arms that is correlated with the study end point can introduce bias. For example, subjects who feel that their condition is not improving may withdraw from a trial and, as a result, the subjects available to evaluate at study end may be the remaining few who experienced a favorable outcome.

It is also recognized *(6)* that bias can come from study reporting in the scientific literature. Studies with positive outcomes are more likely to be published than are studies with negative outcomes. Thus, the historical or background information upon which a study is based might be biased in a particular direction. For this reason there are now national registries of clinical trials such that trials are registered when they are opened (to provide an accounting of the total universe of open trials in a particular field) rather when the results are known and only a subset published. Not all of these types of bias are easily recognized, nor controlled, by investigators.

A Hierarchy of Study Designs

While the randomized controlled clinical trial is regarded as the standard for trial designs, such trials designs are not always applicable in a given setting and there are alternatives to be considered. Most have to do with the selection of control groups to which the experimental intervention is to be compared.

Historical Controls. One approach is the use of external or historical controls. In the case of external or historical controls, all patients to be recruited on a proposed study would receive the new or experimental therapy and their outcomes would be compared to a population that had already been treated by a standard therapy. This results in considerable savings in terms of the number of patients to be accrued, even though the total number of patients may be substantial fraction of the total needed in a randomized controlled trial. For example, such a study would require less than half the number of patients to be treated compared to a randomized trial if only a moderate number of historical controls patients were available. Testing a question of a 20% difference in response rates, assuming the availability of data on 50 historical controls and a historical response rate of 40% would require only 74 patients prospectively treated by an experimental agent (total 124 patients) as compared to a prospective randomized trial which would require 153 patients.

If historical data are valid and available, this is a very efficient design because it requires fewer patients to be accrued prospectively and the newly accrued subjects would all be offered the experimental intervention. The downside of such a design is that the selection of historical controls must be made with extreme caution so as not to bias the study results. Often it is very difficult to know whether bias has been introduced by factors that have not been reported in the historical series or through changes in clinical practice that may affect clinical assessments or outcomes.

Concurrent Controls. A design that avoids this problem is the use of concurrent controls in which participants can serve as their own control. Such designs are desirable if there is less within patient variability in a treatment response than there is between-patient variability. In such cases, outcome estimates will have less variance and the study design will require less accrual. Examples of these designs include cross-over

designs and "N-of-1" designs. These study designs are applicable, however, only in the situation where there is a relatively rapid response to the intervention, the response disappears relatively soon after the intervention is withdrawn and the participant's overall condition does not change over the periods of time in which the intervention occurred or the intervention has been withdrawn. (That is, the condition or the severity of the disease does not change over time.) These designs work well for chronic diseases, but there are many settings in which this assumption cannot be justified or even tested.

Case–Control Designs. A design that is well suited to rare events and rare diseases is the case–control design. In such a design, individuals in whom a certain outcome has been observed (disease severity or particular event) are matched to controls that did not have such an outcome and then the two groups are compared with respect to a particular intervention or exposure. Such designs can be developed from prospective as well as retrospective data collection perspectives. Retrospective data collection is particularly efficient since one can identify just the cases where the events have occurred and matched them to a control where a particular event of interest has not occurred. But it suffers because of the reliance on the quality of historical data. Yet, such designs can be particularly useful in rare diseases in which there is a long lag time between genotype and phenotypic expression. Again the problem is the same as in the case of historical controls where investigators have to be extremely careful in selecting appropriate controls. Therefore, this design is not ranked as high as the randomized controlled trial in terms of the strength of evidence, because of this potential bias.

Cross-Over, "N-of-1," and Factorial Designs. There are a number of different designs which can be employed even when treatment arms are prospectively randomized to reduce sample size requirements. Examples include cross-over designs as well as factorial designs. In the former, participants are randomized to a treatment arm for a period at the end of which the outcome is assessed and then the subjects are "crossed over" to the other treatment. The cross-over design makes the same assumptions as do "N-of-1" trials where participants are randomized to pairs of therapies given in random sequence and a washout period is assumed to eliminate the effect of the treatment after the intervention is withdrawn *(6, 7)*. Cross-over designs use the same patients twice and effectively halve the number of patients that must be enrolled. "N-of-1" designs use the same patients a number of times (generally up to 5) and are even more efficient. The repeated evaluation of a therapy for the same subject also allows the treating physician to draw conclusions about the efficacy of the intervention for a single patient which is very appealing as well.

Factorial designs are similar to cross-over designs but differ importantly in that they essentially involve a double randomization in which two questions are asked in the same participant population. This essentially results conducting two studies at the same time in the same patient population with a sample size savings of an appropriate 50% for both. The sample size requirement for each study is unchanged, however. This type of design also assumes that there is no interaction between the two treatments. By interaction we mean that the effect of treatment A over its comparison group (placebo) is in the same direction regardless of whether the patient received treatment B or not. Again there is an assumption being made that is hard to verify.

Ranking and Selection Designs. Designs for ranking and selection procedures are often helpful and generally require a smaller sample size than randomized controlled trials *(8)*. In ranking and selection designs, the objective is to maximize the likelihood

of selecting the better therapy from a number of therapies as opposed to designing a trial that actually compares therapy directly and measures how much better one is as compared to another. Ranking statistics are also used when information about underlying parametric distributions are unknown. It could be argued that less is learned in such an experimental design and a subsequent experiment is required to measure the actual difference between treatment outcomes. That's because a randomized clinical trial design is to detect a minimally clinical significance between treatments, whereas the ranking statistics only seek to determine which treatment has the better response rate. Yet, the sample size savings can be appreciable as compared to a randomized control trial with less than 25% of the needed accrual to answer almost the same question with the same statistical power.

Randomized Trials. It should also be noted that the choice of end points in a randomized trial can also affect the sample size requirement. For example, a study designed to detect a change in the percentage of cases that respond (a binary outcome, yes or no) to a given treatment versus and alternative (control) treatment will generally have a larger sample size requirement than a study that seeks to detect a 20% change in the value of a continuous outcome measure (e.g., %FEV1). This depends somewhat on the distribution of the outcome measure and its variability (standard deviation) among patients treated on the same (experimental or control) treatment.

There are also some options when designing studies that have time-until-event outcomes, in which the study seeks to determine which treatment delays or prevents the occurrence of an outcome of interest. This might be a study of time until disease progression or overall survival. In these types of study designs, it is the person-years of follow-up that can have a substantial effect of the sample size requirement. For example, a study may take several years to accrue and the study end point is to be assessed at a certain time after the last patient has been accrued. All those patients accrued before the last patient will have been followed for a variable, but longer, period of time. The sample size calculation takes this into account, utilizing all the follow-up data that is available on every patient. If the duration of follow-up is extended for all patients, then the total amount of person-years of follow-up is increased and the sample size is decreased to measure the same effect size. Maintaining the original sample size has the effect of increasing the study power to detect a planned difference in outcome or being able to detect a smaller difference than planned with the original study power.

Interim Analyses. Another consideration in study design is the provision for interim analyses. Interim analysis plans can accompany any type of study design. They generally focus on one or more of the following determinations: (1) are the outcomes observed on the control arm of a trial close to the original planning parameters? (2) do the early results indicate an difference so large as to warrant stopping the study? or (3) do the early results indicate that no difference will be detected if the study would continue as planned?

When studies are designed with control arms, they generally cite data from the literature to estimate the natural history of the disease under standard care assumptions. There is some risk, of course, that the population reported in the literature is unlike that to be prospectively accrued, there may be differences in non-study-related care or outcome ascertainment. Should any of these occur, then the study planning parameters may not hold and there maybe a reason to reconsider the sample size in light of the treatment effect to be measured. Another situation that can occur is when there a large differences that emerge between study arms such that it becomes unethical to continue

to expose the enrolled study participants to the inferior treatment or to offer the possibility of treatment assignment to an inferior treatment for new subjects to be accrued. To make this determination, the study monitoring group must have a high degree of certainty that the difference is real and not simply the randomness of the order in which better or worse outcomes are observed. This high degree of certainty means that the likelihood of falsely concluding there is a difference between the alternative treatments when, in fact, there really is not, is the Type 1 error associated with the study and corresponds to the p value. Thus studies recommended for early termination due to emerging differences generally require much more stringent p values (of the order of $p = 0.001$) than the level of significance for which the overall study is planned (say $p = 0.05$) *(9, 10)*. Terminating a study for lack of a difference between the treatment arms is the mirror of the situation and such a recommendation is based upon the power of the study to detect a difference should there really be one *(11)*. Interim analyses in which there is very little chance of falsely concluding that there is no difference have very low Type 2 error (which is 1 – the study power). Many studies are designed to have 80% power (20% Type 2 error) at study conclusion and interim analyses that conclude the "futility" of continuing would generally do so if the Type 2 error was much greater. There are a number of software packages that are available for calculating stopping rules for interim monitoring designs *(12, 13)*.

Summary

There are many approaches to the design of a trial and many of them can achieve certain economies in terms of the required number of participants that need to be enrolled. However, the options are not without their drawbacks and require investigators to make a number of assumptions, many of which cannot be verified or even tested. It is clear that careful consideration needs to be made regarding those assumptions to find the study design that fits the research question the best. However, in doing so it may be possible to select a clinical trial design that is well suited for a specific rare disease and the clinical question that is to be answered.

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3

Idiopathic and Familial Pulmonary Arterial Hypertension

Jean M. Elwing, Gail H. Deutsch, William C. Nichols, and Timothy D. Le Cras

Abstract Pulmonary arterial hypertension (PAH) is a progressive, fatal disease that is defined hemodynamically. The average life expectancy after diagnosis is short, with death usually due to progressive right ventricular hypertrophy and right heart failure. PAH results from vasoconstriction and structural alterations to the pulmonary vasculature. PAH can be secondary to other disorders, including underlying lung disease or it can be idiopathic without a known predisposing condition. Primary or idiopathic PAH is rare and includes individuals with a family history of disease. This chapter will focus on idiopathic and familial PAH. The discovery and history of the disease, incidence, development of the clinical classification, epidemiology, prognostic factors, and clinical assessment are reviewed. The pathology of vascular remodeling is described, including the potential sequence of events, cell types, and processes involved. The genetics of the disease together with the identification of frequent mutations in the *BMPR2* gene in familial and idiopathic patients is presented. Stresses or pathways that may play a role in triggering PAH in patients with *BMPR2* mutations is reviewed because of the low penetrance of symptomatic disease in families with *BMPR2* mutations. Potential stimuli and pathways that can trigger the disease have been identified from clinical studies of PAH patients and from experimental models of PAH. Current therapies for PAH including general management, pharmacologic, and surgical are reviewed. Future directions in diagnosis, management, pharmacotherapies, genetic studies, pathobiology, and potential cell-based therapies are also discussed.

Keywords: pulmonary arterial hypertension (PAH), familial PAH, idiopathic PAH, vascular remodeling, BMPR-II, *BMPR2*

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Historical Review of Pulmonary Arterial Hypertension

Clinical History

The first case of pulmonary arterial hypertension (PAH) was described more than a century ago by a German physician, Dr. Julius Klob. He reported significant narrowing of the small pulmonary arteries at autopsy in a 59-year-old male whose demise came shortly after the development of lower extremity edema and cyanosis *(1)*. Similar findings were also noted by Ernst von Romberg, a prominent German physician in 1891. He reported a case of a 24-year-old male with unexplained dyspnea and cyanosis who was found to have "extraordinarily widespread, high-grade sclerosis of the pulmonary arteries with consequent hypertrophy of the right half of the heart" at autopsy *(2)*. In 1901, Dr. Abel Ayerza from Argentina further described the clinical syndrome of cyanosis, dyspnea, and polycythemia that was associated with sclerosis of the pulmonary arteries. This syndrome became referred to as Ayerza's disease or "Black Heart Disease" for the next three decades. In the early 1900s, there were several reports in the literature of Ayerza's disease *(3, 4)*. Initially, it was postulated by Dr. F.C. Arrillaga, a student of Dr. Ayerza, that this syndrome was a result of a syphilitic pulmonary endarteritis. This theory was subsequently discounted in 1935 by Dr. Oscar Brenner, a renowned Massachusetts General Hospital pathologist, through an extensive report on 100 patients affected by "sclerosis of the pulmonary arteries." Although syphilis was not found to be a causative agent of the pulmonary vascular changes described by Brenner, he was unable to determine an alternative etiology for these findings *(5)*.

A major advancement in the understanding of PAH came with the advent of invasive hemodynamic assessments in 1929 with the first introduction of a catheter into the right heart. This was performed by a German surgeon, Werner Forssmann, who inserted a urinary catheter into his own antecubital vein and advanced it into his right heart *(6, 7)*. This procedure was not well received by the medical community at that time; thus no further progress in the development of this technique occurred for the next 10 years. In the 1940s, André Frédéric Cournand, a French physiologist and his mentor Dickinson Richards, began to re-explore catheterization of the right heart and invasive pressure measurements of the pulmonary circulation *(8–10)*. In 1956, Forssmann, Cournand, and Richards were awarded a Nobel Prize for the development of right heart catheterization *(11)*.

With the availability of a direct assessment of pulmonary pressures, physiologic study of pulmonary circulation was possible. In the 1950s, Dresdale and colleagues began to evaluate the effect of vasodilators on pulmonary vasoconstriction and pulmonary pressures. Tolazoline, a pulmonary and systemic vasodilator, was found to reduce pulmonary arterial pressures *(12)*. Further studies with acetylcholine, a selective pulmonary vasodilator, showed that this agent lowered pulmonary pressures in the setting of pulmonary vasoconstriction due to hypoxia *(13, 14)* and mitral stenosis *(15)*. These studies initiated our understanding of pulmonary hemodynamics and set the stage for further studies to develop pulmonary vasodilating therapies.

Development of Clinical Classifications

In the 1960s, a significant increase in the awareness of this disease occurred with the epidemic of aminorex-induced PAH. Aminorex fumarate (2-amino-5-phenyl-2-oxazoline) was sold as an over-the-counter appetite suppressant on the Swiss, German,

and Austrian markets between 1965 and 1968. The drug was removed from the market in 1968 due to increased incidence of PAH among its users. Individuals with aminorex-induced PAH were found to have a similar clinical course and histopathology as patients with idiopathic pulmonary arterial hypertension (IPAH) *(16)*. With increased awareness of this disease came a need to reevaluate the diagnosis and clinical classification of PAH.

The 1st World Health Organization (WHO) Meeting was held in Geneva, Switzerland, in 1973 with the objectives to assess the state of the knowledge of PAH and standardize the nomenclature used in this disease. IPAH was referred to as primary pulmonary hypertension (PPH) at that time *(17)* and PPH was the standard terminology utilized until 2003 *(18)*. The next key event in the history of PAH occurred with the creation of the National Institutes of Health (NIH) National Heart, Lung and Blood Institute (NHLBI) registry of patients with PPH. The data collected from 1981 to 1985 on 178 patients with PPH significantly impacted the understanding of the clinical, pathologic, and morphologic features of this disease *(19)*.

With this increased understanding of PAH, the 2nd World Health Organization (WHO) meeting was held in Evian, France, in 1998. This meeting created a comprehensive classification system of pulmonary hypertensive (PH) diseases. Five major categories of PH were identified *(1)*: pulmonary arterial hypertension (PAH) *(2)*, pulmonary venous hypertension *(3)*, PH associated with disorders of the respiratory system or hypoxia *(4)*, PH associated with chronic thrombotic or embolic disease, and *(5)* PH caused by disorders directly affecting the pulmonary vasculature *(20)*.

This clinical classification schema was used until its revision at the 3rd WHO Symposium held in Venice, Italy, in 2003 *(18)*. The 2003 WHO updates were based on increased understanding of the pathogenesis of PH *(21)*. While the 2003 classification maintained the basic architecture of the 1998 guidelines, the terminology of PPH was abandoned and replaced by IPAH. Additionally, pulmonary veno-occlusive disease and pulmonary capillary hemangiomatosis were reclassified into the category of PAH *(18)* (see Figure 3.1).

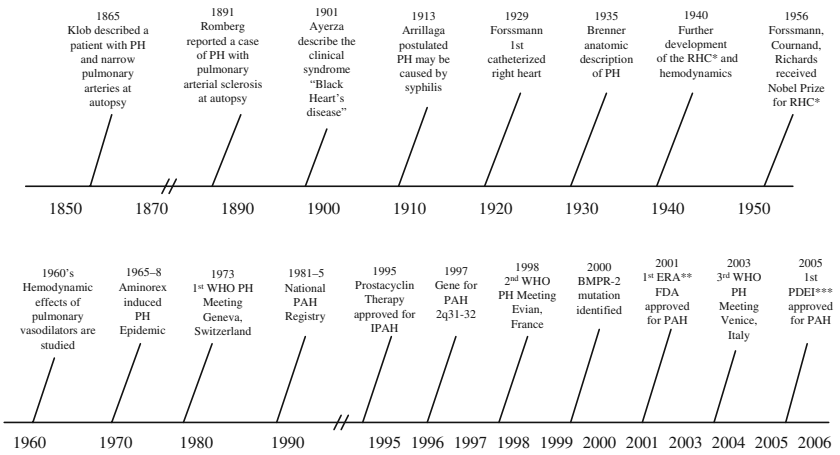


Figure 3.1 Timeline of the clinical, diagnostic, research, and therapeutic advances in pulmonary arterial hypertension over the last 150 years. RHC: right heart catheterization, ERA: endothelin receptor antagonist, PDEI: phosphodiesterase inhibitor

Epidemiology of Pulmonary Arterial Hypertension

Incidence and Natural History

IPAH is a rare disease with an estimated incidence of 1–2 per million individuals *(19, 22, 23)*. Of patients enrolled in the 1981 NIH registry only 6% reported at least one affected relative *(19)*. It is not clear what percentage of IPAH may actually be familial PAH (FPAH), but it most likely is significantly underestimated due to the low and variable (10–20%) genetic penetrance of this disease *(24)*. Although in one study around a quarter of "sporadic" IPAH patients were found to have *BMPR2* mutations *(25)* (see also section on Genetics).

The majority of the current data on the incidence and natural history of IPAH and FPAH have been gathered via patient registries *(19, 26–28)*. The average age of patients enrolled in the 1981–1985 NIH National Heart, Lung and Blood Institute (NHLBI) registry was 36.4 years *(19, 22)*. The registry also confirmed that IPAH predominantly affect females, with a female-to-male ratio of 1.7:1. Additionally, due to nonspecific symptoms of this disease, the average time from onset of symptoms to diagnosis of IPAH was 2 years. This 2-year delay in diagnosis was also seen in the 2002–2003 French national PAH patient registry *(28)*. Additionally, patients were found to have advanced disease at the time of diagnosis with 75% of IPAH patients reporting New York Heart Association (NYHA) functional class III and IV symptoms *(28)* (see Figure 3.1).

Prognostic Factors

The prognosis for PAH prior to effective therapies was very poor. The median survival for patients enrolled in the 1981 NIH registry was 2.8 years. The estimated survival at 1, 3, and 5 years was 68%, 48%, and 34%, respectively *(19)*. Predicted survival has been reported to be improved with the advent of novel PAH-targeted therapies *(29)*; however, a recent meta-analysis study has questioned this *(30)*.

While age *(31)*, smoking history, contraceptive use, pregnancy, presence of antinuclear antibodies, family history of PAH, and gender *(32)* do not appear to affect prognosis in PAH, several other factors do play a role in the outcome of patients with PAH. Elevated right atrial pressure (RAP) *(31–33)*, elevated mean pulmonary arterial pressure (mPAP) *(31–33)*, decreased cardiac index (CI) *(32, 33)*, increased pulmonary vascular resistance (PVR) *(31)*, low diffusing capacity *(32)*, presence of Raynaud's phenomenon *(32)*, decreased exercise tolerance, and advanced NYHA functional class *(32)* have all been associated with a worse prognosis. The NIH registry found that a RAP $\geq$ 20 mmHg, mPAP $\geq$ 85 mmHg, and a CI of $\leq$ 2 l/min/m *(2)* were associated with a reduction in median survival to 1, 12, and 17 months, respectively *(32)*. The NIH registry also revealed that advanced symptoms were associated with a marked reduction in life expectancy. Patients with NYHA function class I and II symptoms had a median survival of 58.6 months, while patients with NYHA functional class III and IV had a median survival of 31.5 and 6 months *(32)*, respectively. Registries have also shown that the two most common causes of death in PAH are sudden death (most likely due to arrhythmias) and right ventricular failure *(31, 32)*. Right ventricular failure accounted for nearly 50% of all PAH-related deaths *(31, 32)*.

Clinical Assessment of Pulmonary Arterial Hypertension

Defining Characteristics

Pulmonary arterial hypertension (PAH) is a progressive, fatal disease that is defined hemodynamically. The widely accepted criteria for PAH is an elevated mean pulmonary arterial pressure ($\geq$ 25 mmHg at rest or $\geq$30 mmHg with exercise) *(19)* associated with a normal pulmonary arterial occlusion pressure (PAOP) ($\leq$15 mmHg) *(34)*. The diagnosis of IPAH/FPAH can be reached only after other etiologies associated with PAH have been excluded, including HIV, scleroderma, congenital heart disease, portal hypertension, or hemaglobinopathies.

Clinical History

Dyspnea is the most common initial symptom of PAH. In the 1981 NIH registry, 98% of patients reported dyspnea at the time of enrollment. Other common symptoms reported at the time of enrollment were fatigue (73%), chest pain (47%), near syncope (41%), syncope (36%), edema (37%), and palpitations (33%) *(19)*. Ten percent of patients also reported a history of Raynaud's phenomenon *(19)*. Patients tended to have advanced symptoms with significant functional limitations at the time of diagnosis of PAH *(19)*. The degree of functional limitation resulting from PAH can be assessed using either the NYHA (see Table 3.1) or WHO functional class system. Seventy-five percent of patients enrolled in the NIH registry reported NYHA III or IV symptoms at the time of diagnosis *(19)*.

Table 3.1 New York Heart Association Functional Classification of PAH. Diagnosis and management of pulmonary arterial hypertension: ACCP Evidence-Based Clinical Practice Guidelines. Data from Rubin *(36)*. Adapted from The Criteria Committee of the New York Heart Association *(319)*.

Class I	No symptoms with ordinary physical activity
Class II	Symptoms with ordinary activity. Slight limitation of activity
Class III	Symptoms with less than ordinary activity. Marked limitation of activity
Class IV	Symptoms with any activity or even at rest

Physical Examination Findings

The physical examination findings in IPAH or FPAH are similar to those seen in any form of PH. Aberrations in the cardiovascular examination are the most common physical examination findings. Patients in the 1981 NIH registry were found to have an accentuated pulmonary component of the second heart sound (P2) 93% of the time. This finding is due to elevated pulmonary pressures leading to forceful closure of the pulmonic valve *(35)*. An early ejection click due to high pressures interrupting the opening of the pulmonary valve may also be heard *(36)*. A right ventricular heave can be appreciated along the left parasternal border due to the increased impulse of the hypertrophied high-pressure right ventricle *(35)*. A right-sided third heart sound (S3) and fourth heart sound (S4) are also commonly present on examination and occurred in 23%

and 38% NIH registry patients, respectively *(19)*. A right-sided S3 is a dull, low-pitched sound occurring in early diastole that is due to decreased right-sided cardiac function and pressure overload *(37)*. The appreciation of an S3 on physical examination has been found to correlate well with an elevated right atrial pressure (RAP 13 mmHg) and a low cardiac index (CI 1.8 l/min/m *(2)*) on hemodynamic assessment *(19)*. A right-sided S4 is a dull, low-pitched heart sound that occurs late in diastole and is a result of increased resistance of ventricular filling following atrial contraction *(37)*.

Several cardiac murmurs are appreciated in PAH. The most common murmur noted is that of tricuspid regurgitation and was present in 40% of patients in the NIH registry *(19)*. The murmur of tricuspid regurgitation is most often holosystolic, best appreciated at the left lower sternal border, and augments with inspiration *(37, 38)*. A Graham Steell murmur due to pulmonic insufficiency may also be appreciated. This finding was recorded in 13% of NIH registry enrollees and correlated statistically with higher pulmonary artery pressures ($\geq$70 mmHg) *(19)*. The Graham Steell murmur is a high-pitched, early diastolic decrescendo murmur noted over the left upper to mid-sternal area resulting from high-velocity regurgitant flow across an incompetent pulmonic valve. Additionally, a pulmonic stenosis murmur may occur as a result of turbulent flow across the pulmonic valve *(36)*.

In cases of advanced pulmonary hypertension, signs of right ventricular failure are seen on physical examination. These signs include an S3 gallop, elevated jugular venous pressure, hepatojugular reflux, a pulsatile liver, ascites, and lower extremity edema. Examination of the jugular venous pulsations may reveal an exaggerated A wave due to the contraction of a pressure overloaded right atrium and/or an exaggerated V wave resulting from elevations in right atrial pressures from ventricular contraction in the setting of an incompetent tricuspid valve *(37)*. The ominous signs of decreased systemic blood pressures, cool extremites, cyanosis, and a narrow pulse pressure may also be seen. These findings are associated with late-stage disease as they are a refection of poor cardiac output with resulting peripheral vasoconstriction *(36)*.

Diagnostic Testing

Patients with PAH may present to medical attention due to symptoms of this disease or due to incidental findings on testing for an unrelated condition. In either of these circumstances, further evaluation is warranted. The first step in the assessment of PAH is to determine if PAH is truly suspected. If there is clinical evidence for PAH on history, physical examination or routine testing with chest radiography, electrocardiography, echocardiography, and then a full evaluation and hemodynamic assessment should be pursued *(39)* (see Figure 3.2).

Electrocardiography

An electrocardiogram (ECG) may provide supporting evidence for the presence of PAH. The 1981 NIH registry revealed that ECG changes were quite common in patients with IPAH. Right axis deviation was seen in 79%, right ventricular hypertrophy in 87%, and right ventricular strain in 74% of patients *(19)*. Atrial arrhythmias such as atrial fibrillation and flutter also can be seen in patients with PAH (see Figure 3.3).

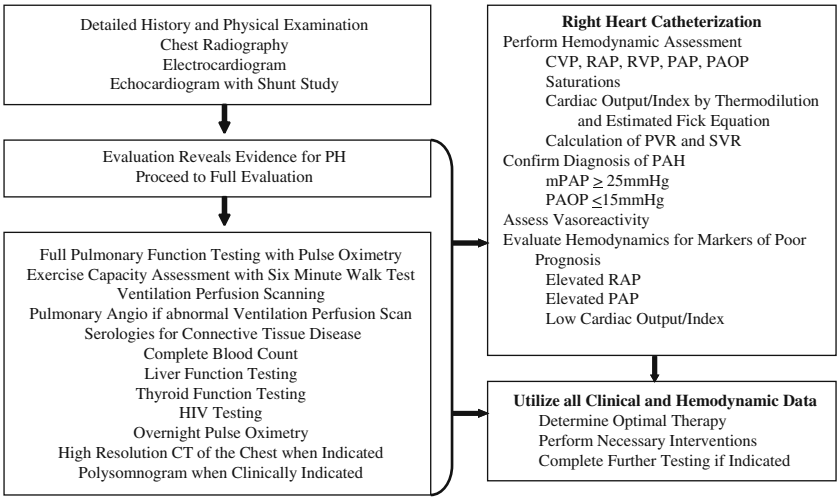


Figure 3.2 Overview of the comprehensive evaluation of pulmonary arterial hypertension. PH: pulmonary hypertension, CVP: central venous pressure, RAP: right atrial pressure, RVP: right ventricular pressure, PAP: pulmonary artery pressure, mPAP: mean pulmonary artery pressure, SVR: systemic vascular resistance, PVR: pulmonary vascular resistance, PAOP: pulmonary artery occlusion pressure

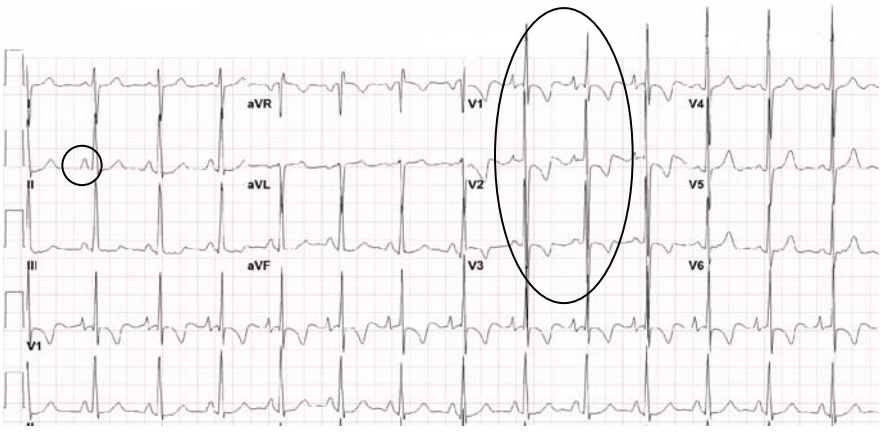
Chest Radiography

Findings on chest radiographs may also support the diagnosis of PAH. The changes seen on chest radiographs in PAH include prominence of the main pulmonary artery (90%), enlarged hilar vessels (80%), and decreased peripheral vascularity (51%) *(19)*.

Doppler Echocardiography

Transthoracic echocardiogram is used to estimate pulmonary artery systolic pressures (PASP). PASP is calculated based on the velocity (v) of the tricuspic regurgitant jet and the right atrial pressure (RAP) using the formula, right ventricular systolic pressure (RVSP) = PASP = 4v *(2)* + RAP *(40)*. The RAP is estimated either by inferior vena cava characteristics *(41, 42)* or by jugular venous distention *(40)*. With trained ultrasonographers and echocardiographers, estimations of PASP can be made in up to 70% of patients *(43)*. The finding of elevated PASP by echocardiogram correlates with the presence of pulmonary hypertension on cardiac catheterization with a high sensitivity (90%) and specificity (75%) *(44)*. When echocardiograms and cardiac catheterization were performed simultaneously, the pulmonary artery pressures have been shown to correlate well (r = 0.96 and SEE = 7 mmHg) *(45)*. Normal PASP does increase with age and body mass index (BMI). The estimated upper limit normal PASP for lower-risk subjects is 37.2 mmHg. PASP >40 mmHg was found in 6% of individuals over 50 years of age and 5% with a BMI >30 kg/m *(2, 46)*. Additional echocardiographic findings that can be seen in PAH include right atrial enlargement, right ventricular dilatation with volume and/or pressure overload, right ventricular hypertrophy, right ventricular dysfunction, right-to-left atrial shunting, and bowing of the intraventricular septum to the left with resulting impairment of left ventricular filling *(42)* (see Figure 3.4).

A

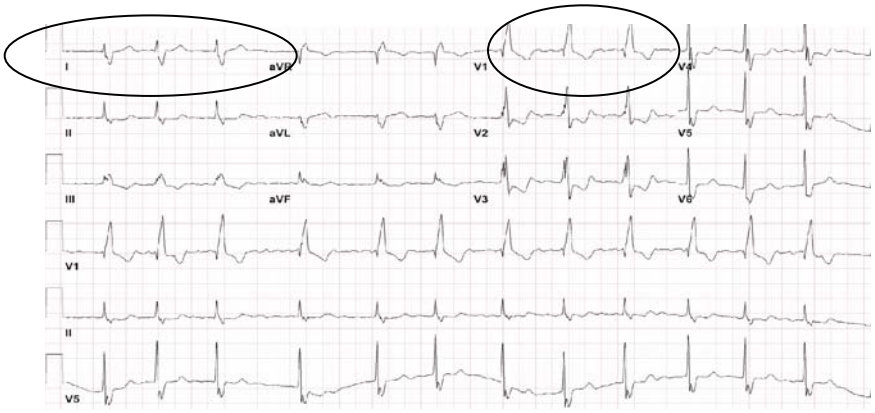


Right atrial Enlargement
Peaked P wave >2.5mm in lead II

Right Ventricular Hypertrophy
Right Axis Deviation
Dominant R wave >5mm in V1
R:S ratio > 1 in V1

Right Ventricular Strain Pattern
ST depression and T wave inversion in V1–V3

B



Atrial fibrillation
Irregularly, Irregular with absent P waves

Right Bundle Branch Block
Large R in V1, Large S in V4
QRS > .12 seconds
RSR' pattern in anterior leads with ST depression and T wave inversion
Wide S wave in V5–V6 and lead 1

Figure 3.3 Electrocardiogram (ECG) of two patients with IPAH and advanced disease. (**a**) Normal sinus rhythm with ECG showing right atrial enlargement, right ventricular hypertrophy, and right ventricular stain pattern. (**b**) Atrial fibrillation/flutter with right bundle branch block

Evaluation of the Etiology of Pulmonary Hypertension

PH can be associated with many common medical conditions. A full diagnostic work-up is recommended for all patients with PH. This evaluation is essential as treatment of PH varies depending on the underlying etiology.

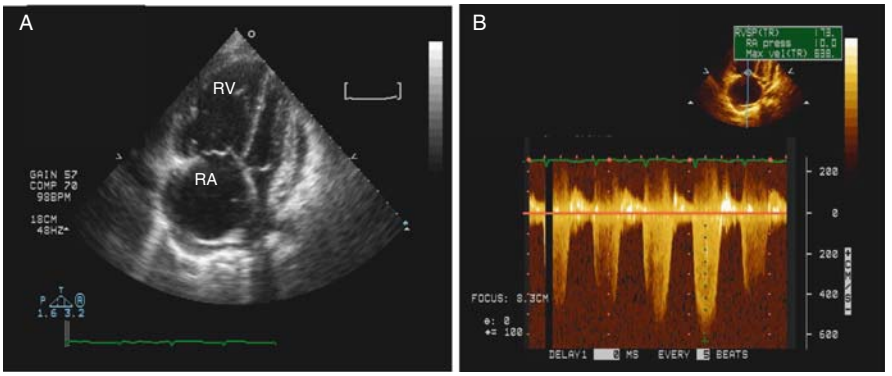


Figure 3.4 Echocardiogram of a young woman with IPAH with right ventricular failure. (**a**) Four-chamber view reveals marked ventricular enlargement with bowing of the intra-ventricular septum to the left impairing left ventricular filling. Right atrium is also markedly enlarged. (**b**) Tricuspid regurgitant jet velocity is used to calculate estimated systolic pulmonary arterial pressure using the equation RVSP= $4v^2$ + RAP

Pulmonary Function Testing

Pulmonary function testing is performed in patients presenting with PH to evaluate for underlying pulmonary disease in the etiology of the elevated pulmonary pressures. Severe obstructive or restrictive abnormalities may indicate this is the case; however, IPAH itself has been associated with mild-to-moderately reduced forced expiratory volume 1 (FEV1) and forced expiratory vital capacity (FEVC/FVC) when compared to age-matched controls *(31)*. IPAH and FPAH patients frequently are found to have reductions in diffusing capacity, but this does not appear to correlate with severity or mortality in this disease *(31, 47)*.

Evaluation for Thromboembolic Disease

An assessment for chronic thromboembolic disease as an etiology of pulmonary hypertension is necessary to determine optimal medical therapy and to identify patients in whom surgical intervention may be curative. Radioisotopic ventilation–perfusion (*V/Q*) scanning is routinely used in this setting *(48)*. The perfusion scan in patients with chronic thromboembolic pulmonary hypertension (CTEPH) is characterized by one or more mismatched segmental defects *(49)*. In contrast, the perfusion in patients with PAH may be normal or reveal multiple small sub-segmental defects *(49, 50)*. If segmental defects are seen on *V/Q* scanning, pulmonary angiography should be performed to assess for chronic thromboembolic disease that is amenable to pulmonary thromboendarterectomy *(51)*.

Radiographic Testing

If there is clinical suspicion or evidence for pulmonary parenchymal disease on a chest radiograph or pulmonary functions, a CT of the chest should be performed. In addition to an evaluation for pulmonary parenchymal disease, findings suggestive of pulmonary hypertension may be seen. Enlargement of the main pulmonary artery has been shown to correlate well with the presence of PH associated with pulmonary parenchymal disease *(52)* as well as in patients with IPAH *(53)* (see Figure 3.5).

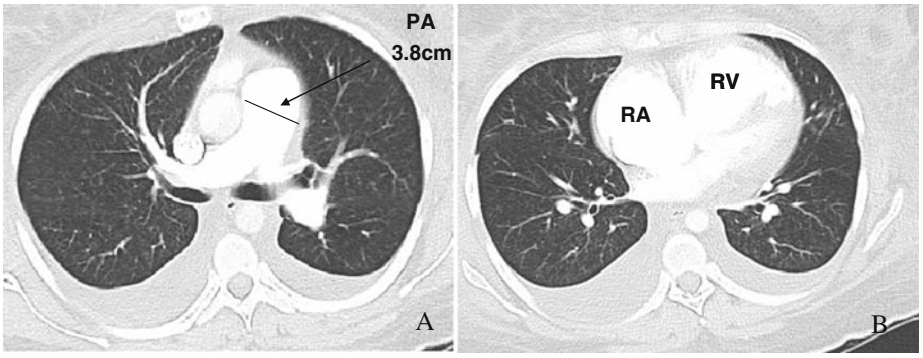


Figure 3.5 Contrast CT finding in a young female patient with IPAH with severe hypoxia and right heart failure. CT with contrast was performed to evaluate for pulmonary emboli. No emboli were seen. (**a**) The scan revealed an enlarged pulmonary artery. (**b**) Severe right atrial and right ventricular dilatation were also seen

Serologic and Laboratory Testing

All patients with PH should undergo routine laboratory testing, including complete blood counts, a liver profile, and thyroid function testing. Serologic testing to evaluate for underlying connective diseases such as systemic sclerosis, systemic lupus erythematosus, and mixed connective tissue disease is also advised. Additionally, testing for HIV is recommended in all patients presenting with PH as the presence of HIV infection significantly impacts management.

MRI

The role for MRI in PH is unclear at the present time. Further study of its utility in this disease is ongoing. When it is performed in patients with PAH, enlarged pulmonary arteries and dilated right-sided cardiac chambers may be seen (see Figure 3.6).

Confirmation of Presence of Pulmonary Arterial Hypertension (PAH)

Right heart catheterization verifies the diagnosis of PAH with direct pressure measurements. As mentioned earlier, the diagnosis of PAH is generally accepted as an elevated mean pulmonary arterial pressure (mPAP; $\geq$ 25 mmHg at rest or $\geq$30 mmHg with exercise) *(19)* associated with a normal pulmonary arterial occlusion pressure (PAOP) ($\leq$15 mmHg) *(34)*. Right heart catheterization provides accurate right atrial, right ventricular, and pulmonary artery pressure measurements. Additional information obtained from cardiac catheterization includes cardiac output/index, left to right shunt assessment, and vasoreactivity testing. Pulmonary hemodynamics and cardiac output/index provide information regarding the severity of hemodynamic impairment and prognosis. Vasoreactivity testing with a short-acting pulmonary vasodilator such as nitric oxide *(54, 55)*, adenosine *(56)*, or epoprostenol *(54)* is performed to guide therapeutic choices. A positive vasoreactivity response is defined as a decrease in mPAP of more than 10 mmHg, with final mPAP of less than or equal to 40 mmHg, when associated with a normal or high cardiac output *(39)*. IPAH or FPAH patients with a positive response to acute vasodilator challenge(s) may be candidates for chronic calcium channel blocker therapy *(57)*.

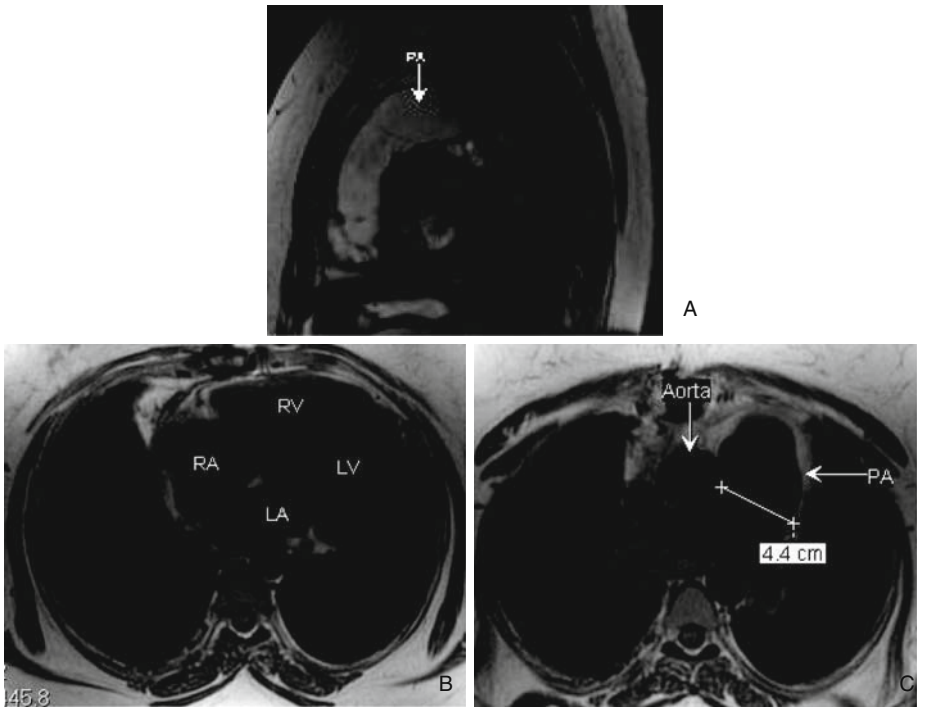


Figure 3.6 MRI of a young woman with IPAH with dyspnea and right heart failure. (**a**) Sagittal images reveal markedly an enlarged main pulmonary artery. (**b**) Enlargement of the right-sided cardiac chambers is seen. (**c**) Pulmonary artery is markedly enlarged

Pathology

Pulmonary hypertension results from vasoconstriction and structural alterations to the pulmonary vasculature. The process of pulmonary vascular remodeling involves all layers of the vessel wall, including the endothelium, media, and adventitia (see Figure 3.7). Although the plexiform lesion is the pathologic hallmark of IPAH and FPAH, the entire spectrum of changes are seen within this disorder and are identical to those seen in other forms of severe pulmonary hypertension *(58–61)*. As well as plexiform lesions, typical findings include medial hypertrophy of the smooth muscle wall, extension of smooth muscle into normally nonmuscularized vessels, intimal fibrosis, adventitial thickening, and thrombosis of small arteries *(62)*. Alteration of the pulmonary veins, including veno-occlusive disease, may also be seen. Despite a clinical diagnosis of IPAH or FPAH, plexiform lesions are frequently not seen and medial hypertrophy with intimal fibrosis is the primary manifestation at lung biopsy *(62, 63)*.

Insight into the cellular mechanisms that are associated with severe hypertension has been gained from phenotypic assessment of the vascular lesions in patients with primary and secondary pulmonary hypertension. Although the sequence of events that result in plexiform lesions are not well understood, immunohistochemistry with three-dimensional vascular reconstruction has indicated that they are composed predominantly of endothelial cells and develop in small-to-medium pulmonary

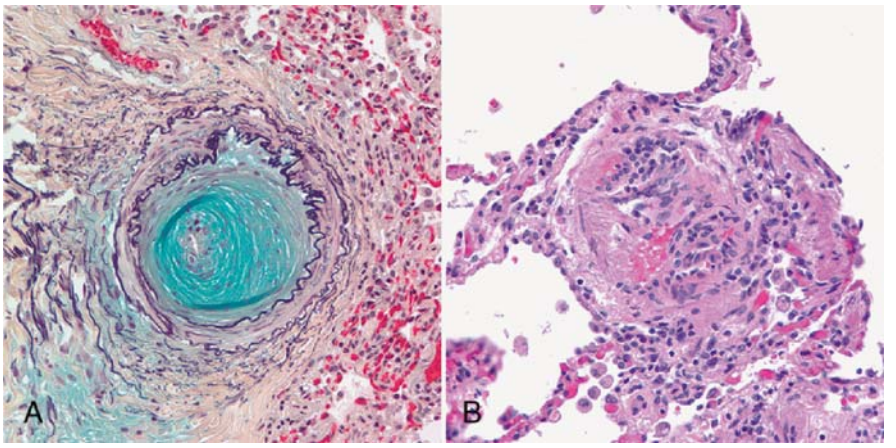


Figure 3.7 Pathology of vascular lesions in FPAH and IPAH. (**a**) Concentric intimal fibrosis in severe pulmonary hypertension. Movat pentachrome stain highlights the laminar fibrosis (*blue-green*) that severely narrows the lumen of an artery (×200). (**b**) Plexiform lesion from a patient with IPAH. The cellular lesion is composed of interlacing slit-like vascular channels (×200)

arteries just distal to branch points *(64, 65)*. Concentric intimal fibrotic lesions are frequently proximal to plexiform lesions, suggestive of a topographical relationship. The specific distribution of these occlusive lesions distal to arteriolar bifurcations indicates that shear stress and/or turbulent flow may influence their pathogenesis.

There is evidence to suggest that severe pulmonary arteriopathy in patients with IPAH is driven by dysregulated endothelial cell growth, akin to angiogenesis or neoplasia *(66)*. The endothelial cell proliferation in plexiform lesions is monoclonal in patients with primary but not secondary forms of pulmonary hypertension, and these cells appear phenotypically altered with loss of tumor suppressor proteins and abnormal expression of growth and apoptosis genes *(65, 67–69)*. Of interest, there is a high prevalence of human herpes virus 8 in patients with IPAH (see section on Pathogenesis later) and plexiform lesions share a histological and immunohistochemical resemblance to cutaneous Kaposi's sarcoma lesions *(70)*. Endothelial cell proliferation is largely mediated by vascular endothelial growth factor signaling (VEGF), the components of which are upregulated in plexiform lesions *(65, 66)*. Blockade of the VEGF receptor in combination with chronic hypoxia in rats induces endothelial apoptosis followed by endothelial cell proliferation and pulmonary hypertension *(71)*. The authors propose that in susceptible individuals high shear stress at sites of vessel branching selects for apoptosis-resistant endothelial cells that proliferate and ultimately form plexogenic lesions *(72)*.

In comparison to the endothelium, there has been less focus on characterizing the molecules associated with smooth muscle and fibroblastic remodeling of the pulmonary vasculature in patients with IPAH. As discussed below, pulmonary endothelial cells produce a number of important mediators that influence vascular tone and smooth muscle cell proliferation, and many of these factors have been examined in patient samples. Notably, the vasoconstriction and vascular remodeling compounds, endothelin (ET-1), serotonin transporter (5-HTT), thromboxane A_2, and angiotensin-converting enzyme

(catalyzes activation of angiotensin I to angiotensin II), have all been shown to be increased in advanced lesions of IPAH and may exert their effect through the angiopoietin/tyrosine kinase-2 pathway *(73–78)*. Further studies are needed to better understand the epistatic relationship between the different signaling molecules and possible interactions with the genetic mutations known to predispose to IPAH (see below).

Genetics

History

In 1954, Dresdale and colleagues described the occurrence of pulmonary hypertension among several family members *(12)*. While this is generally considered the first report of FPAH, Clarke and coworkers may actually have first described FPAH in 1927 *(79)*. After the description by Dresdale in 1954, slow progress was made in understanding the genetics of FPAH during the next 30 years with individual reports describing 13 different families in the United States. In 1984, Loyd and colleagues published a follow-up analysis of these 13 families including clinical descriptions of 8 new cases in 9 of the families as well as a new, 14th family *(80)*. This report also described inheritance patterns, which included father-to-son transmission as well as vertical transmission (affected individuals in successive generations) suggesting an autosomal dominant pattern of inheritance.

In 1981, the Division of Lung Diseases of the National Heart, Lung, and Blood Institute of the NIH initiated the Patient Registry for the Characterization of Primary Pulmonary Hypertension *(19, 32)*. The goals of the registry were to obtain and evaluate data on the natural history, pathogenesis, and treatment of PAH. Thirty-two centers in the United States entered 187 PAH patients between 1981 and 1985. Of these 187 patients, 12 reported a first-order blood relative as also having the disease resulting in a prevalence of 6.4% for FPAH among IPAH cases. The clinical and pathologic features of these 12 patients and 24 other patients with FPAH were identical to those of patients with IPAH *(19, 80)*. This report of the NIH-sponsored national prospective study on IPAH in the United States remains the benchmark study of IPAH to this date. Results from a national PAH registry in France were recently reported, which enrolled a total of 674 adult PAH patients between 2002 and 2003. Of these, 39.2% (264 patients) were classified as IPAH and 3.9% *(26)* as FPAH. Thus, of the idiopathic cases in this study (IPAH + FPAH), 26 of 290 (9%) reported an affected relative, which is similar to the NIH study of 1987 (6.4%) *(28)*.

To date, well over 100 families have been identified in the United States with at least two affected individuals. The largest registry of families in the United States is that of Dr. James Loyd and colleagues at Vanderbilt University, which currently contains 100 families *(81)*. Of the 3,750 total individuals followed up, 352 subjects meet criteria for PAH. Studies by Loyd and colleagues have confirmed that FPAH is an inherited autosomal dominant disorder. In addition, these studies have shown that FPAH presents with reduced penetrance. While it occasionally affects most or all members in a sibship, more often than not only a few individuals among several at-risk family members are affected. The penetrance varies widely between different families, ranging anywhere from 20% to as high as 80%. Loyd and colleagues studied more than 429 at-risk members of 24 families with FPAH, 99 who were affected and another 25 who were obligate carriers as part of the National Registry for FPAH *(82)*. More females had the gene

(84 versus 40 males) and more females with the gene developed the disease (72 of 84 females [86%] versus 27 of 40 males [68%] for a 2.7:1 gender ratio of affected patients). The mean age at death between females and males did not differ. The age correction for penetrance shows that by age 10, 10% of individuals known to carry the gene developed the disease, while at age 70, 92% of people with the FPAH gene had the disease *(82)*. The mean age at death decreased in successive generations, suggesting the phenomenon of "genetic anticipation" and the possibility of expansion of a triplet repeat as the cause of the disease *(83)*.

Identification of BMPR2 Gene Mutations

To determine the genetics of FPAH, two independent groups performed a genome-wide screen using microsatellite markers spaced approximately every 10 cM and DNA samples isolated from individuals in families in which IPAH was segregating *(84, 85)*. The study of Nichols et al. used samples from six large families, which included 19 affected individuals and 58 unaffected individuals, and found initial evidence for linkage on chromosome 2q31–32 *(84)*. There was no evidence of genetic heterogeneity, suggesting that the same gene causes FPAH. Similar findings were reported by Morse et al. using linkage analysis in two FPAH families *(85)*. Refinement of the candidate interval, reported by Machado et al. *(86)*, was accomplished through haplotype analysis of additional affected individuals in the United States and the United Kingdom and a complete physical map was constructed allowing the placement of 17 known genes and 64 ESTs in the candidate interval. Since there were no obvious functional candidates in the candidate interval, sequence analysis was initiated of those genes for which the intron/exon boundaries were known. At the same time, a gene whose complete gene structure was not yet known, but for which the 3′ half could be determined via BLAST similarity searching of its cDNA, was considered as a potential functional candidate. This was the gene for BMPR-II, a type II TGF-β receptor. BMPR-II was first identified using TGF-β type I receptors as bait in a yeast two-hybrid screen *(87)*. BMPR-II was considered a potential functional candidate as expression of TGF-β is upregulated in remodeling pulmonary arteries, and heterozygous mutations in *ENG* and *ALK1* (encoding components of the TGF-β receptor complex) result in hereditary hemorrhagic telangiectasia *(88, 89)*.

DNA sequence analysis of the *BMPR2* gene (which encodes the BMPR-II protein) has identified around 150 distinct mutations that alter the *BMPR2* coding sequence in PAH patients. Both FPAH and IPAH patients from a wide range of ethnic groups, including Americans, Europeans, Japanese, Chinese, Israeli Jews, and Indians, have undergone extensive analyses of the *BMPR2* coding region and intron/exon boundaries *(90)*. Most recently, systematic analyses for whole exon deletions/duplications of *BMPR2* have been completed using multiplex ligation-dependent probe amplification (MLPA) and quantitative real-time PCR. Using these methods, Nichols et al. and others have identified these types of mutations in a number of FPAH and IPAH patients *(91, 92)*. Mutations have been identified in at least 70% of recognized FPAH cases and in 11–40% of IPAH patients *(90–95)*. The spectrum of *BMPR2* defects includes all major mutation classes with single-nucleotide substitutions resulting in nonsense (∼33%), missense (∼30%), or splice site mutations (∼6%); small insertions or deletions/duplications (∼25%); or partial gene deletions or duplications (∼6%). Approx-

imately 70% of *BMPR2* mutations in FPAH or IPAH patients are predicted to cause premature truncation of the *BMPR2* transcript. At least 25 of the 150 or so mutations identified to date have been observed in more than one family, with the most frequent type of recurrent mutation resulting from the substitution of an arginine codon (CGA) with a nonsense codon (TGA) *(90)*. The distribution and types of mutations identified in the *BMPR2* gene in both FPAH and IPAH patients are shown in Figure 3.8.

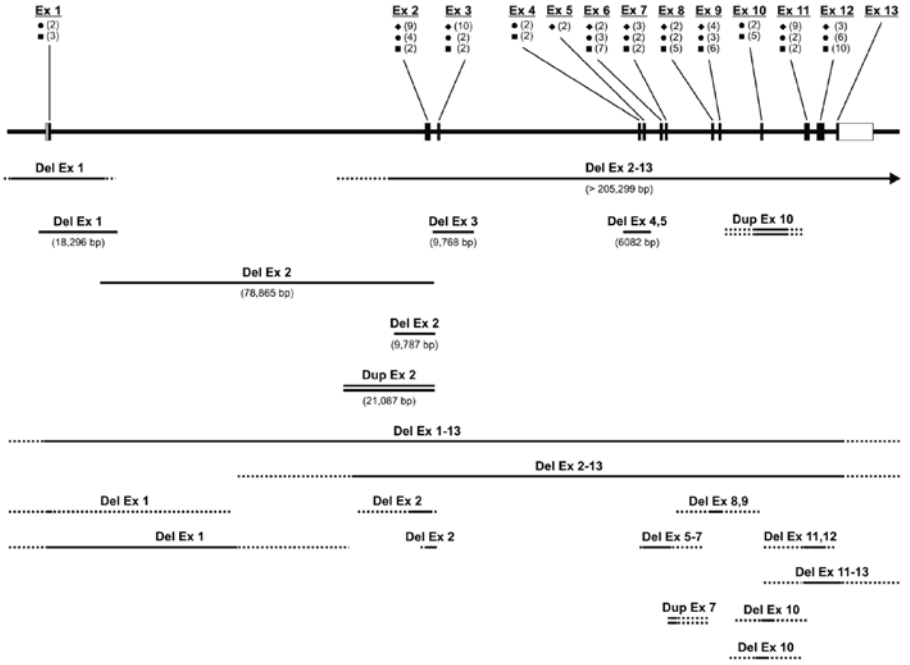


Figure 3.8 Distribution of *BMPR2* mutations in FPAH and IPAH. Proportional representation of *BMPR2* with exons indicated by *black boxes* along the gene. Untranslated portions of exon 1 and exon 13 (*white boxes*) are also shown. Below each exon label are the number of missense (♦), nonsense (●), and frameshift (■) mutations found in each exon. Large-scale exonic deletions/duplications are labeled and shown below *BMPR2* with *solid lines* indicating regions of definite deletion/duplication and *dotted lines* representing regions in which breakpoints are yet to be determined. Deletion/duplication sizes in base pairs are shown in parentheses where known

For the majority of IPAH patients, it remains unknown as to whether *BMPR2* mutations are inherited from a nonpenetrant parent, or represent a de novo mutation. In a very small sample of IPAH patients harboring *BMPR2* mutations, both parents were available for DNA study. In four out of six cases, the mutation was inherited from a nonpenetrant parent suggesting that the familial form of the disorder represents a higher percentage of IPAH patients than the reported 10% *(90, 96)*. This also suggests that additional family members are at risk for carrying *BMPR2* mutations and therefore at risk for developing PAH. Additional studies by Loyd and coworkers at Vanderbilt University of 3,750 individuals in 100 families have identified 2,256 individuals at risk for developing the

disease. Of those, 352 are affected giving a penetrance of 15.6% for FPAH. Of the six most heavily affected families, 24.2% of first-degree relatives of the PAH patients were also diagnosed with the disease *(81)*. However, this study does not differentiate between *BMPR2* mutation-positive individuals and at-risk individuals who may not have been tested for a *BMPR2* mutation. Recent analysis by Loyd and colleagues *(93)* of a very large pedigree harboring a known *BMPR2* mutation has identified 23 affected individuals and 30 unaffected obligate mutation carriers, with a disease penetrance of 43.4% in this family.

Genetic Testing and Counseling

The reduced disease penetrance of FPAH (and IPAH), either with or without a known *BMPR2* mutation, represents a challenge with regard to genetic counseling/clinical recommendations of these families/individuals. Siblings or children of patients who have FPAH or of obligate disease carriers (i.e., have an affected child and are in the bloodline for the disease but are themselves as yet unaffected) have an overall 50% chance of inheriting the abnormal gene. However, since the penetrance by some estimates is approximately 25%, this yields an estimated risk of 12.5% for expressing disease. As stated above, penetrance for individual families has been shown to vary anywhere from as low as 20% to as high as 80%. Therefore, the estimated risk for expressing disease will also vary among families. Current recommendations for asymptomatic family members of individuals who have FPAH is to undergo echocardiographic screening at 3- to 5-year intervals *(35)*. Genetic testing for mutations in *BMPR2* is now available clinically. Therefore, patients who have IPAH or FPAH, as well as their families, should be instructed about the availability of genetic testing and the potential risk for family members to develop PAH. However, genetic testing should only be provided after professional genetic counseling. Screening for a *BMPR2* mutation is most efficient in an affected individual so that the specific mutation in their family can be identified. Once the specific mutation is identified, clinical genetic testing of at-risk unaffected relatives can be conducted for the known mutation. Given the vast number of potential mutations in the large *BMPR2* gene, genetic testing of relatives of a PAH patient has no rationale unless a mutation is first identified in the patient. The identification of a germline *BMPR2* mutation in an IPAH patient can be alarming since this signifies that additional family members may also be harboring this mutation and are thus at-risk for developing the disease. This converts the concept of the disease from that of a rare event into that of a potentially familial disease. These family members should be offered counseling about their risk and the availability of testing for the known *BMPR2* mutation.

Potential Modifiers and Other Genes

Additional genetic factors have been reported to be associated with PAH either as modifiers acting in conjunction with a *BMPR2* mutation or as disease-producing mutations in other genes. Common genetic polymorphisms in the serotonin (5-hydroxytryptamine) transporter (SERT or 5-HTT) have been implicated in disease pathogenesis by Eddahibi and colleagues *(75, 97, 98)*. They reported increased growth of pulmonary artery smooth muscle cells (PASMC) from PAH patients compared with controls when stimulated with serotonin. The investigators attributed these mitogenic effects to increased 5-HTT expression *(99)*. They proposed a clinical link to the molecular cause for 5-HTT

overexpression based on a polymorphic variant in the 5-HTT gene promoter. Homozygosity for a long promoter variant (L allele) was present in approximately 65% of 89 severe PAH patients, as compared to only 27% of 84 control subjects without PAH *(75)*. Previous studies by these same investigators have demonstrated increased expression of the L allele as compared to the short promoter variant (S allele) in FPAH patients. Genetic abnormalities of 5-HTT may lead to an imbalance of cellular processes that facilitates abnormal PASMC proliferation and the development of PAH *(100)*. Machado and colleagues recently examined the role of polymorphic variation within the 5-HTT gene in 528 PAH patients and 353 control subjects. However, they found no significant differences in the frequency of 5-HTT alleles in either FPAH or IPAH patients as compared to controls *(101)*. Likewise, no differences were detected with regard to presence or absence of a BMPR2 mutation, age of disease onset, or gender. These results suggest that polymorphic variation at the 5-HTT locus is not likely to contribute to phenotypic expression of PAH. In a similar study by Willers and colleagues *(102)*, results were comparable to those of Machado et al. *(101)*, although Willers et al. *(102)* did report an earlier age at diagnosis for those FPAH patients homozygous for the L allele, as compared to those FPAH patients who did not have this genotype suggesting an as-yet unappreciated interaction between BMPR2 mutations and 5-HTT polymorphisms that affect disease expression.

Pulmonary hypertension that is clinically and histologically identical to FPAH and IPAH has recently been described in multiple kindreds with hereditary hemorrhagic telangiectacia (HHT) *(103–105)*. HTT is a vascular dysplasia characterized by mucocutaneous telangiectasias that cause recurrent epistaxis, gastrointestinal bleeding, and arteriovenous malformations of the pulmonary, hepatic, and cerebral circulations *(89)*. Defects in other components of the TGF-β pathway – activin receptor-like kinase 1 (*ALK1*) on chromosome 12 and endoglin (*ENG*) on chromosome 9 – have been identified in HHT patients *(106, 107)*. Molecular analysis of PAH/HHT patients by Trembath and colleagues, as well as Harrison and colleagues, has failed to identify any associated *BMPR2* mutations but instead have identified *ALK1* mutations segregating in these families *(103, 104)*. More recently, *ENG* mutations have been identified in unrelated PAH/HTT patients *(108, 109)*. The finding of identifiable genetic abnormalities of the TGF-β pathway in patients with both PAH and HHT suggests a common molecular pathway precipitating pulmonary vascular disease. While no direct interaction between the gene products of *BMPR2* and either *ALK1* or *ENG* has been elucidated, each receptor mediates signaling through the Smad family of coactivators suggesting an interaction among these receptors at some level *(110)*.

Pathogenesis

The pathogenesis of PAH remains poorly understood, although mutations in *BMPR2* have been identified in FPAH and a portion of IPAH cases as discussed earlier (see section on Genetics). A major enigma in the field is the reduced penetrance of *BMPR2* mutations, in that only a subgroup of individuals with mutations develop symptomatic PAH. In addition, cases of FPAH and particularly IPAH occur with no identifiable mutations in *BMPR2*. Hence, the identification of additional mechanisms and "triggers" that cause individuals with *BMPR2* mutations to develop symptomatic PAH is an area of intense interest. This section will review the current understanding of factors and

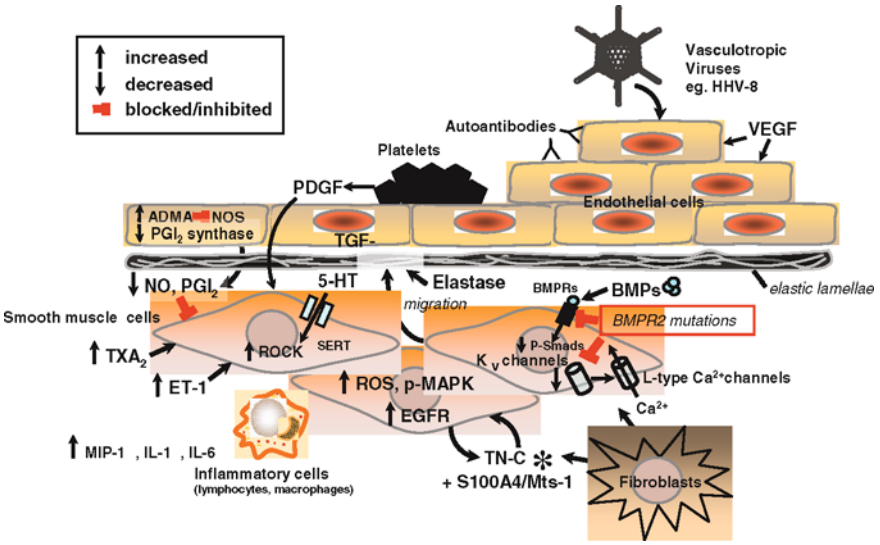


Figure 3.9 Pathways and factors implicated in the pathobiology and pathogenesis of FPAH and IPAH. Schematic shows pathways and factors that have been associated or linked to FPAH and IPAH. The role of these pathways in the pathobiology/pathogenesis of FPAH and IPAH is in many cases uncertain. The role of mutations in the BMPR2 receptor are, however, increasingly better understood, although the reduced penetrance suggests that environmental factors, epigenic factors, or modifier genes play a role in triggering the disease process. The schematic identifies many of the pathways and factors that have been studied to date as potential triggers or disease modifiers

pathways that may play a role in the pathogenesis of IPAH and FPAH (see Figure 3.9 for summary schematic). In many cases, data from experimental models of PAH have provided insights into the pathogenesis of PAH and will be discussed in relation to clinical data from IPAH and FPAH patients. As discussed earlier (see section on Pathology) pathologic processes that are believed to contribute to the development and progression of PAH include vasoconstriction, remodeling of pulmonary arteries, inflammation, aberrant apoptosis, and in situ thrombosis. In addition, dysregulated cellular processes that contribute to the remodeling of vessels include excessive proliferation, migration, and reduced apoptosis. The role of these factors and pathways must be considered in the context of their role in these pathological and dysregulated cellular processes.

Endothelial Dysfunction

Endothelial dysfunction has long been thought to play an important role in the pathogenesis of PAH *(111)*. Endothelial dysfunction can lead to altered or abnormal production of vasoactive mediators, which play a critical role in regulating vascular tone, structure, and homeostasis *(111, 112)*. Vasoactive mediators include nitric oxide (NO), prostacyclin, thromboxane, endothelin-1 (ET-1), and serotonin. Many of these mediators have direct effects on smooth muscle cells, regulating their contractile, proliferative, and phenotypic state. These mediators can also have effects on other cells in the vascular wall, including fibroblasts and endothelial cells, and can alter coagulability.

Nitric Oxide

Nitric oxide (NO) is produced from the amino acid L-arginine and molecular oxygen in a reaction catalyzed by NO synthase (NOS) enzymes, of which there are three isoforms *(113)*. Endogenously produced NO plays an important role in the transition of the pulmonary circulation at birth and regulation of pulmonary vascular tone *(114, 115)*. NO induces smooth muscle relaxation by activating guanylate cyclase and increasing cyclic GMP levels in smooth muscle cells. In addition, NO can also inhibit smooth muscle cell proliferation and platelet aggregation *(112, 116–118)*. Animal studies, including genetic ablation of the endothelial nitric oxide synthase (eNOS) isoform in mice and the attenuating effects of inhaled NO in chronic hypoxia-induced PAH, have suggested an important role for NO in PAH *(119, 120)*. Giad and Saleh *(121)* reported reduced or no detectable expression of eNOS in the pulmonary arteries of PAH patients, although this has been disputed *(122)*. In patients with plexigenic arteriopathy, arterial expression of eNOS inversely correlated with the severity of the histological changes and total pulmonary resistance. Measurements of whole-body NO production in patients with IPAH/FPAH (after intravenous infusion of radiolabeled L-arginine) showed lower excretion of nitrite and nitrate in IPAH/FPAH patients, suggesting that either NO production was reduced or NO metabolism was increased *(123)*. Another potential mechanism for reduced NO production in PAH is increased production of a naturally occurring inhibitor of NO synthase, asymmetrical dimethylarginine (ADMA). Kielstein et al. *(124)* reported that plasma ADMA levels were higher in IPAH patients, correlated with indices of right ventricle (RV) dysfunction, and were an independent predictor of survival. In addition, acute infusion of ADMA into healthy volunteers increased pulmonary vascular resistance and decreased stroke volume, strongly supporting a role for ADMA in PAH. Reduction in dimethylarginine dimethylaminohydrolase (DDAH) can lead to increases in ADMA levels, as this enzyme catalyzes the hydrolysis of ADMA to dimethylamine and L-citrulline. In addition, endothelial injury or dysfunction may also play a role as endothelial cells can produce ADMA *(125, 126)*. Increased levels of ADMA may provide an explanation for the so-called arginine paradox, that is that treatment with L-arginine attenuates PAH in some circumstances, despite cellular levels of L-arginine being well above the K_m for NOS *(127)*. In this situation increasing L-arginine levels would reduce the inhibitory effects of ADMA on NOS.

Prostacyclin

Endothelial production of prostacyclin, a product of the arachidonic pathway, causes vasodilatation of underlying smooth muscle cells by stimulating cAMP formation. Prostacyclin, like NO, can also inhibit smooth muscle cell proliferation and platelet aggregation *(128, 129)*. PAH patients have been reported to have reduced urinary levels of prostacyclin metabolites *(78)*, which may be due to reduced prostacyclin synthase expression levels in the pulmonary arteries *(130)*. Hence these data suggest that this pathway is dysregulated and prostacyclin production reduced in PAH. A variety of animal studies support a role for altered prostacyclin levels in the pathogenesis of PAH *(129)*, including overexpression of prostacyclin synthase in transgenic mice, which provided protection from hypoxia-induced PAH *(131)*. Prostacyclin derivatives were developed early on for treatment of PAH, mainly as a result of data from experimental studies, and have now become a front-line therapy for PAH (see section on Current Therapies for Pulmonary Hypertension).

Thromboxane

Thromboxane (TXA_2) is a potent vasoconstrictor that can also increase platelet aggregation. Like prostacyclin, TXA_2 is also a product of the arachidonic pathway and is produced by endothelial cells. Studies that reported reductions in prostacyclin metabolites in the urine of patients with IPAH also found increased levels of a stable TXA_2 metabolite *(78)*. Together with animal data, these studies suggest that an imbalance in the ratio of prostacyclin to TXA_2 might contribute to dysregulated vascular tone and remodeling in PAH *(78, 132–135)*. In addition, this imbalance could also favor increased platelet aggregation leading to in situ thrombosis, increasing release of platelet-derived growth factors and other mediators, and so further promoting vasoconstriction and remodeling.

Endothelin

The endothelin (ET) family of peptides includes three members, ET-1, -2, and -3, which have been implicated in a variety of pathologic conditions *(136, 137)*. ET-1 is the best studied and is a potent vasoconstrictor and can also promote smooth muscle cell proliferation, platelet aggregation, fibrosis, and inflammation in a variety of conditions *(138–140)*. There are two receptors for ET-1: ET_A and ET_B *(137)*. Activation of ET_B receptors on endothelial cells stimulates vasodilatation (which may be mediated by NO and prostacyclin) *(141, 142)*. Whereas activation of ET_B and ET_A receptors on smooth muscle cells causes vasoconstriction *(143)*. ET-1 levels are elevated in patients with PAH *(74, 144)*, however, the inducing pathways and mechanisms are unclear. In patients with IPAH/FPAH and PAH related to Eisenmenger syndrome, the pulmonary-to-venous ratio of ET-1 is increased and ET-1 levels strongly correlate with pulmonary vascular resistance *(74, 145, 146)*. While ET-1 expression has rarely been detected in pulmonary arteries of control subjects, in IPAH/FPAH patients increased ET-1 expression was detected in endothelial cells of arteries with medial thickening and intimal fibrosis *(74)*. ET-1 has been shown to play an important role in the pathogenesis of adult, neonatal, and fetal models of PAH *(147–158)*. Hypoxia is a potent inducer of ET-1 and also ET receptor expression in animal models *(159)*. Treatment with ET receptor antagonists reduced the severity of PAH in experimental models *(150–153, 155, 156, 160)*, providing strong support for use of these type of drugs in clinical disease. Bosentan, a combined ET_A and ET_B receptor antagonist, is currently used for treatment of PAH patients (see section on Current Therapies for Pulmonary Hypertension).

Growth Factors

A number of different growth factors, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and epidermal growth factor receptor (EGFR) ligands, have been shown to play a role in the pathogenesis of PAH in experimental models *(66, 71, 161–166)*. However, the role of many of these growth factors in patients with IPAH and FPAH remains unclear. In adult, neonatal, and fetal animals, inhibition of VEGF signaling caused severe PAH along with structural changes in the lung *(71, 162, 167, 168)*. EGF receptor signaling is induced by a variety of injurious stimuli, oxidant stress, and inflammatory mediators *(169, 170)* and inhibition of the EGF receptor-attenuated PAH in the monocrotaline-induced model of PAH (*163*). PDGF receptor blockade-attenuated PAH in the fetal sheep model of PAH induced by ductus arteriosus ligation *(161)*.

Serotonin

Serotonin (5-hydroxytryptamine; 5-HT) is a neurotransmitter and can cause potent vasoconstriction and stimulate smooth muscle cell proliferation *(171–175)*. Serotonin can either enter the cell via the 5-HT transporter (5-HTT) *(176)* or can activate cell surface receptors *(177, 178)*. Entry via the 5-HTT into smooth muscle cells activates MAP kinase, GATA4-mediated transcription, and cell proliferation, due in part to increased production of reactive oxygen species (ROS) *(171, 174, 179, 180)*. IPAH has been associated with the use of appetite suppressants aminorex and dexfenfluramine, which increase levels of serotonin *(181–183)*. An epidemic of PAH occurred in Europe in patients who took aminorex and PAH was also seen with dexfenfluramine use in France and the United States *(23, 184, 185)*. Increased plasma serotonin levels have been reported in patients with IPAH/FPAH and may be due to abnormal platelet storage *(186)*. The fawn-hooded rat has higher circulating 5-HT levels, due to abnormal platelet storage, and a high susceptibility to PAH, particularly at high altitude *(187)*. Eddahbibi and colleagues *(188)* have demonstrated that the growth response of pulmonary artery smooth muscle cells (PASMC) from PAH patients to 5-HT was higher than cells from normal subjects and was related to increased 5-HTT expression. Serotonin also increased the susceptibility of BMPR-II-deficient mice to PAH, suggesting a link between BMPR2 mutations and serotonin in the pathogenesis of FPAH *(189)*. A number of other studies support a role for this pathway in PAH, including exacerbation of hypoxia-induced PAH with 5-HT treatment *(99)* and increased production of 5-HT by endothelial cells from IPAH patients *(76)*.

Bone Morphogenic Proteins (BMPs), Transforming Growth Factor-β (TGF-β), and Smad Signaling

As discussed earlier, *BMPR2* gene mutations have been identified in at least 70% of FPAH and IPAH patients. However, the role of this pathway in the pathogenesis of PAH in patients remains unclear, in large due to the low penetrance of the disease in individuals with BMPR2 mutations. BMPs are members of the TGF-β superfamily, but are distinguished from other TGF-β family members by having seven rather than nine cysteines. BMPs, originally named for their ability to induce bone formation, can regulate cell division, apoptosis, migration, and differentiation in a variety of cells and tissues. While BMPs are secreted, their diffusing capacity is influenced by their N-terminal amino acid composition, which determines their ability to bind to proteoglycans. BMPs bind to BMP receptors (BMPRs), of which there are type I and II receptors. Binding BMPs to BMPRII receptors causes phosphorylation and transactivation of BMPRI receptors in an activation complex. BMPRI receptors then activate the transcription factors Smads 1 and 5, which bind Smad 4 (C-Smad), forming a transcription factor complex. This complex enters the nucleus and activates expression of genes that regulate the cellular processes described above.

Important insights into the role of BMP2 mutations in the pathogenesis of PAH have come from the work by Morrell et al. *(190)*, who examined the proliferative responses of pulmonary artery smooth muscle cells (PASMC) from IPAH patients, patients with secondary PAH (SPH), and normal controls. In PASMC from control patients, BMPs (BMP-2, -4, and -7) and TGF-β_1 inhibited basal and serum-stimulated cell proliferation. In contrast, BMPs and TGF-β_1 failed to suppress proliferation of PASMC from IPAH patients, but not SPH patients. A *BMPR2* mutation was found in one of the five IPAH

patients used to isolate PASMC. This study suggests that normally the BMP/BMPR system plays a role in suppressing growth of PASMC, although effects on PASMC contraction have also been identified (see "Ion channels" below). A subsequent study by the same group *(191)* examined the signaling pathways downstream of BMPRs in PASMC from normal subjects and patients with *BMPR2* mutations. In normal PASMC, BMP-4 activated Smad1 as well as $p38^{MAPK}$ and ERK1/2. Smad signaling was antiproliferative, whereas $p38^{MAPK}$ and ERK1/2 signaling were pro-proliferative. In PASMC from PAH patients with *BMPR2* mutations, Smad1 signaling was defective and the cells were unresponsive to the growth-suppressive effects of BMP4. In addition, the pulmonary vasculature of patients with FPAH and IPAH was found to be deficient in the activated form of Smad1 *(191)* suggesting that defective Smad signaling and unopposed $p38^{MAPK}$ and ERK1/2 signaling underlie the abnormal proliferation of PASMC in patients with BMPR2 mutations.

Recently, interactions between the BMP receptor and the serotonin pathways have been studied. Chronic infusion of serotonin caused PAH in $BMPR2^{+/-}$ mice, which was further increased with exposure to chronic hypoxia *(189)*. The response to chronic hypoxia alone was similar in $BMPR2^{+/-}$ mice and wild-type mice, suggesting that serotonin induces different pathways than hypoxia, increasing the susceptibility to PAH with *BMPR2* haploinsufficiency. However, as mentioned above (see section on Genetics) the role of SERT (5-HTT) polymorphisms in PAH patients remains unclear.

Vasoactive Intestinal Peptide (VIP)

VIP a potent vasodilator is believed to mediate nonadrenergic noncholinergic relaxation in the pulmonary circulation *(192)*. Male mice lacking VIP develop moderate PAH, with vascular remodeling and perivascular inflammatory infiltrates *(193)*. In addition, VIP has been shown to inhibit the proliferation of PASMC from patients with IPAH *(193)*. Alterations in the VIP gene have been found in IPAH patients *(194)*. VIP-containing nerves are normally abundant in the walls of pulmonary arteries *(192)*, whereas they were undetectable in IPAH patients. Further support for a protective role for VIP came from a study showing that daily inhalation of VIP for 3 months improved exercise tolerance and pulmonary hemodynamics *(195)*. A variety of other peptides, including ANP, adrenomedulin, and pituitary adenylate cyclase-activating peptide (PACAP) also stimulate pulmonary vasodilation and have been studied in experimental models of PAH *(196)* although their role in PAH patients is unclear.

Tenascin-C (TN-C)

TN-C, an extracellular matrix glycoprotein, is highly expressed in the lesions of patients with FPAH *(197, 198)*. TN-C is also increased in experimental models of PAH *(199, 200)* and in children with PAH due to congential heart defects *(197)*. PASMC from FPAH patients express elevated levels of TN-C and the homeobox transcription factor Prx, which stimulates TN-C gene expression *(201)*. TN-C promotes PASMC proliferation and survival, at least partly by increasing EGF receptor signaling *(202)*. ERK1/2 activity was also greater in PASMC from FPAH patients and ERK1/2 inhibition stimulated Smad nuclear localization and inhibited TN-C expression *(198)*. Interestingly, inhibition of Smad signaling in normal PASMC using a kinase-deficient BMPR1b

receptor led to increases in TN-C *(198)*. Collectively, these data suggest a role for TN-C in the pathobiology of vascular remodeling and elevated levels of TN-C have been seen in patients with *BMPR2* mutations *(197, 198)*.

Elastase

Serine elastases degrade extracellular matrix (ECM), leading to the release of ECM-bound growth factors, as well as activation of matrix metalloproteinases *(203)*. Rabinovitch and colleagues have reported increased serine elastases in hypoxia and monocrotaline-induced models of PAH *(203, 204)*. Increases in elastase activity help stimulate smooth muscle cell proliferation by facilitating MMP-mediated clustering of $\alpha_v\beta_3$-integrins, activation of EGFR signaling, and increased production of TN-C *(202)*. Importantly, treatment with a serine protease inhibitor and overexpression of the serine protease inhibitor elafin reversed monocrotaline-induced PAH in rats and protected mice from hypoxia-induced PAH, respectively *(203, 204)*. These experimental studies suggest that elastases could play an important role in vascular remodeling in PAH.

S100A4

S100A4, also known as metastasin-1 (Mts-1), is a calcium-binding protein that is well known for its role in tumor metastasis. Rabinovitch and colleagues found that about 5% of transgenic mice overexpressing S100A4/Mts1 (under the control of the HMG-CoA reductase promoter) developed plexigenic arteriopathy, neo-intimal remodeling, and PAH *(205)* and that S100A4 was increased in the smooth muscle cells of remodeling vessels in children with PAH secondary to congenital heart defects *(205)*. In cultured PASMC, serotonin increased S100A4 expression through a ROS/MAPK/GATA4-dependent mechanism, suggesting that S100A4 may be downstream and contribute to the pathogenesis of serotonin-mediated PAH *(180)*. In addition, S100A4 was also shown to activate the RAGE receptor and so may stimulate PASMC migration, as well as proliferation in remodeling arteries *(180)*.

Inflammation

Early studies by Voelkel and Tuder suggested a role for inflammation in vascular remodeling in IPAH *(206, 207)*. Increased numbers of inflammatory cells, including lymphocytes and macrophages, were found in plexiform lesions *(64)*. In addition, macrophage inflammatory protein 1α (MIP1 α), IL-1β, and IL-6 were also elevated in lung biopsy samples and serum from patients with severe IPAH *(208)*. Autoimmunity may also play a role, as elevated levels of auto-antibodies, including antiendothelial cell antibodies, have been detected in IPAH patients *(209, 210)*. This has led to the suggestion that immune dysfunction, related to abnormal regulatory T-cell activity, causes inflammation and hence plays a role in the pathogenesis of vascular remodeling in PAH.

Viral Infection

The association of human immunodeficiency virus 1 (HIV-1) infection with severe PAH is well known *(211, 212)*. Human herpes virus 8 (HHV-8) infection is increased

in HIV-1 patients with IPAH/FPAH and in some cases of Castleman's disease *(213, 214)*. Voelkel and colleagues detected a high rate of HHV-8 infection in lung tissue and microdissected cells from the plexiform lesions of IPAH/FPAH patients, but a much lower rate of infection in SPH patients *(70)*. Interestingly, Rabinovitch and colleagues have recently reported that viral infection increases the incidence of PAH in S100A4/Mts-1 transgenic mice *(215)*, through a mechanism that involved the breakdown of elastin and elastin peptide-mediated inflammation. Hence, viral infection and viral-induced inflammation may play a role in the pathogenesis of vascular remodeling and PAH.

Ion Channels

Voltage-gated potassium (Kv) channels regulate the resting potential of vascular smooth muscle cells and play a direct role in hypoxic pulmonary vasoconstriction *(216, 217)*. PASMC from IPAH/FPAH patients have been reported to have dysfunctional Kv channels *(218)*. Archer, Weir, and colleagues have shown that inactivation of Kv channels results in membrane depolarization, calcium influx via activation of voltage-gated (L-type) calcium channels, and pulmonary vasoconstriction *(217, 219)*. Hypoxia and dexfenfluramine both inhibit Kv channels in vascular smooth muscle cells *(220)* and in vivo gene transfer of Kv1.5 channels has been shown to attenuate PAH and restore hypoxic vasoconstriction in chronically hypoxic rats *(221)*. Rodman and colleagues have reported that BMP2 treatment increases Kv1.5 channel expression in cultured human PASMC and that Kv1.5 protein levels were reduced in dominant-negative *BMPR2* transgenic mice with PAH *(222)*. RV systolic pressures normalized when the dominant-negative *BMPR2* transgenic mice were treated with nifedipine, an L-type calcium channel blocker, suggesting that activation of L-type calcium channels causes PAH in these mice. These data suggest that *BMPR2* mutations might cause pulmonary vasoconstriction through reductions in Kv channel expression in PASMC and calcium influx through L-type calcium channels. Hence it has been suggested that *BMPR2* mutations may cause vasoconstriction through this mechanism, which could precede and then lead to vascular remodeling in patients with FPAH *(222)*.

Rho-Kinase

Vascular smooth muscle tone is determined by the balance between the activities of myosin light-chain kinase and myosin light-chain phosphatase, which favor constriction and dilation, respectively. Rho-kinase (ROK or ROCK) is a downstream target of the small GTPase RhoA, which regulates a variety of different cellular functions, including cell adhesion and motility, actin cytoskeleton organization, smooth muscle contraction, and gene expression. Rho-kinase is a major regulator of myosin light-chain phosphatase and when activated inhibits myosin light-chain phosphatase, which promotes vasoconstriction *(223)*. ROCK signaling mediates vasoconstriction induced by hypoxia and a number of stimuli *(224)*. ROCK signaling is known to be involved in other pathologic conditions, including renal vasoconstriction *(225)*, systemic vascular remodeling, and cardiac hypertrophy *(226–229)*, and Fasudil has been used for treatment of patients with intractable severe coronary spasm after coronary artery bypass surgery *(230)*, vasospastic angina *(231)*, and patients with effort-induced angina *(232)*. ROCK plays a role in the constrictor and proliferative responses to 5-HT *(233)* and ET-1 *(234)* and synthe-

sis of TN-C by smooth muscle cells *(235)*. Importantly, in vivo studies showed that ROCK inhibitors reduced PAH in a number of experimental models, including hypoxia *(236)*, monocrotaline *(237, 238)*, high flow *(239, 240)*, SU5416 treatment plus hypoxia *(241)*, and in fawn-hooded rats *(242)*. A few studies have reported beneficial responses in patients with severe PAH (including some with IPAH) treated acutely with the ROCK inhibitor, Fasudil, further supporting a role for Rho-kinase in PAH *(243, 244)*.

Current Therapies for Pulmonary Hypertension

General Measures

The management of patients with pulmonary hypertension is complex and frequently challenging. Several general measures should be considered in all patients with IPAH or FPAH. Physical activity should be encouraged but should be limited by symptoms of chest pain, severe dyspnea, pre-syncope, or syncope. Air travel should be avoided if possible due to increased risk of pulmonary vasoconstriction with decreased oxygen tension at high altitude. If air travel is necessary, the use of supplemental oxygen should be considered. Control, prevention, and treatment of infections are imperative. Vaccinations for influenza and pneumonia are recommended *(245)*. Close monitoring and prompt treatment is imperative for indwelling central venous catheter infections. Pulmonary infections should be assessed and treated in a timely fashion. Since anemia is not well tolerated in patients with PAH, anemia should be assessed quickly and treated. In a meta-analysis of data collected between 1978 and 1996, IPAH in women was associated with 30% mortality with pregnancy *(246)*; therefore, women with PAH are strongly advised to avoid pregnancy. The American Heart Association and American College of Cardiology recommends termination of pregnancy in women with PAH *(245)*. Contraception is recommended for all women of childbearing age. Currently, there is no consensus on the safest form of contraception, but it is believed that estrogen-containing therapies should be avoided whenever possible. Additionally, it is also recommended to avoid hormone-replacement therapy in postmenopausal women with PAH *(247)*.

Conventional Therapies

Warfarin, oxygen, diuretics, and digoxin are often times referred to as conventional therapy in PAH. In situ microscopic thrombosis has been documented in IPAH. Also, patients with PAH-associated right ventricular failure and venous stasis are at increased risk of the development of venous thrombosis. Therefore, anticoagulation is recommended for IPAH/FPAH patients, unless there is a contraindication to this therapy, as it has been shown to be associated with improved survival *(248, 249)*. Hypoxemia is a potent pulmonary vasoconstrictor; therefore, oxygen therapy is recommended in hypoxemic patients with PAH *(245)*. In general, supplemental oxygen is administered to keep saturations >90% at all times. Diuretic therapy is used in patients with right ventricular failure and volume overload. However, over diuresis should be avoided as PAH patients may be preload dependent and develop hypotension, renal failure, and syncope with excessive diuresis *(245)*.

Calcium Channel Blockers

Calcium channel-blocking agent has been used in the treatment of pulmonary arterial hypertension over the last 40 years *(250)*. A select group of patients with IPAH/FPAH (<10%) are candidates for calcium channel blocker therapy and a portion of these patients have a long-term clinical response to therapy *(57)*. This group is defined by patients with IPAH/FPAH who have a significant response to a vasoreactivity challenge with nitric oxide *(54, 55)*, intravenous epoprostenol *(54)*, or intravenous adenosine *(56)*. A positive vasodilator response is generally considered as a decrease in mPAP by at least 10 mmHg or an mPAP of $\leq$ 40 mmHg with a normal or high cardiac output *(39)*. A trial of long-acting nifedipine, diltiazem, or amlodipine should be considered in patients with IPAH/FPAH with a positive vasoreactivity challenge and without any contraindications. Verapamil should be avoided due to its negative inotropic effects. A long-term response to calcium channel blockers is defined as a sustained hemodynamic improvement for at least 1 year on monotherapy with calcium channel blockers with achievement of NYHA I–II functional status. If patients do not experience improvement in symptoms and hemodynamics, alternative PAH-directed therapy should be instituted *(34)*.

Targeted Pulmonary Arterial Hypertension Therapies

Targeted therapies for PAH have been developed over the last two decades. The prostacyclin, endothelin, and nitric oxide pathways are targets of current FDA-approved therapies for PAH (see Figure 3.10).

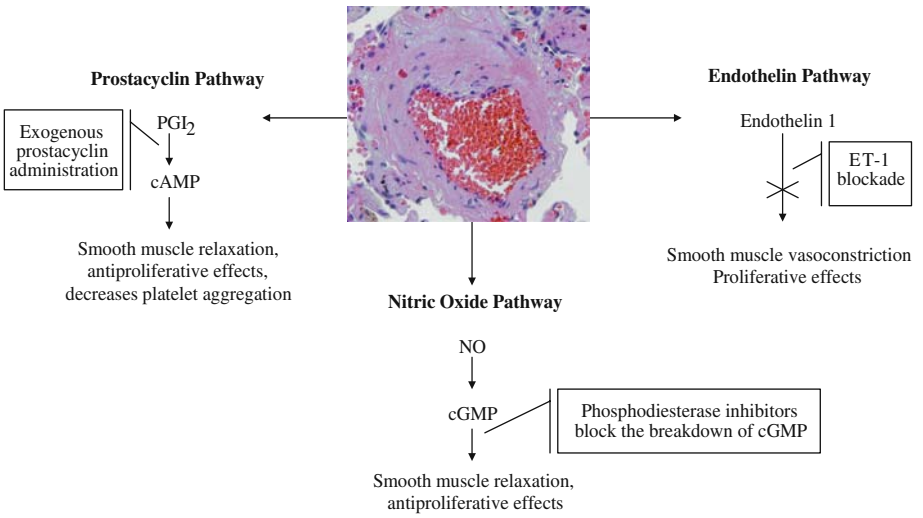


Figure 3.10 Current targeted pathways for therapy in pulmonary arterial hypertension

Prostanoids

Prostacyclins act via a G protein-coupled receptor (GPCR) pathway by increasing 3′–5′-cyclic adenosine monophosphate (cAMP) leading to smooth muscle vasorelax-

ation and inhibition of proliferation *(251, 252)*. Exogenous prostacyclins have been used in the treatment of PAH for the last two decades. Prostanoids can be delivered via the intravenous, subcutaneous, or inhaled route. Epoprostenol and treprostinil are both administered intravenously.

Intravenous epoprostenol has been shown to improve exercise capacity, quality of life, hemodynamics, and survival in a 12-week open-labeled trial comparing epoprostenol with conventional therapy in 81 patients with advanced IPAH (NYHA functional classes III and IV) *(253)*. The epoprostenol-treated group experienced a 47 m increase in 6-minute walk distance (6MWD) while those treated conventionally had a 66 m decrease *(253)* during the trial.

Intravenous treprostinil has also been studied in a 12-week open-labeled trial of 16 functional class III and IV PAH patients and was associated with an improvement in functional capacity, pulmonary hemodynamics, and exercise capacity and a 82 m increase in 6MWD *(254)*. In addition, Treprostinil can also be used subcutaneously and in a 12-week, double-blind, placebo-controlled multicenter trial in 470 patients with PAH, improvements in dyspnea, hemodynamics, and exercise capacity were seen *(86)*. Eighty-five percent of patients using treprostinil did report infusion site pain; however, only 8% of patients discontinued the medication because of this *(255)*.

Iloprost is currently the only approved prostanoid, which can be delivered via inhalation. It is administered through a specialized nebulized system six to nine times daily. Iloprost has been studied as monotherapy and in combination with other PAH therapies. In a 3-month randomized, double-blind, placebo-controlled trial in 203 patients with NYHA functional class III or IV PAH and CTEPH, monotherapy with inhaled iloprost was found to improve functional class and exercise tolerance (36 m increase in 6MWD) in treated patients *(256)*. The addition of iloprost to bosentan, an oral endothelin blocker, in 67 patients with functional class III PAH was associated with increased exercise tolerance, functional class, clinical worsening, and hemodynamics *(257)*. To date, the long-term benefit of iloprost therapy has not been clearly determined *(258, 259)*, thus further studies are needed.

Endothelin Receptor Antagonists

As discussed earlier, endothelin-1 (ET-1) is a vasoconstricting peptide that acts on the ET_A and ET_B receptors *(260)*. Currently, three oral endothelin antagonists are being used and/or studied for the treatment of PAH and include bosentan, sitaxsentan, and ambrisentan. Bosentan is an oral dual endothelin receptor (ET_A and ET_B) antagonist and was the first endothelin antagonist approved for use in PAH. In a multicenter, randomized double-blind, placebo-controlled study of 32 patients with PAH, bosentan therapy was associated with improved exercise tolerance, functional class, and pulmonary hemodynamics. The bosentan treatment group experienced an average of a 70 m increase in 6MWD over the 12-week period *(261)*. In an additional 16-week double-blind, placebo-controlled study of 213 functional class III and IV PAH patients, treatment with bosentan leads to increased exercise tolerance on 6MWD by 36 m and a delay in time to clinical worsening *(262)*. It appears that survival is also impacted by treatment with bosentan. McLaughlin et al. reported a 96% 12-month and 89% 24-month survival in PAH patients initially treated with bosentan monotherapy *(263)*. The survival rates in bosentan-treated patients exceeded the historical controls as the NIH registry reported a 69% 12-month and 57% 24-month survival in untreated patients.

Sitaxsentan is a selective ET_A receptor antagonist that is not yet approved by the FDA for the treatment of PAH. In 2004, Barst et al. studied 178 NYHA functional class II–IV PAH patients in a multicenter, randomized trial comparing sitaxsentan to placebo. Sitaxsentan treatment was associated with a 35 m increase in 6WMD. Additionally, improved functional class and pulmonary hemodynamics were seen in the treated group *(264)*. A second 18-week double-blind, placebo-controlled trial to evaluate sitaxsentan evaluated 247 PAH patients and found patients treated with 100 mg of sitaxsentan daily experienced a 31.4 m increase in 6MWD *(265)*. Three percent of patients treated with 100 mg sitaxsentan daily experienced a three-fold increase in serum aminotransferases.

Ambrisentan, a second selective ET_A receptor antagonist, was recently approved for use in PAH in the United States *(266)*. In a 12-week double-blind, dose-ranging study, 64 functional class II–III patients with PAH were randomized to receive 1, 2.5, 5, or 10 mg of ambrisentan followed by 12 weeks of open-label ambrisentan. At 12 weeks, all doses of ambrisentan resulted in a statistically significant increase in 6MWD (33.9–38.1 m). Improved Borg dyspnea index, functional class, pulmonary hemodynamic, and cardiac index were also reported. Overall, this medication was well tolerated and side effects were unrelated to dose. Patients (3.1%) did experience a three-fold elevation in serum aminotransferases. Two phase III trials have been completed to evaluate ambrisentan; publication of these results are pending at the moment *(34)*.

Phosphodiesterase Inhibitors

As discussed earlier, nitric oxide is a well-known pulmonary vascular vasodilator and antiproliferative agent. Nitric oxide acts via 3′–5′-cyclic guanosine monophosphate (cGMP) to induce vasorelaxation *(267)*. Phosphodiesterase type-5 inhibitors decrease the degradation of 3′–5′-cyclic guanosine monophosphate (cGMP) in the lung, which allows for sustained pulmonary vascular smooth muscle relaxation *(268)*. Sildenafil is a potent phosphodiesterase type-5 inhibitor that was previously approved for the treatment of erectile dysfunction. During the last decade there have been several reports of its successful use in PAH *(269–271)*. A 12-week randomized, double-blind trial of 278 PAH was completed comparing placebo to therapy with 20, 40, or 80 mg of sildenafil three times daily. Treatment with sildenafil was associated with an increase in 6MWD but was not dose dependent. Additionally, the treated group was found to have statistically significant improvements in pulmonary hemodynamics and functional class. Use of sildenafil was associated with flushing, dyspepsia, and diarrhea. Treatment with sildenafil did not result in decrease in clinical worsening. A total of 222 patients who completed the 12-week randomized study entered a long-term extension study. Monotherapy with sildenafil at 80 mg three times daily for 1 year was associated with a mean increase in 6MWD of 51 m *(272)*.

Interventional Procedures

In the setting of severe PAH with right heart failure unresponsive to medical therapies, surgical intervention with septostomy or lung transplant may be indicated. Atrial septostomy has been performed in end-stage PAH patients and is often used as a bridge to transplant. This procedure creates a right-to-left shunt in an attempt to decompress the right heart and increase cardiac output. Septostomy does lead to a decrease in oxygenation; however, due to the improved cardiac output there is an increase in

systemic oxygen transport. Overall, the procedure is associated with 5–15% mortality. If the procedure is tolerated, septostomy is associated with improved symptoms and hemodynamics *(273)*.

Future Directions

Diagnosis and Management

Pulmonary hypertension evaluation, treatment, and management are rapidly evolving. Despite increased awareness and education efforts, patient identification and evaluation continue to be delayed *(29)*. Currently, the diagnosis is made based on clinical suspicion and routine testing. Future advances in echocardiography may improve the diagnostic yield of this test *(274, 275)*. Additionally, phase-contrast magnetic resonance (MR) imaging shows promise as a noninvasive tool to assess pulmonary arterial flow parameters to estimate pulmonary arterial pressures and pulmonary vascular resistance. In a retrospective review on 59 patients who underwent right heart catheterization and MR scanning, Sanz et al. found that a phase-contrast MR had a sensitivity of 92.9% and a specificity of 82.4% for the detection of PAH *(276)*. Biomarkers such as B-type natriuretic peptide (BNP) may have a role in the diagnosis and management of PAH. BNP is a known marker of right ventricular dysfunction *(277)*. Elevations in BNP are associated with worse prognosis *(278)*. Additionally, BNP decreases with effective treatment of PAH *(279)*. Endothelin-1 (ET-1) is also a biomarker that is known to be elevated in PAH *(146)*; however, its clinical utility is unclear at the present time. Other biomarkers that are currently being evaluated in PAH are isoprostanes (prostaglandin-like compounds formed in vivo from the free radical-initiated peroxidation of arachidonic acid), asymmetric dimethylarginine (ADMA), C-reactive protein (CRP) *(280)*, vascular endothelial growth factor (VEGF) *(281–284)*, platelet-derived growth factor (PDGF) *(164–166)*, and cardiac troponin T *(280)*. Future study is required to determine if these biomarkers could be useful tools in the diagnosis and management of PAH.

Pharmacotherapies

Several potential targets exist for the development of future pharmacotherapies in PAH. Potassium channel openers *(285)*, arginine therapy *(286)*, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors *(287, 288)*, serotonin (5-HT) antagonists *(289)*, serotonin transporter (5-HTT) inhibitors *(290)*, adrenomedullin *(291–294)*, nitrites *(295, 296)*, tyrosine kinase inhibitors *(163, 164)*, Rho kinase inhibitors *(224)*, and peroxisome proliferator-activated receptor gamma (PPARγ) *(297)* are all promising agents. Selected future targets are mentioned below. Additional reading of the comprehensive reviews of this rapidly advancing area in PAH is recommended *(298, 299)*.

Potassium channels modify vascular tone and proliferation. Inhibition of one or more of the voltage-gated potassium channels (Kv) in the pulmonary artery smooth muscle cells leads to opening voltage-gated calcium channels leading to an influx of cytosolic Ca^{2+} and thus smooth muscle constriction *(216)*. Kv1.5 or Kv2.1 channels are downregulated in pulmonary arterial smooth muscle cells from patients with PAH *(218)* as well as in a rat model with hypoxia-induced PH *(300)*. Augmentation of potassium channel opening or upregulation of these channels may have a therapeutic role

in PAH. Oral dichloracetate is known to increase the expression and function of Kv2.1 channels *(285)*. This agent has been studied in animals and found to decrease vascular remodeling and PVR in rats with hypoxic pulmonary hypertension *(285)*. Further study is necessary to evaluate the potential role for oral dichloracetate and potassium channel openers in the treatment of PAH.

The HMG-CoA reductase inhibitors, statins, have potent antiproliferative and anti-inflammatory effects *(301)*. Additionally, statins associated with a suppression of endothelial and vascular smooth muscle cell neo-intimal responses to vascular injury *(302)*. Statins are also thought to promote vascular repair and augment nitric oxide production via stabilization of nitric oxide synthase *(303, 304)*. Statins have been shown reverse monocrotaline-induced pulmonary hypertension in rats *(287, 288)*; however, further evaluation of this therapy in humans with PAH is required.

Platelet-derived growth factor has been shown to be elevated in animal models of PH *(166, 305)* as well as humans with PAH *(164)*. Furthermore, pulmonary hypertensive changes in both monocrotaline and hypoxia-induced rat models of PH could be ameliorated with the PDGF antagonist STI571 *(164)*. Case reports of treatment PAH with the tyrosine kinase inhibitor, imatinib *(306–308)*, are promising; however, controlled trials are needed to the safety and efficacy of this agent in PAH.

Genetics

Given that *BMPR2* mutations (and *ALK1* and *ENG* mutations) have been identified in less than 80% of FPAH cases, the question arises as to the nature of the genetic defect in the remaining families. While some of them may harbor as-yet undetected *BMPR2* mutations, it is likely that other genetic factors may play a primary role in disease pathogenesis. Observed differences in disease penetrance, age of onset, and especially gender in FPAH and IPAH suggest the involvement of additional genetic modifiers, which contribute to the disease. Due to the unavailability of relatively large families in whom no BMPR2 mutations have been identified, genetic linkage studies to identify additional genetic factors are likely to prove difficult. Therefore, future studies may be facilitated by the collection of large cohorts of seemingly IPAH patients to determine any novel genetic associations that may contribute to disease pathogenesis. Whole-genome association studies involving the genotyping of hundreds of thousands of single-nucleotide polymorphisms in patient cohorts are currently underway to identify genetic factors for many diseases *(309, 310)*. Similar studies in a cohort of a thousand or more IPAH patients may help identify new genes contributing to the pathogenesis of PAH. The role of gene mutations or polymorphisms in the vasoactive mediators (NO, PGI2, TXA_2, ET-1, 5-HT) and factors (TGF-α, elastase, TN-C, S100A4/Mts1) mentioned above is unclear. In addition, while these mediators/factors contribute to the pathophysiology of PAH, whether alterations in these mediators/factors contribute to the pathogenesis of PAH or are just downstream consequences of the disease process, albeit exacerbating the pathophysiology, remains unclear.

Pathobiology and Cell-Based Therapies

While the pathology of IPAH has been well studied in adults it remains unclear whether children have a similar histological phenotype. For example, the plexiform lesions in young children with IPH are frequently more muscularized without endothelial cell

proliferation and have a more prominent expansion of the fibroblastic adventitial layer (G. Deutsch, unpublished data). Further characterization of the pathology of IPAH and FPAH is essential to validate mouse models and therapeutic strategies. Novel findings are that transdifferentiation of endothelial cells *(311)* may contribute to vascular remodeling and suggest that cells in lesions may not simply arise by proliferation.

New directions treatment of PAH include cell-based therapy with endothelial progenitor cells or cells engineered to express elevated levels of vasodilators, such as NO. Experimental studies in animal models have provided positive results and preliminary studies in humans have been encouraging *(312–318)*. A better understanding of the pathogenesis, particularly triggers and disease/genetic modifiers, will help the identification of novel targets and possibly preventative therapies.

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4

Lymphangioleiomyomatosis

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Abstract Lymphangioleiomyomatosis is a rare, cystic lung disease of women that most commonly presents with progressive dyspnea on exertion and recurrent pneumothorax in the third or fourth decade of life *(1)*. LAM can also be associated with abdominal and thoracic lymphadenopathy, renal and extrarenal angiomyolipomas, and chylous fluid collections in the abdomen, chest, or pericardium. LAM occurs almost exclusively in females, for reasons that are not understood, but biopsy-documented LAM in males has also been reported. Cystic changes consistent with LAM are found in about 30–40% of women who have the heritable disease tuberous sclerosis complex (TSC), a neurocutaneous tumor suppressor syndrome. TSC-associated LAM (TSC-LAM) is frequently asymptomatic. LAM also occurs in patients who do not have TSC, and despite an estimated prevalence that is 10-fold lower than that of TSC-LAM, these "sporadic LAM or S-LAM" patients generally outnumber TSC-LAM patients 6:1 in pulmonary clinics and registries around the world. S-LAM is also associated with TSC mutations, but they are found only in the neoplastic lesions in the lung, kidney, and lymphatics, and not in normal tissues or in the circulating blood cells. The histopathologic hallmark of LAM in the lung is interstitial expansion with benign-appearing smooth muscle cells, which infiltrate all lung structures, including alveolar septa, airways, blood vessels, lymphatics, and pleura. The origin of the invading cells is unknown, but available evidence suggests an extrapulmonary source. Two metastatic mechanisms have been proposed: dissemination from angiomyolipomas and pulmonary microvascular dissemination of LAM cell clusters originating in the lymphatics and gaining access to the venous circulation at the level of the thoracic duct. The prognosis in LAM depends on the mode of presentation and is more favorable in patients who are ascertained through screening, pneumothorax or incidental findings on studies obtained for other purposes rather than through shortness of breath. There are currently no treatments which are known to be effective. Antagonism of estrogen action, using progestins or GnRh agonists, is the most commonly employed empiric therapeutic strategy. Advances in our understanding of the molecular pathogenesis of LAM have far outstripped progress in the

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clinical arena, and clinical trials directed at molecular targets identified through basic investigation are underway in the United States and Europe.

Keywords: interstitial lung disease, chylous effusion, HMB-45, pneumothorax, tumor suppressor syndrome, perivascular epithelioid cell tumor, sirolimus

Introduction

Lymphangioleiomyomatosis was first described in 1918 in a tuberous sclerosis patient who presented with bilateral pneumothoraces *(2)*. The first report of a case of LAM in patient that did not have tuberous sclerosis appeared in Germany in 1937 *(3)*. The nomenclature of LAM was confused for decades to follow, reported under names of lymphangiopericytoma, lymphangiomyoma, leiomyomatosis, lymphangiomatous malformation, and intrathoracic angiomyomatous hyperplasia, before the sentinel reports by Cornog and Enterline *(4)* and Corrin, Leibow and Friedman *(5)*. The clinical, radiologic, and pathologic descriptions of LAM in those papers brought clarity to the field and set the stage for future advances. Well before their time, they recognized the lymphatic origins of LAM, the close association between LAM and TSC, and the nonmalignant, neoplastic nature of the LAM lesion.

Epidemiology

The major sources of epidemiologic data in LAM are the National Heart Lung and Blood Institute (NHLBI) LAM Registry from the National Institutes of Health (NIH) *(6)*; The Japanese Ministry LAM Registry *(7)*; large case series from France *(8)*, the United Kingdom *(9, 10)*, Japan *(11)*, and Korea *(12)*; and patient foundations around the world. All of these are based on unvalidated data collected remotely by questionnaires from either the physician or the patient, except for the NHLBI Registry, which was a prospective study. For this reason, much of what follows is based on the NIH study. The NHLBI Registry patients were seen over a 3-year period at 1 of 6 sites around the United States (Stanford, Mayo Clinic, Cleveland Clinic, NHLBI, Tufts, National Jewish Hospital), and data was collected longitudinally for 5 years in a standardized manner. To date, only the baseline data from that study has been published. All 243 of the NHLBI Registry registrants were women, and 13 patients were excluded based on prior lung transplant. The average age at onset of symptoms was 38.9 ± 0.73 years and the average age at LAM diagnosis was 41.0 ± 0.65 years, which is similar to that reported from France (36.3 years/39.3 years) *(8)*, but much later than that in the United Kingdom (31 years/35 years) *(10)* or Japan (31.6 years/34 years) *(7)*. Earlier recognition in Japan is likely related to routine annual chest X-ray screening of all working citizens for tuberculosis and the convention that first pneumothoraces in patients without apparent lung disease are frequently evaluated with chest CT scanning (but not in the United States, Europe, or Australia). There are four cases of biopsy-documented LAM in men in the literature: three in men who had definite or probable TSC *(13–15)* and one in a man who had no evidence of TSC *(16)*. Other non-biopsy-documented cases of male TSC patients with cystic changes that are typical for LAM have been reported,

Table 4.1 Vital Status of LAM Patients Registered with LAM Foundation.

	Foreign	United States	Total
Living	389	816	1,205
Deceased	39	171	218
Total	428	987	1,423

but subclinical LAM in men with TSC is almost certainly a rare occurrence. Screening of approximately 20 male TSC patients at the NIH *(17)* and in Cincinnati (unpublished observation) did not reveal a single case.

The prevalence of LAM is difficult to estimate. As of this writing, the LAM Foundation has registered over 1,400 patients, including 838 from the United States and 362 from foreign countries (personal communication, Jill Raleigh, CEO, The LAM Foundation) (Table 4.1). To date, international LAM organizations have collectively registered at least 700 patients, with the largest concentrations of identified patients in Europe (approximately 400) and Japan (approximately 200). There is some overlap between the foreign LAM patients registered with the US LAM Foundation and the foreign LAM patients registered with foreign foundations. Given approximately 816 living registered LAM patients in the United States, and the current US population of 303 million, the minimum prevalence of LAM in the United States is estimated to be approximately 2.7 per million. As can be seen in Table 4.2, LAM prevalence calculated in this way varies between 1 and 3 per million in most developed countries. This compares favorably with the prevalence of LAM determined by questionnaires sent

Table 4.2 International LAM Patient Populations and Organisations.

	Name	Living	Deceased	Total	Pop. (10^6)	Living per million
United States	LAM F	816	206	1,022	303	2.7
Japan	J-LAM	173	–	173	128	1.4
Germany	LAM Selbsthilfe	106	7	113	82	1.3
France	FLAM	142	8	150	64	1.5
UK	LAM Action	120	18	138	60	2.0
Brazil	ALAMBRA	68	10	78	187	0.4
Canada	LAM Canada	61	3	64	33	1.8
Australia	LARA	50	8	58	21	2.4
Italy	A.I. LAM	40	–	40	59	0.7
China	LAM China	41	5	46	1,321	.03
Spain	AELAM	57	3	60	45	1.1
Romania	LAM Romania	14	1	15	21	1.6
Korea	LAM Korea	28	–	28	49	0.6
New Zealand	NZ LAM Trust	13	2	15	4	3.3
Austria	LAM Austria	22	–	22	8	2.8
Netherlands	Tante Meila	15	–	15	17	0.9
Norway	LAM Norway	15	–	15	4.6	3.3
Total		1,781	271	2,052	2,407	

to all pulmonary physicians in England *(10)*, France *(8)*, and Japan *(11)*, requesting information about their patients with LAM. These are certainly underestimates, since LAM is difficult to diagnose, not all patients or physicians respond to questionnaires, and not all LAM patients are seen by pulmonologists or register with LAM organizations. In addition, we know that there is a large, undiagnosed population of women with tuberous sclerosis who have LAM. Only 14.8% of patients in the NHLBI Registry *(6)* and 11% of the LAM patients registered with the LAM Foundation report that they have TSC (Table 4.3). We know from several studies that cystic change consistent with LAM is present in 30–40% of women with TSC *(17–19)* and that the estimated prevalence of TSC in the population is approximately 1 in 12,500 *(20)*. These data suggest that TSC-LAM affects approximately 250,000 women worldwide and 15,000 in the United States, much greater numbers than the 2,000–2,500 or so LAM patients who are known to be registered with international LAM organizations. It is clear from screening studies that TSC-LAM is often subclinical and mild, and may be less of a health priority for patients who are suffering from other manifestations of TSC than for S-LAM patients.

Table 4.3 Clinical Characteristics of TSC-LAM and S-LAM Patients Registered with the LAM Foundation.

	TSC-LAM	S-LAM
N	123	897
AML	72%	22%
Lung transplant	7%	10%
Supplemental O_2	23%	20%
Pneumothorax	49%	42%
Chylothorax	1%	12%
Uterine fibroids	17%	12%

Genetic Basis and Molecular Pathology

Overview

LAM pathogenesis appears to involve one of the most unusual pathogenic mechanisms in human disease: the metastasis of histologically benign cells *(21)*. Making this mechanism even more remarkable and fascinating is the fact that this metastasis occurs almost exclusively in women. The molecular basis of LAM revolves around four key questions: What genetic factors contribute to LAM pathogenesis? Why does LAM occur exclusively in women? What is the cell of origin of LAM? and Why is LAM associated with cystic lung destruction?

The last decade has resulted in breathtaking progress in elucidating LAM pathogenesis *(22)*. LAM research serves as a shining example of "bench to bedside" disease-oriented research, with key translational discoveries in human tissue specimens alongside discoveries in model organisms including *Drosophila melanogaster* and rodents leading to clinical trials with targeted therapeutic approaches.

Tuberous Sclerosis Complex-Associated LAM

Tuberous sclerosis complex (TSC) is a tumor suppressor gene syndrome characterized by benign tumors in multiple organs, seizures, mental retardation, and autism. TSC

exhibits autosomal dominant inheritance with 95% penetrance. However, only approximately 20% of TSC patients have a positive family history of TSC. The remaining 80% of cases represent de novo mutations in either of the two genes known to be associated with TSC, *TSC1* or *TSC2*. *TSC2*, which was cloned in 1993 and is located on chromosome 16p13, has 41 exons and produces a 5.5-kb mRNA transcript *(23)*. *TSC1*, on chromosome 9q34, was cloned in 1997 and has 21 coding exons *(24)*. The 8.6-kb TSC1 mRNA transcript contains a small 5′ and a large 4-kb 3′ un-translated region.

The most frequently occurring tumors in TSC patients include cerebral cortical tubers, facial angiofibromas, cardiac rhabdomyomas, and renal angiomyolipomas. Multiple bilateral renal angiomyolipomas occur in the majority of TSC patients, with an onset in childhood *(25)*. Angiomyolipomas are benign lesions composed of three distinct cell types: smooth muscles, fat, and vascular cells. Genetic studies have revealed that all three cell types within angiomyolipomas arise from a common precursor cell, in contrast to virtually all other blood vessel-filled tumors, in which the vessels are recruited by the tumor and therefore arise separately *(26)*.

TSC1 and *TSC2* are tumor suppressor genes, which in the classic "two-hit" tumor suppressor gene model are associated with disease when a germline mutation inactivates one allele and a second inactivating mutation occurs in somatic tissues *(27)*. Often the somatic, "second hit" mutation involves loss of the chromosomal region containing the entire wild-type copy of *TSC1* or *TSC2*. This chromosomal loss is detected when heterozygous DNA markers present in normal DNA are found to be homozygous in tumor DNA, which is referred to as loss of heterozygosity (LOH). LOH for either *TSC1* or *TSC2* has been detected in the majority of angiomyolipomas and rhabdomyomas from TSC patients *(28)* and in LAM cells from TSC patients *(29)*.

Radiographic evidence of LAM is present in about one-third of women with TSC, although only a fraction of these women have clinically significant pulmonary symptoms *(17–19)*. Germline mutations in both *TSC1* and *TSC2* are associated with LAM in TSC *(19, 30–34)*. The mutations in women with TSC and LAM are found throughout the genes and include the two most frequent *TSC2* mutations (R611Q and an 18-base-pair inframe deletion in exon 40) and a missense mutation in the last exon (exon 41) of *TSC2 (30)*. Therefore, there is no evidence for a genotype–phenotype correlation.

Sporadic LAM

Sporadic LAM refers to the form of this lung disease in women who do not have clinical manifestations of TSC and do not have germline *TSC* gene mutations *(35)*. About 30–60% of women with sporadic LAM have renal angiomyolipomas *(36, 37)*. One of the first clues to the pathogenesis of sporadic LAM was the finding of TSC2 LOH in angiomyolipomas from women with the sporadic form of LAM *(38)*. Subsequently, inactivating mutations in the remaining allele were detected, implicating *TSC2* inactivation in the pathogenesis of the angiomyolipomas. Remarkably, the identical *TSC2* mutations in the angiomyolipomas were also present in microdissected pulmonary LAM cells of five sporadic LAM patients but not in normal DNA from the kidney, lung, or peripheral blood mononuclear cells of these patients *(32)*. These data indicated for the first time that somatic *TSC2* mutations are a cause of sporadic LAM. These mutational results were confirmed in Japanese sporadic LAM patients *(33)*. The pattern of the mutations – present in the LAM and angiomyolipoma cells, but not in any normal cell types – suggested that LAM cells may spread or metastasize to the lungs from

the angiomyolipoma or another site *(32)*. Finally, the detection of TSC2 LOH in the sporadic LAM cells proved that these cells, like other tumor cells in TSC, fit the two-hit tumor suppressor gene model.

Strong additional support for the "benign metastasis" model of LAM pathogenesis arose from studies of women with the sporadic form of LAM who had recurrent LAM after lung transplantation. We and others also found that recurrent LAM after lung transplantation is derived from the patient's original LAM cells *(39, 40)*, consistent with a metastatic mechanism. LAM cells carrying *TSC2* mutations have also been detected in mediastinal lymph nodes and circulating in the blood of women with LAM *(41)*. Taken together, these data support a model in which the pathogenesis of LAM involves the metastasis of benign cells; the fact that LAM occurs primarily in women suggests that the metastasis is estrogen driven. Mutations have been detected in LAM cells from patients with the sporadic form of LAM *(29, 32–34, 41)*.

It is important to emphasize that, as yet, the fraction of patients with the sporadic form of LAM who carry *TSC1* or *TSC2* mutations is unknown. While this is an obviously important question, it is experimentally very difficult for two reasons: first, LAM cells are tightly intermingled with reactive pneumocytes and other cells that do not carry mutations, and therefore mutational analyses using conventional mutation detection techniques such as sequencing require microdissection of the LAM cells. Second, as discussed earlier, mutations can occur throughout *TSC1* and *TSC2*, requiring the sequencing of more than 60 exons on DNA prepared from the microdissected cells. While we do not yet know the fraction of sporadic LAM that carries *TSC1* or *TSC2* mutations, the majority of LAM and angiomyolipoma specimens that have been studied show immunohistochemical evidence of hyperactivation of the mTOR pathway, suggesting that mTOR activation is a unifying event in LAM pathogenesis *(42)*. Activating mutations in Rheb and Rheb-Like protein (RLP) were not detected in angiomyolipomas from women with LAM *(43)*.

LAM and the mTOR Signaling Cascade

The pace and trajectory of LAM research has been dramatically accelerated by the genetic relationship of LAM to TSC. *TSC2* encodes tuberin, a 200-kDa protein with a domain near the carboxyl terminus containing GTPase-activating protein (GAP) homology. GAP proteins convert members of the Ras superfamily from their active, GTP-bound state to their inactive, GDP-bound state. *TSC1* encodes hamartin, a 140-kDa protein with no homology to tuberin. The first critical clues for the function of hamartin and tuberin came from studies in *Drosophila*. Mutations in *dTsc1* and *dTsc2* (the *Drosophila TSC1* and *TSC2* homologues, respectively) were found to result in an increase in cell size, through activation of dTOR (*Drosophila* target of rapamycin) *(44)*. mTOR (the mammalian target of rapamycin) forms two functionally distinct complexes: mTOR complex 1 (TORC1) and mTOR complex 2 (TORC2) *(45, 46)* (Figure 4.1). TORC2 contains mTOR, GbL, and Rictor, and controls the actin cytoskeleton, whereas TORC1 contains mTOR, GbL, and Raptor, and controls protein synthesis and cell growth *(47)*. Rapamycin specifically inhibits TORC1.

In normal cells, the protein products of *TSC1* and *TSC2*, hamartin and tuberin, respectively, form heterodimers *(48)* that regulate TORC1 *(49–51)* in response to growth factors, the cell cycle, and nutrient availability. mTOR and its substrates, p70 ribosomal protein S6 kinase (p70S6K) and 4EBP1, are components of cellular pathways

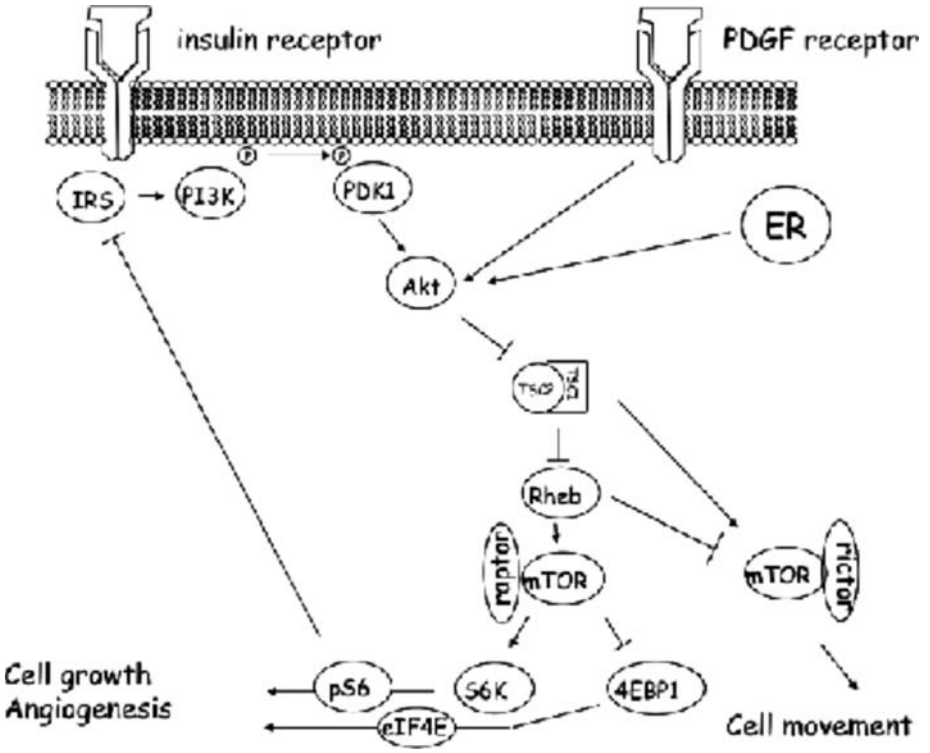

Figure 4.1 Signaling cascade that is regulated by the tuberous sclerosis proteins, hamartin (TSC1), and tuberin (TSC2). Binding of extracellular ligands by cell surface receptors results in activation of downstream targets. In the case of insulin, binding to the insulin receptor results in phosphorylation of IRS, followed by PKD, PI3K, and Akt. Phosphorylation of tuberin by Akt results in inactivation of the domain which maintains Rheb in the "off" state. The unrestrained Rheb activity results in activation of downstream targets including mTOR, which together with raptor forms mTORC1 and activates S6 and eIF4E and promotes cell growth. The phosphorylated hamartin tuberin complex promotes and Rheb inhibits the activation of mTOR complexed with rictor, which is involved in cytoskeletal dynamics and cell movement

that regulate protein synthesis, cell size, and cell proliferation *(52, 53)*. Regulation of mTOR is achieved via tuberin's GTPase-activating domain, which stimulates the inactivation of the small GTPase Rheb (Ras homologue enriched in brain) *(54–59)*. Rheb, like other Ras family members, cycles between an active GTP-bound and an inactive GDP-bound state. Phosphorylation of tuberin by Akt (protein kinase B), p90 ribosomal S6 kinase (RSK) 1, ERK2 (MAPK) *(60)*, or MK2 *(61)* (which is downstream of p38 MAPK) releases tuberin's inhibition of p70S6K *(49, 62–64)*. Hamartin is inhibited in mitotic cells by cyclin-dependent kinase 1(CDK1) *(65)*. In contrast to these inhibitory phosphorylations, tuberin is activated in the setting of glucose or energy deprivation by AMP kinase (AMPK) *(59)*.

Mutations in either *TSC1* or *TSC2* result in activation of the mTOR/Raptor complex 1 pathway (TORC1), which is believed to play a critical role in LAM pathogenesis. Hyperphosphorylation of p70S6K and/or its substrate ribosomal protein S6 has been shown in cells from LAM and TSC patients *(26, 66–68)*, consistent with activation of the mTOR signaling pathway. The precise mechanisms through which loss of *TSC2*

leads to the proliferation of LAM cells are not fully understood. It is known that expression of *TSC2* in LAM-derived cells inhibits their growth, migration, and invasion *(69, 70)*.

A key area of uncertainty is whether Rheb has TORC1-independent targets that are disease relevant. Rheb is known to inhibit the activity of B-Raf kinase and C-Raf kinase, resulting in downregulation of the Raf/MEK/MAPK signaling cascade. This activity of Rheb is unaffected by rapamycin, and therefore TORC1 independent. The role of B-Raf/C-Raf inhibition in LAM pathogenesis is not yet known. One hypothesis is that reactivation of C-Raf by estrogen contributes to the female predominance of LAM. Other investigators have studied rapamycin-independent functions of *TSC2*, including Finlay, who found that RhoA is activated in TSC2-null cells in a rapamycin-independent manner *(76)*.

The Cell of Origin of LAM

The genetic data discussed above indicate that LAM cells spread to the lung through a metastatic mechanism. If LAM cells arise outside the lung, where do they originate? The cell of origin of LAM is almost certainly closely related to the cell of origin of angiomyolipomas, since LAM cells are identical to the smooth muscle cell component of angiomyolipomas at the histologic, immunohistochemical, and electron microscopic levels. LAM cells histologically resemble immature smooth muscle cells, yet their distinctive expression of melanocyte-associated proteins, including the melanocytic transcription factor (MITF) *(22, 77)*, indicates that their origin is not from a simple smooth muscle cell precursor. It has been recognized for decades that LAM cells are immunoreactive to HMB-45, a monoclonal antibody to the melanoma-associated surface antigen gp-100. In fact, HMB-45 immunoreactivity is widely used to diagnose LAM, since few other tumors of human diseases are HMB-45 positive: melanoma, angiomyolipomas, sugar cell tumors. At the electron microscopic level, the HMB-45 positivity appears to result from the presence of pre-melanosomes in the cytoplasm of LAM cells.

The expression of HMB-45 by this group of tumor types has led them to be designated as "perivascular epithelioid cell tumors" or "PEComas." Yet the origin of the putative perivascular epithelioid cell remains unknown. PEComas of the uterus and soft tissues have been reported *(78–80)*, more frequently in women and in patients with TSC, as well as in patients with S-LAM and TSC-LAM. The expression of melanocytic and other neural crest lineage makers has led to the speculation that LAM cells may be of neural crest origin *(77)*.

Lymphangiogenesis is believed to play a role in LAM pathogenesis, and serum vascular endothelial growth factor D (VEGF-D) has been reported to be elevated up to 30-fold in patients with LAM *(71)*. LAM cell clusters enveloped by lymphatic endothelial cells can be identified in the chylous pleural and ascitic fluid from LAM patients, and LAM cell clusters can be identified in lymphatic channels in lymph nodes *(72, 73)*. In one theory of LAM pathogenesis, LAM cell clusters migrate up the thoracic duct, become deposited in internal jugular vein, and are distributed throughout the lung via the pulmonary artery *(72)*. Chylous complications, thoracic duct enlargement, and lymphangiomyomas are uncommon in TSC-LAM *(74)*, suggesting that metastasis in that subset may occur through a different mechanism, perhaps from the angiomyolipoma

through the renal vein into the lung. There have been several case reports of tumor extension into the renal vein and right atrium *(75)* from benign angiomyolipomas. Once lodged in the distal pulmonary microvasculature, LAM cells may gain access to the interstitial space.

Why Does LAM Occur in Women?

There are at least two possible explanations for the striking female predisposition of LAM: either the cell of origin is expressed only by women and naturally travels to the lung, or the cell of origin is expressed by both men and women but is induced to proliferate and travel to the lungs only in women, under the influence of hormonal stimuli. Two factors appear to minimize the possibility that only women express the cell of origin. First, there are occasional reports of LAM in men, and second, both men and women with TSC develop angiomyolipomas at a similar frequency, and since the smooth muscle cells of angiomyolipomas and LAM are virtually identical, this suggests that both men and women have the elusive cell of origin.

The second possibility that LAM cells proliferate and metastasize in response to female hormones would make sense in that LAM is a disease primarily of young–adult women, and the anecdotes of that LAM become significantly more severe during pregnancy. The carboxy terminus of tuberin interacts with the estrogen receptor (ER) *(81)* and tuberin is found to function in vitro as a transcriptional co-repressor of the estrogen receptor *(82)*, resulting in a twofold decrease in ERE-luciferase reporter response. Finlay et al. confirmed the interaction between ER alpha and tuberin, and showed that re-expression of tuberin in tuberin-null ELT-3 cells (from rat uterine leiomyoma) abrogated estradiol (E2)-induced growth in vitro *(83)*. York et al. recently showed that tuberin and ER alpha interact at endogenous expression levels in multiple cell types *(84)*. Additional work is clearly needed to understand whether and how these data are related to LAM pathogenesis.

During the past decade, it has been increasingly appreciated that E2 triggers rapid, non-genomic signaling cascades that contribute to growth, survival, and migration. These events occur in seconds to minutes and can be activated by ERs that lack a nuclear localization signal or that are targeted to the plasma membrane *(85)*, thereby clearly dissociating them from nuclear transcriptional activity *(86)*. The non-genomic actions of E2 appear to be mediated by a pool of ER localized to the plasma membrane *(85, 87, 88)*. Three of the best understood non-genomic actions of E2 are activation of p38 MAPK, p42/44 MAPK, and PI3K. Activation of p38 MAPK and MAPKAP-2 (MK2) occurs within 10 min of E2 treatment in endothelial cells expressing endogenous ER *(87, 88)*. Activation of p42/44 MAPK (ERK 1/2) occurs within 10 min of E2 treatment of human lung myofibroblasts expressing endogenous ER *(89)*. Activation of phosphatidylinositol 3-kinase (PI3K), leading to activation of the protein kinase Akt, occurs within 5 min of E2 treatment *(90, 91)* in endothelial cells and in other cell types including MCF-7 breast cancer cells *(92, 93)*. Because signaling cascades initiated by membrane-localized ER also stimulate transcription, the term "non-genomic" to describe the cytoplasmic effects of E2 is somewhat misleading. For example, in vascular endothelial cells treated with E2 for 40 min with and without the PI3K inhibitor LY294002, at least 250 genes are increased by at least twofold in a PI3K-dependent manner, including the genes for the transcription factors Myc and Jun *(94)*.

In primary cells derived from an S-LAM angiomyolipoma, which were shown to have bi-allelic TSC2 inactivation (an inactivating mutation (R611Q) in one *TSC2* allele and loss of heterozygosity of the other allele), estrogen stimulated cell growth. This proliferation was associated with increased phosphorylation of p42/44 MAPK at 5 min and increased expression of c-myc at 4 h. These findings are consistent with the activation of both genomic and non-genomic signaling pathways *(95)*.

The TSC/Rheb/mTOR pathway plays a critical role in the regulation of estrogen-induced proliferation signals. In MCF-7 cells, 17β-estradiol (E_2) rapidly increased the phosphorylation of downstream targets of mTOR: p70 ribosomal protein S6 kinase (S6K), ribosomal protein S6, and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). The PI3K inhibitor wortmannin and the mTOR inhibitor rapamycin blocked E_2-induced activation of S6K. E_2 rapidly (within 5 min) stimulated tuberin phosphorylation at T1462, a site at which Akt phosphorylates and inactivates tuberin. E_2 also rapidly decreased the inactive, GDP-bound form of Rheb. Finally, we found that siRNA downregulation of endogenous Rheb blocked the E_2-stimulated proliferation of MCF-7 cells, demonstrating that Rheb is a key determinant of E_2-dependent cell growth.

Whether these effects of estrogen have relevance to LAM is unknown. In cells carrying bi-allelic inactivation of *TSC1* or *TSC2*, presumably this pathway would not be active. However, the proportion of LAM cells carrying bi-allelic inactivation is not yet defined. It was recently proposed that in cells with mutational inactivation of one copy of *TSC2*, inactivation of the remaining wild-type tuberin could be mediated by tuberin phosphorylation rather than *TSC2* mutation, leading to tumorigenesis *(95a)*. Therefore, phosphorylation and inactivation of tuberin as a result of E_2 stimulation could promote the proliferation of LAM cells carrying a single mutation in *TSC2* and account in part for the strong female predisposition of LAM. Further studies are needed to test this hypothesis in LAM-derived cells. Additional estrogen-linked mechanisms may also be involved in LAM pathogenesis, since tuberin has been found to interact with estrogen receptor alpha (ERa) and to function in vitro as a transcriptional co-repressor of the estrogen receptor *(81, 82)*.

Why Is LAM Associated with Cystic Lung Disease?

The pathogenesis of cystic lung disease in LAM is incompletely understood, in part because of the lack of a robust LAM animal model. It is known that LAM cells express matrix metalloproteinases (MMPs), including MMP2 and MT1-MMP, and that the expression of MMPs may be lower after treatment with anti-hormonal agents *(96)*. Increased expression of serum response factor (SRF) has been demonstrated in LAM cells, which may lead to MMP activation *(97)* and downregulation of tissue inhibitor of metalloproteinase (TIMP)-3 in LAM cells *(98)* and thereby contribute to the tissue destruction in LAM. A simple explanation, therefore, is that expression of MMPs is entirely responsible for the cyst formation. However, while many malignant tumor cells that metastasize to the lungs express MMPs, cystic degeneration of the surrounding lung parenchyma is rarely observed in cancer, suggesting that other mechanisms may contribute to the cyst formation. Identifying these mechanisms will be critical to LAM therapy, especially at early stages, in order to prevent the loss of lung parenchyma which may represent an irreversible change.

Studies of Birt–Hogg–Dubé (BHD) syndrome may yield clues to the pathogenesis of cyst formation in LAM. BHD is an autosomal dominant disorder characterized by hamartomas of skin follicles, lung cysts, spontaneous pneumothorax, and renal cell carcinoma *(99–101)*. The BHD gene was cloned in 2002 and encodes folliculin, which has no significant homology to other human proteins *(102)*. It has recently been discovered that BHD functions in the TOR pathway in *Schizosaccharomyces pombe (103)* and in mammalian cells *(104, 105)*. Surprisingly, in *S. pombe* the BHD homologue functions as an activator of Tor2 (one of the two homologues of TOR), in contrast to the TSC1/TSC2 homologues, which function as inhibitors of Tor2. The precise relationship between BHD and mTOR in mammalian cell is not yet clear, but it is tempting to speculate that in BHD, inappropriate mTOR inhibition leads to lung cysts, while in TSC, inappropriate mTOR activation leads to LAM cell proliferation and cysts. One possible mechanism for these apparent contradictory results involves the balance between mTOR's two distinct complexes in mammalian cells, mTORC1 (mTOR and raptor) and mTORC2 (mTOR, rictor, and SIN1). Inhibition of mTORC1 with rapamycin alters the stoichiometry between mTORC1 and mTORC2 in a cell-type-specific manner, with loss of mTOR–raptor binding at early time points and loss of mTOR–rictor binding at later time points *(45)*, indicating that the balance between mTOR activation and inhibition is tightly regulated.

Clinical Presentation

The average interval between the onset of symptoms and diagnosis in LAM varies between 2.4 years in Japan *(7)* to 3.0 years in France *(8)* and 3.5 years in the United States *(6)*. This delay is frequently related to the failure of the physician consulted to consider the diagnosis, and in many cases patients are first told that they have asthma or chronic obstructive lung disease. The most common initial manifestations of LAM are pneumothorax and progressive dyspnea on exertion *(106)*. Pulmonary symptoms were the presenting features of the disease in 86.5% of patients in the NHLBI Registry, including pneumothorax in 35% *(6)*. Over the course of illness, pneumothorax eventually occurred in about 55% of Registry patients, lower than the average of 65% from other series, perhaps because the Registry selected for patients who were comfortable with air travel to enrolling sites. Even in patients with pneumothorax as the presenting sign, the diagnosis is often delayed. Almoosa reported that the average number of pneumothoraces prior to the diagnosis of LAM is 2.2 *(107)*. Other symptoms and signs reported by the NHLBI registrants included cough (31%), wheezing (46.5%), angiomyolipoma (38%), hemoptysis (30%), chylous effusion (21%), and chylous ascites (4.3%). Chest pain has been reported in 32–50% of patients in other series but was not mentioned in the NHLBI series.

Renal angiomyolipomas are much more common in patients with TSC-LAM (92%) than in patients with S-LAM (32%), while lymphangioleiomyomas are more common in S-LAM (29%) than TSC-LAM (9%) *(108)*. These findings raise the interesting possibility that the source of metastatic cells may differ in patients with S-LAM and TSC-LAM. Cystic lymphangiomyomas in the abdomen may vary in size over the course of the day, with erect posture and with dietary variation. Increasingly, LAM is discovered in asymptomatic patients who are found to have lymphadenopathy, abdominal masses, or cystic changes in the lung on CTs of the abdomen or the chest that are obtained for other reasons.

Physical Examination

The physical examination in LAM is often nonspecific *(106)*. Crackles or wheezes are heard in a minority of patients, and clubbing is distinctly uncommon. Elevated neck veins, a right ventricular heave, or a tricuspid regurgitant murmur may suggest pulmonary hypertension and should trigger an evaluation including echocardiogram and possibly right heart catheterization. Careful dermatologic, ocular, and dental surveys should be performed for evidence of TSC, including facial angiofibromas, subungual fibromas, shagreen patches, dental pitting, and hypomelanotic macules (including ash leaf and confetti configurations). A Wood's lamp may examination of the skin be useful for identifying the latter.

Diagnosis

The diagnosis of pulmonary LAM is considered definite in the presence of typical cystic changes on HRCT and either a positive biopsy from lung, tissue, or lymph node, or a compelling clinical context such as known tuberous sclerosis, known angiomyolipoma, or chylothorax (with LAM cell clusters – see "Pathology"). Skin manifestations of TSC (see above) will be present in most but not all patients with TSC-LAM, but not in patients with S-LAM. There are two common diagnostic scenarios: (1) symptomatic women without a prior chest CT and (2) symptomatic or asymptomatic women with cystic changes on chest CT or chest cuts of the abdominal CT.

Symptomatic Women Without a Prior Chest CT

The most common pitfall in making the diagnosis of LAM is failure to consider the diagnosis in women who present with progressive, unexplained dyspnea on exertion or a sentinel pneumothorax. Exercise-induced desaturation and unresponsiveness to conventional therapy for obstructive lung disease should certainly trigger further evaluation in a young woman with a minimal or negative smoking history. Perhaps the most effective systematic approach to earlier diagnosis of LAM would be institution of guidelines to obtain a HRCT in all nonsmoking women with pneumothorax. The argument against this practice is that primary spontaneous pneumothorax is a far more common etiology for pneumothorax in a young woman. The incidence of primary spontaneous pneumothorax in women is about 1.2 cases per 100,000 per year *(109)*. If extrapolated to the entire population of the United States, one could anticipate approximately 1,800 cases in women per annum. Over a 30-year period, the expected number of PSP events in women in the United States would be 54,000. In the same time interval, if the 850 known living US patients remained constant and each suffered an average of three pneumothoraces, the number of pneumothoraces related to LAM would be 2,550. Therefore, LAM would be responsible for approximately 5% of apparent primary spontaneous pneumothoraces in women. This crude analysis does not account for secondary forms of pneumothorax but gives some sense of scale of how often a screening CT in women with apparent primary spontaneous pneumothorax and no apparent underlying lung disease would identify LAM. The specificity of a screening CT scan would be enhanced by targeting nonsmoking women or those with minimal tobacco use, since 80% of patients with PSP smoke and the incidence of PSP is correlated with cigarette consumption. At 1–12 cigarettes/day the risk of pneumothorax is fourfold higher than that in nonsmokers; at 13–22 cigarettes/day it is 14-fold higher than that in nonsmokers and at

>22 cigarettes/day it is 68 times higher than that in nonsmokers *(109)*. On the basis of these data, the LAM Foundation Pleural Disease Consensus Group recommends obtaining a chest CT in nonsmoking women or women with limited tobacco exposure at the time of the first pneumothorax. To be effective, this recommendation must be accepted by the pulmonary community and reach emergency medicine and primary care physicians, who most commonly see undiagnosed LAM patients.

Symptomatic or Asymptomatic Patient with Cystic Change on a High-Resolution Chest CT

For pulmonary physicians, the most common diagnostic dilemma is the evaluation of patients who have been referred for recurrent pneumothorax or with cystic change in the lung on HRCT scan of the chest, identified either during evaluation of pulmonary symptoms or found incidentally, such as on the chest cuts of an abdominal CT. The HRCT is the single most useful diagnostic tool, and in the hands of expert radiologists, it is reported to be 72% accurate in differentiating LAM from other cystic lung diseases, perhaps up to 88% accurate when the radiologist is "confident" *(110)*. Armed with a typical HRCT and other information about the patient, such as the age, gender, smoking history, the clinician who is familiar with LAM can likely be more than 90% sure of the diagnosis. Given the lack of effective interventions, this degree of certainty may be sufficient in some circumstances, such as in asymptomatic patients, those with early disease, those who are less troubled by diagnostic ambiguity, and those who are not considering pregnancy, use of estrogen-containing medications, or clinical trials. In cases where diagnostic certainty is required or desired, there are several possible approaches that may obviate the need for surgical biopsy. These include (1) obtaining a dedicated CT, ultrasound, or MRI of the abdomen to screen for angiomyolipomas, which can be identified with certainty based on the presence of fat within the tumor. Cystic lymphangiomyomas are also consistent with LAM and certainly enhance diagnostic certainty but can be confused with necrotic nodes, (2) pleural tap and evaluation of LAM cell clusters in patients with pleural effusions (see "Pathology"), and (3) detailed evaluation of the skin, CNS (including head CT or MRI), and eyes by physicians who are knowledgeable about the manifestations of tuberous sclerosis, which may reveal evidence for TSC. If none of these approaches are informative, video-assisted thoracoscopic biopsy is the preferred diagnostic modality. Transbronchial biopsy has occasionally been definitive, but the small sample obtained is often insufficient *(111)*. Genetic testing for tuberous sclerosis mutations is commercially available but is quite expensive and will only be positive in TSC-LAM (because S-LAM patients do not have mutations in circulating leukocytes). Serum VEGF-D is elevated in the serum of patients with LAM but not in serum of patients without other cystic and chylous lung diseases, and if validated, may be useful diagnostically *(112)*. Detection of circulating LAM cells demonstrating LOH by fluorescence in situ hybridization (FISH), though technically difficult, is another promising diagnostic approach.

Pathology

On gross examination the lungs are enlarged and diffusely cystic. The dilated airspaces range in size from a few millimeters to 2.0 cm in diameter *(5, 113)*. Microscopic examination of the lung reveals foci of smooth muscle cell infiltration of the lung parenchyma, airways, lymphatics, pleura, and blood vessels, associated with areas of thin-walled cys-

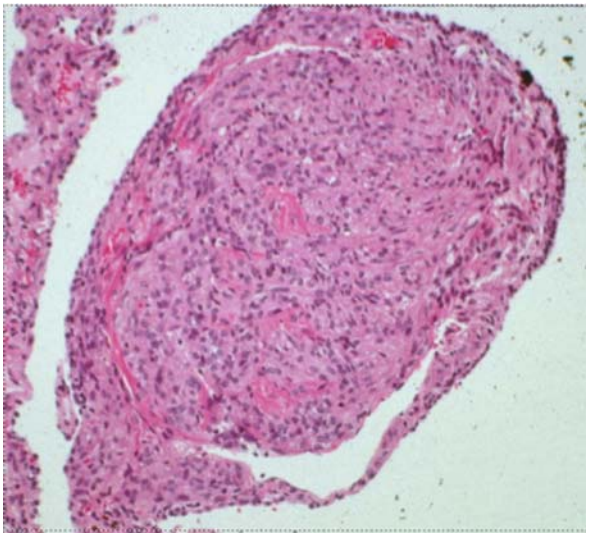

Figure 4.2 LAM histopathology – LAM nodules are composed of haphazardly arranged spindle-shaped epithelioid cells with abundant eosinophilic cytoplasm

tic change (Figure 4.2). The lesions are composed of actin-positive, spindle-shaped cells which stain abundantly with proliferative markers such as proliferating cellular nuclear antigen (PCNA) and less abundant cuboidal epithelioid cells which stain with a monoclonal antibody called HMB-45 *(114)*. This immunohistochemical study is very useful diagnostically, since other smooth muscle-predominant lesions in the lung do not react with the antibody *(115)*. Estrogen and progesterone receptors may also be present in some LAM lesions *(116, 117)*, but not in normal lung tissue *(118)*. Unlike the dilated airspaces in emphysema, the cystic spaces are lined with hyperplastic type II cells *(119)*. Diffuse nodular proliferation of type II cells indicative of MMPH may occur in patients with TSC, in the presence or the absence of LAM *(120)*. Clusters of cells in the chylous pleural fluid of patients with LAM were first described by Valensi *(121)* in 1973. Later, Itami demonstrated that the clusters originated in the dilated lymphatic system and were composed of alpha smooth muscle, actin-positive spindle cells enveloped by a single layer of endothelial cells *(122)*. He suggested that LAM cells clusters could be used diagnostically, to obviate the need for biopsy in patients with chylous manifestations of LAM. In 2004, Kumaska et al. reported abundant lymphangiogenesis in the lymphatic systems of patients with LAM *(73)*. The LAM cell clusters described previously were composed of a spherical collection of LAM cells expressing HMB-45 and VEGF-D, enveloped by a single layer of lymphatic endothelial cells expressing markers podoplanin and the receptor for VEGF-D, VEGF-R3 *(72)*. His group also reported the marked elevation of serum VEGF-D in patients with LAM *(71)*. The serum VEGF-D level does not appear to be elevated in the serum of other chylous and cystic lung diseases that can mimic LAM, such as emphysema or Langerhans cell histiocytosis, and may be useful diagnostically *(112)*.

Physiology

Quality controlled lung function data was collected prospectively by the NHLBI Registry *(6)*. Spirometry revealed obstructive changes in about 57% of patients, restrictive changes in about 21%, and normal results in about 34%*(6)*. Hyperinflation was unusual, present in about 6%. The average residual volume was 125% of predicted value when measured by plethysmography but was 103% of predicted value when determined by gas dilution methods. These data suggest that a significant proportion of gas trapped in the chest is not in communication with the airway. Reduction in DLCO and increase in residual volume are generally considered to be the earliest physiologic manifestations of LAM, and it is not unusual for DLCO to be reduced out of proportion of FEV1 *(123)*. Cardiopulmonary exercise testing in patients with LAM reveals that exercise-induced desaturation is often present, even in patients with normal or near-normal DLCO and FEV1 *(124)*.

Radiology

The chest radiograph is often normal early in the disease. Bilateral and symmetric reticulonodular infiltrates, cysts and bullae or a honeycomb appearance, may evolve over time but are virtually never specific enough to suggest LAM in the absence of other data. The high-resolution CT scan of the chest is the most useful and the most sensitive radiographic test. The HRCT reveals thin-walled cysts of sizes varying from a few millimeters to several centimeters in all lung distributions (Figure 4.3). The morphology of the cysts is useful in differentiating LAM from other cystic lung diseases. The presence of an internal septa or a "centrilobular dot" consistent with a vessel is frequently seen in emphysema, but never in LAM. The number of cysts varies in LAM from a few to complete replacement of the normal lung tissue. The abdominal CT may reveal angiomyolipomas in the kidney, liver, spleen, or adrenal and cystic lymphangiomyomas (Figure 4.4).

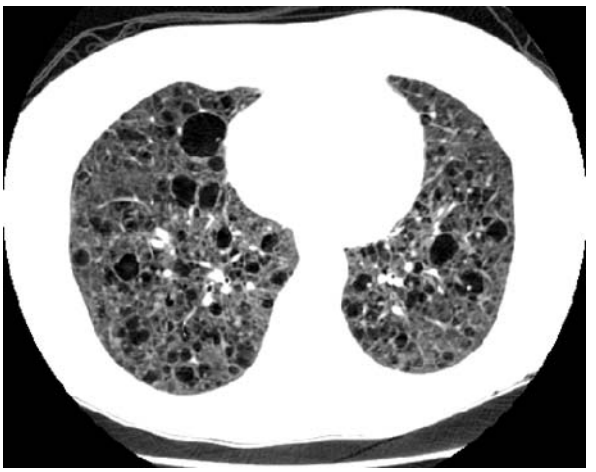

Figure 4.3 HRCT of the lung in a patient with LAM. High-resolution CT scan reveals scattered cysts ranging in size from a few millimeters to a few centimeters, some of which are about the pleura

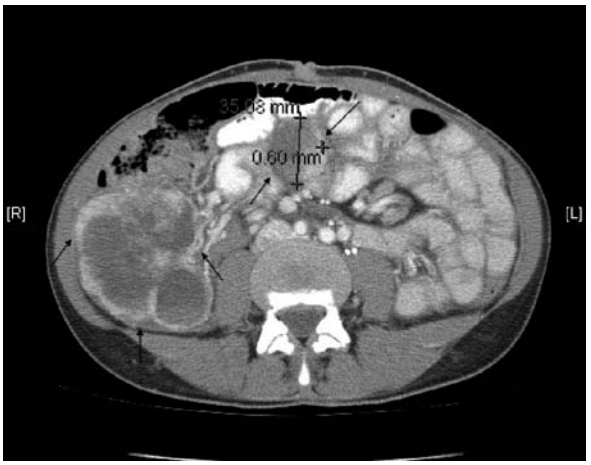

Figure 4.4 Abdominal CT in a patient with LAM. The abdominal CT in a TSC-LAM patient reveals two large cystic lymphangiomyomas (*arrows*)

Clinical Course and Management

Pulmonary Function

The average rate of decline in FEV1 and DLCO in 275 patients studied in a single lab at the NHLBI was 75 cm^3 ± 9 ml and 0.69 ± 0.07 ml/min/mmHg, respectively *(125)*. In other series from Europe, the rate of decline in FEV1 was considerably higher, estimated at approximately 100–120 cm^3/year *(8, 126, 127)*. There was some evidence in these studies that rate of decline in lung function correlates with initial DLCO, with menopausal status, and with progesterone treatment.

Renal Angiomyolipomas, Lymphadenopathy, and Lymphagiomyomas

Renal angiomyolipomas may require embolization or cauterization if bleeding occurs, which is thought to be more common when the diameter of the tumor exceeds 4 cm *(128)*. Others feel that the extent of aneurysmal change determines bleeding risk. Nephron-sparing partial resections may be required for very large tumors *(129)*. Nephrectomy should be considered only when all options for more conservative measures have been exhausted.

Pleural Complications

Of those NHLBI Registry participants who had a history of an initial pneumothorax, the average number of recurrences was 3.4 *(6)*, higher than the 2.0 recurrences (3.0 ± 2.6 pneumothoraces per patient, total) reported in Japan *(7)*. The LAM Foundation Pleural Consensus Group advocated the use of a pleurodesis procedure on the first pneumothorax, given the >70% chance of recurrence *(107)*. Chemical sclerosis, mechanical abrasion, talc poudrage, and pleurectomy have all been effective in patients with LAM. The failure rate with chemical and surgical pleurodesis is high, on the order of 35%, for reasons that are not understood. Although prior pleural procedures can increase

perioperative bleeding in transplant patients, they do not appear to affect candidacy or survival *(130)*. Chyle does not generally cause pleural inflammation or fibrosis, and small chylous effusions often require no intervention once the diagnosis of LAM is made. Shortness of breath may mandate drainage, however, and in some cases repeatedly. Pleural symphysis may be required to prevent nutritional and lymphocyte deficiencies that can result from repeated taps or persistent drainage. Chemical pleurodesis is generally an effective therapy for chylothorax, as is mechanical abrasion and talc poudrage *(131)*.

Screening and Follow-Up

Several screening studies have revealed that 30–40% of patients with TSC have cystic changes in their lung consistent with LAM *(17–19)*, and the Tuberous Sclerosis Association recommends that women with TSC be screened by HRCT at least once after reaching the age of 18 *(132)*. It is reasonable to consider screening asymptomatic women with TSC with pulmonary function tests, including spirometry, lung volumes, and diffusing capacity for carbon monoxide, every 1–3 years. The wisdom and appropriate interval of periodic screening with HRCT beyond the initial scan is debated because of the lifetime radiation risk. In our clinic, women with TSC and no known cystic change are scanned with HRCT at an interval of every 3–5 years. In patients with S-LAM or symptomatic TSC-LAM, the interval for follow-up testing varies with severity but in general PFTs are obtained every 6–12 months and HRCTs are repeated every 1–5 years.

Treatment

Most of the current treatment strategies for LAM are based on antagonism of estrogen action and are empiric and unproven. The results of a small series of patients treated with progestins *(8, 127, 133)*, GnRh agonists *(134–136)*, and oophorectomy *(137)* are inconclusive and conflicting. A large retrospective study of the effect of progestin therapy on the rate of decline in pulmonary function revealed no effect on FEV1 and perhaps an acceleration in the rate of decline in DLCO *(133)*.

Clinical Trials

The only completed controlled trial involving patients with LAM was the Cincinnati Angiomyolipoma Sirolimus Trial, which included lung function measures as secondary endpoints *(138)*. Twenty-three patients with angiomyolipomas and either tuberous sclerosis or LAM or both were treated for 1 year with escalating doses of sirolimus. By the fourth month, all patients were receiving doses of the drug which produced serum levels of 10–15 ng/ml. Renal tumor volume measured by MRI revealed a 50% reduction in tumor size at the end of the first year, but the kidney tumor size returned to 85% of the original volume over the course of the following year. Average FEV1 and FVC improved by 118 and 394 cm^3 on drug, and the residual volume fell by 400 cm^3. Although FEV1 and FVC began to decline again off drug, these values remained significantly above baseline at 1 year. The reduction in residual volume was also durable through the 1 year point. The total lung capacity, diffusing capacity, and, most significantly, the 6-min walk test distance did not change on sirolimus. There were a number of side effects, including six hospitalizations while patients were on the drug. To explore

the possibility that sirolimus has a beneficial effect on lung function, a larger placebo-controlled trial called the Multicenter International LAM Efficacy of Sirolimus Trial (MILES) was launched in December 2006 (NCT000414648). As of this writing, the efficacy and the safety of mTOR inhibitor therapy for LAM remain unclear.

While these results are potentially encouraging, they also highlight that our knowledge of how best to treat LAM is incomplete and raise two obvious questions: (1) Why did the angiomyolipomas only partially regress? There are at least four possible explanations. One simple explanation is tissue and cellular penetration. Angiomyolipomas are highly vascular tumors, but these vessels are often highly dysmorphic, with aneurysmal dilatations. Blood flow within an angiomyolipoma is likely to be chaotic. A second explanation is that rapamycin reached the majority of the cells within the angiomyolipoma, inhibited TORC, resulting in cell shrinkage but not cell death, thereby also explaining the regrowth of the tumor following therapy. A third explanation is that rapamycin reached the cells, inhibited TORC1, and activated pathways that promote cell growth and tumorigenesis. It is known that cells lacking TSC1 or TSC2 have feedback inhibition of the Akt and PDGFR pathways. A final possibility, and a critical one in the pathogenesis of both LAM and TSC, is that rapamycin reached the cells within the angiomyolipomas but that Rheb has other disease-relevant targets, beyond TORC1. While these four possibilities are not mutually exclusive, each would lead to a different targeted approach: better drug delivery, vs. targeting cell death pathways, vs. targeting the pathways that are re-activated by rapamycin, vs. targeting other pathways activated by Rheb.

(2) Why did the angiomyolipomas return to their original volume so quickly? One possibility is that the major effect of sirolimus is a reduction in cell size. In this model, the tumor would shrink by virtue of the aggregate effect of each cell contracting to approximately 50% of its original volume. Mammalian cells do indeed shrink by approximately 40% upon exposure to sirolimus. One way to address this question is by measuring the size of the tumor soon after sirolimus is withdrawn. A second possibility is that sirolimus resulted in apoptosis and cell drop out and that new growth rapidly filled the void until contact inhibition ensued. It will be difficult to distinguish between these possibilities without biopsies.

Transplantation

The United Network for Organ Sharing has recorded 126 transplants for LAM from 1989 through 2007, including 77 double-lung transplants and 49 single-lung transplants. The 1-, 3-, and 5-year survival for single- and double-lung transplants was 87, 73, and 61% and 92, 83, and 77%, respectively[1]. These survival rates are equal to or better than those of other disease groups transplanted in the same time frame. Although the question of bilateral vs. unilateral transplantation has not been directly studied in LAM, bilateral lung transplantation produces slightly better functional outcomes in other obstructive lung diseases such as emphysema *(139)*. However, double-lung transplantation is not always feasible due to the limited availability of organs and the urgency of the procedure in some patients. With other obstructive lung diseases, referral for lung transplantation is considered as FEV1 approaches 30% of the predicted value. However, the average percent predicted FEV1 at transplant for LAM during the 1989–2007 period was 36%, compared to 24% for emphysema, 22% for alpha 1-antitrypsin deficiency, and 28% for cystic fibrosis. This is consistent with our

clinical experience that a subset of LAM patients develop disabling dyspnea with well-preserved pulmonary mechanics, often in association with a low DLCO, and require transplant evaluation before the typical 30% FEV1 threshold is reached. There have been three case reports of recurrence of LAM in the donor allograft *(39, 40, 140, 141)*. The recurrences did not appear to contribute to death in any of these patients, and at the present time we do not feel that recurrence should be considered in judging the candidacy of patients. More than half of LAM patients who have undergone lung transplantation have had a prior history of a pleural fusion procedure, and although postoperative bleeding risk is increased, the operative mortality and the long-term survival do not appear to be affected *(107)*.

Challenges and Future Directions

Progress in LAM research has been hampered by the lack of an animal model that recapitulates LAM and by the difficulties in growing LAM cells in culture. Mice carrying heterozygous TSC1 or TSC2 deletions develop epithelial tumors and cysts of the kidney and hemangiomas of the liver but do not develop renal angiomyolipomas or LAM *(67)*. LAM-derived cells in culture include a mixture of cell types, and the LAM cells appear to undergo senescence after several passages (which is not surprising given that they are histologically benign and usually slow growing). Since most tissue specimens are acquired at the time of lung transplantation and therefore represent end-stage disease, the proportion of LAM cells varies greatly between cultures and between passages of a given culture. While the detection of TSC1 or TSC2 mutations in cultured LAM cells has been proposed as a "gold standard," these mutations can be challenging and expensive to detect.

Despite these challenges, remarkable advances in the pathogenesis of LAM have occurred since the year 2000 when TSC2 mutations were found in LAM cells. It is expected that continued basic, translational, and clinical research will lead to highly effective, targeted therapies for women with LAM. Progress in LAM would be greatly facilitated by the development of rodent models of LAM, allowing testing of therapeutic strategies in different stages of LAM progression, and the development of quantitative biomarkers and/or imaging parameters of LAM progression. These tools would streamline the design of clinical trials and allow multiple single and combinatorial agents to be tested in an efficient manner. The efficient design of clinical trials is critical, since the number of available patients is small, and many different potential therapeutic approaches have already been proposed, including statins *(76)*, estrogen antagonists, interferon gamma *(142, 143)*, and matrix metalloproteinase inhibitors.

Notes

1 Based on OPTN data as of January 31, 2008." " This work was supported in part by Health Resources and Services Administration contract 234-2005-370011C. The content is the responsibility of the authors alone and does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations implies endorsement by the US Government."

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5

Autoimmune Pulmonary Alveolar Proteinosis

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Abstract Pulmonary alveolar proteinosis (PAP) is a rare syndrome characterized by accumulation of surfactant lipids and proteins in pulmonary alveoli that can result in progressive impairment in gas exchange and respiratory insufficiency. The serendipitous discovery of PAP in GM-CSF-deficient mice and subsequent identification that neutralizing GM-CSF autoantibodies are strongly associated with PAP in humans led to our current concepts of the pathogenesis of PAP and the central role GM-CSF and alveolar macrophages play in surfactant homeostasis in health and disease. PAP comprises part of a spectrum of disorders of surfactant homeostasis that includes disorders of surfactant clearance and disorders of surfactant production. The former are caused by disruption of GM-CSF signaling (primary PAP) or by an underlying disease that impairs alveolar macrophage functions including surfactant catabolism (secondary PAP). Disorders of surfactant production are caused by inborn errors of surfactant metabolism (surfactant metabolic dysfunction disorders), e.g., mutations in the *SFTPB*, *SFTPC*, or *ABCA3* genes. Important differences in clinical presentation, natural history, pathogenesis, and surfactant function suggest that these latter diseases should be considered separately from PAP rather than as a form of the same syndrome. The overall prevalence of PAP is approximately 6–8 per million. Ninety percent of cases are specifically associated with high levels of GM-CSF autoantibodies, which has diagnostic importance and has led to common use of the term autoimmune PAP to replace other terms including idiopathic PAP. Autoimmune PAP typically presents as dyspnea of insidious onset; however, up to one third of individuals may be asymptomatic. Whole lung lavage remains the most effective therapy but GM-CSF inhalation therapy is a promising alternative currently in clinical evaluation. Progress in understanding PAP pathogenesis and the role of GM-CSF in surfactant homeostasis and in inflammatory and autoimmune diseases are important benefits derived from integration of basic science, clinical medicine, and translational research. Future studies will focus on pathogenesis, development of improved therapies for PAP and the role of GM-CSF in health and disease.

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Introduction and Definitions

Surfactant plays a critical role in the lungs by reducing surface tension at the alveolar wall–liquid–air interface, thereby preventing alveolar collapse. It is also important in lung host defense serving to both stimulate clearance of microbial pathogens and regulate inflammatory responses in the lungs. Surfactant is composed of 90% lipids, primarily phosphatidylcholine and phosphatidylglycerol, and 10% proteins. These proteins include two that are hydrophilic (surfactant protein (SP)-A and SP-D) and two that are hydrophobic (SP-B and SP-C). SP-A and SP-D are members of the collectin protein family and have essential roles in the opsonization, killing, and clearance of bacteria within the alveolar space as well as immunomodulation of inflammatory cell recruitment and activation *(1)*. SP-B and SP-C have essential surface active properties important to mechanical stabilization of the alveolus *(2)*.

Surfactant is synthesized, processed to mature components, and stored in cytoplasmic organelles called lamellar bodies in alveolar type II cells *(3)*. It is secreted into the alveolar space forming tubular myelin, from which surfactant phospholipids contribute to the formation of mono- and multilayers at the air–liquid interface. These films reduce the surface tension caused by the aqueous fluid layer lining the alveolar walls that, in the absence of surfactant, is sufficient to cause alveolar collapse as occurs in premature infants whose lungs are too immature to make surfactant. After use, surfactant is expelled from the film at the air–liquid interface as small aggregates and is either taken up and recycled by alveolar epithelium or taken up and catabolized by alveolar macrophages. Catabolism of surfactant lipids and proteins in alveolar macrophages requires the presence of GM-CSF in the lungs *(4, 5)*, which acts via the transcription factor PU.1 to stimulate catabolism of surfactant lipids and proteins *(6)*. GM-CSF also stimulates a number of other functions of alveolar macrophages including adhesion, expression of cell-surface receptors, phagocytosis, microbial killing, cytokine signaling, and others, which together support the conclusion that GM-CSF is critical for the terminal differentiation of alveolar macrophages *(6)*.

PAP is a syndrome defined histologically as the accumulation of surfactant lipids and surfactant proteins within pulmonary alveoli. The rarity of syndrome and the varied nature of the disorders associated with its development have hampered progress in elucidating pathogenesis. Further, it can occur as a predominant accumulation of surfactant within otherwise normal appearing alveoli or as a relatively smaller and variable degree of surfactant accumulation within grossly distorted alveoli. Disorders of surfactant homeostasis occur in individuals of all ages, involve widely differing pathogenic mechanisms, and have markedly different clinical presentation, natural history, prognosis, and response to therapies. While several forms of PAP and a separate group of PAP-like disorders are now recognized, the variable and overlapping use of multiple terms in the medical literature obfuscates distinction among them. Some of the alternative terms used include pulmonary alveolar proteinosis (PAP), pulmonary alveolar lipoproteinosis, pulmonary alveolar phospholipidosis, pulmonary alveolar phospholipoproteinosis, idiopathic PAP, acquired PAP, and congenital PAP. These terms have been used to represent differences in the age of onset (e.g., congenital vs acquired), the biochemical nature of the accumulated material, or the lack of pathogenic

understanding (alveolar phospholipidosis vs idiopathic PAP). Consequently, before proceeding, the terms to be used to describe each clinical form of the PAP syndrome and related clinical disorders will first be defined.

Anticipating pathogenesis, disorders of surfactant homeostasis can be defined in the context of abnormalities in either the production or the clearance of surfactant. Disorders of surfactant clearance comprise the majority of individuals with the PAP syndrome. Further, they have a characteristic histological appearance comprised primarily of alveoli filled with lipoproteinaceous material (surfactant). The alveolar wall is intact, thin, and normal appearing and, although not usually evaluated, the accumulated surfactant is functional. Occasionally, fibrosis may be present, especially in advanced discase.

In marked contrast, disorders of surfactant production (see below) are far less common, typically occur in neonates and children, and are associated with significant alveolar wall distortion and varying degrees of accumulation of dysfunctional surfactant *(7–10)*. Further, the clinical course, prognosis, and response to therapy are also quite distinct. While these disorders are sometimes referred to as congenital PAP, our growing understanding suggests they are more usefully considered as distinct from PAP rather than as a clinical variant of PAP.

Disorders of surfactant clearance include two groups of diseases: (1) primary PAP in which the syndrome is caused by a primary abnormality in GM-CSF signaling and (2) secondary PAP in which PAP occurs as a consequence of another disease (Table 5.1). In primary PAP, surfactant catabolism by alveolar macrophages is impaired by GM-CSF signaling dysfunction occurring either as a consequence of high levels of neutralizing GM-CSF autoantibodies (autoimmune PAP), or function-disrupting mutations in the genes encoding the GM-CSF receptor (e.g., *CSF2RA* and *CSF2RB*) or (in mice) in the gene encoding GM-CSF (*CSF-2*). In secondary PAP, surfactant catabolism is impaired by any one of a number of underlying diseases (Table 5.1). Disorders of surfactant production will not be referred to as PAP, but rather as pulmonary surfactant metabolic dysfunction disorders. It is sometimes useful to refer to PAP categorically by the age of onset,

Table 5.1 Classification of PAP and other disorders of surfactant homeostasis.

GM-CSF signaling dysfunction (Primary PAP)
Autoimmune PAP
CSF2RA mutations
CSF2RB mutations
GM-CSF deficiency (not yet identified in humans)
Secondary PAP
Hematologic and other malignancies
Immune deficiency syndromes
Inhalation exposure
Chronic infections
Lysinuric protein intolerance
Drug-induced PAP
Disorders of surfactant production
SFTPB mutations
SFTPC mutations
ABCA3 mutations
Diseases with PAP-Like histology (GM-CSF autoantibody negative)

especially when the pathogenesis has not been established. Thus, we will use the term congenital PAP to refer to the occurrence of the PAP syndrome in a neonate or child when consistent with the presence of the PAP syndrome-causing abnormality from birth. Acquired PAP will refer to the occurrence of the PAP syndrome in a previously healthy adolescent or adult individual. Acquired PAP is categorized as primary PAP when occurring in the absence of an underlying disease associated with PAP and as secondary PAP when it occurs in an individual with another underlying disease associated with or known to cause PAP.

The remainder of this chapter will review recent advances that have raised PAP from obscurity to clarity in little more than a decade and have defined critical roles for the alveolar macrophage and granulocyte/macrophage colony-stimulating factor (GM-CSF) in pulmonary surfactant homeostasis and innate immunity. We will focus on the common clinical form of the syndrome, autoimmune PAP, and comment on differences with other clinical forms where appropriate.

Epidemiology

Current data regarding the epidemiology of PAP derive from several reports including a meta-analysis of 410 identifiably separate patients representing most or all cases of PAP reported in the medical literature from its initial description in 1958 through 1999 *(11)*, a recent contemporaneous cohort of 223 autoimmune patients in Japan *(12)*, and a smaller study of 15 cases from Israel *(13)*.

Autoimmune PAP comprises ~90% of all individuals with PAP, occurs in a worldwide distribution, and affects older children and adults. It occurs more frequently in men than women (ratio ~2:1) and usually presents in the second to fifth decades, although it has been observed in children as young as 8 years of age. The incidence is approximately 0.36–0.49 cases per million individuals *(11–13)*. However, this may be an underestimate since in the largest study of a contemporaneous population to date, 31% of individuals were asymptomatic *(12)*. The prevalence is reported to be 3.7–6.2 cases per million individuals *(11–13)* and is also likely to be an underestimate for the same reason. No ethnic predominance has been reported *(14)* and African-American patients account for 17% of the reported cases in North America *(15)*.

Smoking is associated with development of PAP and the higher incidence in men has been linked to the increased frequency of smoking among men compared to women *(11)*. Inhalation exposure to toxic dusts (e.g., titanium, silica) is reported to be associated with development of PAP *(11)*; however, a recent study in Japan did not confirm a strong association with environmental exposure *(12)*. Interestingly, 80% of Japanese women with autoimmune PAP were never smokers and had no history of pulmonary exposures.

Other forms of primary PAP are extremely rare and only a few cases have been reported. These include individuals with abnormalities in the GM-CSF receptor β chain *(10)* and a family with mutations in the GM-CSF receptor α chain *(16)*. No individuals with PAP and *CSF2* mutations have yet been identified in humans *(17)*, although, as noted above and described below, this occurs in mice *(18, 19)*.

Secondary PAP represents 8–9% of individuals with the syndrome *(11)*. The age distribution of affected individuals follows that of the underlying disease responsible for causing PAP in a given individual. A number of diseases are reported as being

associated with the development of secondary PAP (Table 5.1), including hematologic and other malignancies *(20, 21)*, immune deficiency syndromes *(22)*, inhalation exposure *(23)*, chronic infections *(22)*, lysinuric protein intolerance *(24–26)*, or certain drugs *(27)*.

Surfactant metabolic dysfunction disorders are associated with hereditary mutations in the genes encoding SP-B *(7, 9, 28, 29)*, SP-C *(8, 30–32)*, or ABCA3 *(33)*. Such mutations account for many but not all such cases, and future studies are expected to identify additional genetic diseases associated with surfactant metabolic dysfunction. The gene frequency of a common form of SP-B deficiency (i.e., caused by the 121ins2 *SFTPB* allele) is approximately one mutation per 1,000–3,000 individuals *(28)*.

Molecular Pathogenesis

Rosen et al., in their initial description *(34)* established that the material accumulating in the lungs of individuals with PAP was composed primarily of phospholipids with a lesser amount of protein and very little carbohydrate. Subsequent studies established this material to be surfactant *(35)* and demonstrated that alveolar macrophages in PAP were also filled with surfactant and had functional abnormalities such as defects in chemotaxis *(36)*, adhesion *(36)*, phagocytosis *(37)*, microbicidal activity *(36)*, and phagolysosome fusion *(38)*. An early theory, the "overstuffed alveolar macrophage," held that the abnormal surfactant accumulation *caused* the alveolar macrophage dysfunction observed in PAP *(39)*. However, support for this theory was weakened by the observation that BAL fluid from PAP patients reproduced some of the abnormalities in normal alveolar macrophages from healthy individuals *(40, 41)*. Identification of a soluble inhibitory factor from patients with "idiopathic PAP" (now autoimmune PAP) that blocked mitogen-stimulated proliferation of normal allogeneic and autologous monocytes suggested that a circulating factor might be involved *(42)*. Notwithstanding, for three decades, the pathogenesis remained unclear as to whether the surfactant accumulation in PAP was due to increased production or decreased clearance of normal surfactant or to the presence of abnormal surfactant. However, ultrastructural *(43, 44)*, biochemical *(45, 46)*, and functional *(47)* analysis of the PAP material and data from murine PAP models *(3)* strongly suggest the pathogenesis of autoimmune PAP is caused by reduced clearance rather than overproduction or abnormal surfactant lipid or protein *(48)*.

The serendipitous discovery that genetically modified mice deficient in GM-CSF develop a lung phenotype essentially identical to that in individuals with the common form of PAP provided a critically important clue about disease pathogenesis. Together with extensive subsequent studies, this observation provided an important "roadmap" for studying the pathogenesis and therapy of PAP in humans. Hence, animal models of PAP will be discussed prior to further consideration of the pathogenesis of PAP in humans.

Animal Models

GM-CSF, a small glycoprotein cytokine expressed similarly in humans and mice, was discovered in the 1970s and intensely studied for several decades thereafter. Prior to 1994, it was considered primarily as a regulator of hematopoietic cell growth *(49–51)*.

However, in 1994, two groups independently discovered that mice deficient in GM-CSF develop PAP *(18, 19)*. One initial report established that surfactant accumulation was not due to increased production *(19)*. A subsequent report demonstrated that surfactant accumulation was due to impaired catabolism of surfactant lipids and proteins by alveolar macrophages *(5)*. Since uptake of surfactant was not affected, this abnormality was also responsible for the abnormally large, foamy appearance of these cells *(52)*. The absence of gross hematological abnormalities in these mice suggested that GM-CSF may not be critical for basal hematopoiesis in healthy, uninfected mice.

Correction of PAP by expression of GM-CSF in the lungs but not by systemic GM-CSF administration established the lung as the site of action for GM-CSF-mediated regulation of surfactant homeostasis *(53)* but did not determine its cellular target (i.e., alveolar macrophages vs epithelial cells). This was answered with another murine model of PAP (GM-CSF receptor β chain-deficient mice *(54, 55)*). Transplantation of normal bone marrow into these GM-CSF receptor-deficient mice reversed the PAP phenotype, identifying alveolar macrophages as the cellular target of GM-CSF "therapy" of PAP in mice *(56)*.

The observation that both PAP patients and GM-CSF-deficient mice had increased mortality from infections suggested that GM-CSF was also important in immunity in humans and mice *(11, 57)*. Reports demonstrated an increased susceptibility to pulmonary infection by bacterial *(58)*, fungal *(59)*, and mycobacterial *(60)* pathogens and impaired pulmonary clearance of bacterial, fungal, and viral pathogens *(58, 59, 61)*. Alveolar macrophages from GM-CSF-deficient mice had multiple abnormalities in host defense functions including cell adhesion, cell-surface pathogen recognition receptor expression, nonspecific and receptor-mediated phagocytosis, superoxide production, microbial killing, and proinflammatory cytokine secretion *(6, 58, 59, 61–63)*. Restoration of GM-CSF specifically in the lungs reversed the microbial susceptibility, pulmonary surfactant clearance, and abnormal alveolar macrophage functions demonstrating that GM-CSF is critical in alveolar macrophage-mediated lung host defense.

The diversity of alveolar macrophage abnormalities in GM-CSF-deficient mice suggested GM-CSF may be required for alveolar macrophage differentiation and that its critical site of action was within the lung itself. This hypothesis was strongly supported by the observation that PU.1 was markedly decreased in alveolar macrophages in these mice *(6)*. PU.1 is a "master" myeloid cell transcription factor that regulates many genes in macrophages and stimulates myeloid cell differentiation *(64)*. It was further confirmed by retroviral expression of PU.1 in cultured alveolar macrophages from GM-CSF-deficient mice, which restored defective surfactant catabolism and the other abnormal alveolar macrophage functions listed above *(3, 6, 60, 62)*. Data supporting the concept that GM-CSF acts locally in the lung to preserve lung surfactant homeostasis and lung host defense include the observations that the PAP phenotype can be corrected by (1) pulmonary but not systemic GM-CSF gene transfer *(65)*, (2) pulmonary but not systemic GM-CSF protein replacement *(66)*, and because (3) pulmonary and blood pools of GM-CSF appear to be compartmentalized by a "lung-blood barrier" *(53)*.

The pathogenesis of secondary PAP is less well studied and poorly understood. Notwithstanding, it appears to be caused by any one of a number of other underlying disorders that reduce either the numbers or functions of alveolar macrophages *(20, 21, 67–69)*. An animal model of secondary PAP has demonstrated that depletion of alveolar macrophages is associated with accumulation of surfactant *(70)*.

Disruption of Surfactant Homeostasis in Autoimmune PAP

The identification of PAP in GM-CSF-deficient mice prompted a reevaluation of the pathogenesis of PAP in humans with respect to abnormalities of GM-CSF and GM-CSF signaling. One early experimental approach evaluated the concept by testing the therapeutic efficacy of empiric administration of recombinant human GM-CSF in a single patient with "idiopathic PAP" (now autoimmune PAP), which resulted in radiographic, physiologic, and symptomatic improvement *(71)*. Importantly, GM-CSF was not absent in the BAL fluid and serum in these patients *(72)*. However, subsequent studies demonstrated that GM-CSF bioactivity was undetectable *(73)* and that leukocyte mobilization response to GM-CSF administration was blunted *(74)*.

Reexamination of the soluble inhibitory factor present in patients with "idiopathic" PAP (now autoimmune PAP) resulted in a critical observation linking the pathogenesis of human PAP to GM-CSF. BAL fluid from PAP patients inhibited the binding of GM-CSF to cellular receptors and GM-CSF-dependent cellular proliferation *(75)*. The soluble inhibitory factor turned out to be polyclonal anti-GM-CSF immunoglobulin of the IgG subclass *(76)*. Importantly, high levels of these autoantibodies were present in all cases of "idiopathic PAP" but not in any cases of secondary PAP, "congenital PAP" (actually disorders of surfactant homeostasis occurring in neonates due to surfactant metabolic dysfunction disorders), other lung disorders or in normal individuals *(76)*. Further, the autoantibodies present in both blood and lungs, and bind GM-CSF with very high affinity *(73)*. Because GM-CSF autoantibodies are present in PAP patients at levels far exceeding normal circulating GM-CSF levels (by up to 50,000 fold), they virtually eliminate GM-CSF bioactivity in vivo *(73a)*. Recently, PAP was reproduced in healthy, Non human primates injected with PAP patient-derived GM-CSF autoantibodies. New Reference = Human GM-CSF autoantibodies and reproduction.

Multiple lines of evidence suggest that GM-CSF regulates myeloid cells similarly in humans and mice. Guided by studies in GM-CSF-deficient mice, GM-CSF was also found to regulate PU.1 in human alveolar macrophages *(77)*. Thus, GM-CSF likely regulates alveolar macrophage terminal differentiation, surfactant homeostasis, and lung host defense in humans via stimulating expression of PU.1 in alveolar macrophages. The absence of GM-CSF signaling results in a pattern of abnormalities of pulmonary cytokine expression that is similar in autoimmune PAP in humans and GM-CSF-deficient mice. For example, the macrophage growth and differentiation factor, macrophage colony-stimulating factor (M-CSF), is similarly elevated in both human and murine forms of PAP *(78, 79)*. Similarly, monocyte chemotactic protein 1 is elevated in the lungs of both *(63, 80)*. The mechanism of these abnormalities of cytokine expression is currently unknown. However, the very similar pattern of alveolar macrophage abnormalities in autoimmune PAP and GM-CSF-deficient mice, two settings in which GM-CSF signaling is disrupted by very different mechanisms in different species, strongly suggests a common molecular pathophysiology, i.e., that disruption of GM-CSF signaling blocks alveolar macrophage terminal differentiation and impairment of multiple functions including surfactant catabolism. Although baseline blood neutrophil counts are normal in patients with autoimmune PAP and GM-CSF-deficient mice, both have defects in neutrophil functions, including impaired adherence, production of reactive oxygen species, phagocytosis, bacterial killing *(81)*. Importantly, the pattern of neutrophil defects was strikingly similar, suggesting a common mechanism of regulation in man and mice. Further, these results demonstrate GM-CSF is also

important systemically in determining the baseline functional capacity of circulating neutrophils.

Genetic Basis of Congenital PAP and Disorders of Surfactant Production

Autoimmune PAP occurs in previously healthy individuals and no monogenic disease components or linkage studies have been identified. However, in one series of 15 patients diagnosed with PAP in Israel between 1976 and 1997, ethnic and familial clustering and the rarity of cases among Ashkenazi Jews were interpreted as suggesting a genetic predisposition *(13)*. GM-CSF autoantibody testing was not available at the time of that report, so the proportion of autoimmune PAP in the report was not determined. Further, at least one case appears to be a disorder caused by surfactant metabolic dysfunction.

No data are available describing any associations with human leukocyte antigen or other candidate genes. Seven (two male and five female) of 410 cases of PAP published by Seymour were found to have had co-existing autoimmune disorders or positive autoimmune serology *(11)*. The autoimmune abnormalities included clinical rheumatoid arthritis in two cases, positive smooth-muscle antibodies in two cases (one with positive rheumatoid factor), immunoglobulin A nephropathy, multiple sclerosis, and possible celiac disease *(11)*. From the recent Japanese report, 3 of 223 cases with autoimmune PAP had other autoimmune diseases including polymyalgia rheumatica, hemolytic anemia, and Wegener's granulomatosis *(15)*.

Congenital PAP has been described in association with individuals with abnormalities in the GM-CSF receptor β chain *(10)* and a family with mutations in the GM-CSF receptor α chain *(16)*. No individuals with PAP caused by *CSF2* mutations have yet been identified in humans *(17)* although, as already described, this occurs in mice *(18, 19)*.

Taken together, the observations in patients with PAP and high levels of neutralizing GM-CSF autoantibodies, congenital PAP associated with GM-CSF receptor β chain deficiency, function-altering *CSF2RA* mutations, and mice deficient in GM-CSF or the GM-CSF receptor β chain support the concept that primary PAP is caused by disruption of GM-CSF signaling to alveolar macrophages in the lungs in both man and mice. They suggest a common cellular and molecular pathophysiology in man and mouse wherein disruption of GM-CSF signaling impairs alveolar macrophage terminal differentiation and the ability to catabolize surfactant lipids and proteins, thus disrupting surfactant homeostasis. Observations in patients with leukemia and reduced numbers of alveolar macrophages following intensive chemotherapy or the absence of GM-CSF receptors myeloid cells and rats depleted of alveolar macrophages support the concept that secondary PAP is caused by a reduction in either the number or functions of alveolar macrophages.

Clinical Presentation and Course

Autoimmune PAP typically presents in previously healthy adult individuals as progressive exertional dyspnea of insidious onset. When present, cough is usually nonproductive or associated with scant whitish sputum. Less commonly, fever, chest pain or hemoptysis, and weight loss may also occur, especially if secondary infection is present. In most cases, the history does not reveal evidence of significant prior exposure to

pulmonary toxins, e.g., metal dusts. A history of smoking is present in many but not all individuals. The physical exam can be unremarkable, but reveals mild inspiratory crackles in 50% of cases, cyanosis is present in severe cases, and digital clubbing is rare. A recent report from Japan showed that 31% of PAP patients are asymptomatic *(12)*.

The clinical course of autoimmune PAP is variable with patients falling into three categories: stable persistent symptoms, progressive deterioration, or spontaneous improvement *(34)*. In the former, persistent symptoms may vary in intensity over time and respond to therapeutic whole lung lavage but subsequently recur. In others, the intensity of the disease is greater and patients have a progressive decline in pulmonary function despite treatment. A meta-analysis of 303 reported cases by Seymour showed that significant spontaneous improvement occurred in only 8% of PAP patients *(11)*. A retrospective analysis of 343 previously reported PAP cases indicated the 5-year survival in individuals not undergoing whole lung lavage therapy was 85 ± 5% *(11)*. Further, the study also showed that 72% of mortality was directly due to respiratory failure from PAP and 18% was indirectly due to PAP due to uncontrolled infections. However, a recent cross-sectional study of 223 patients with autoimmune PAP appeared to have a lower mortality rate (no mortality was observed during the 5 year period of study), although the study was not actually designed to address mortality. Further studies are needed to determine if there are regional or ethnic differences in mortality in individuals with autoimmune PAP or if the differences represent an evolution in the care of these patients.

Individuals with acquired PAP are at risk for secondary infections from a variety of microbial pathogens including common pathogens (*Streptococcus*, *Klebsiella*, *Hemophilus*, *Staphylococcus*, *Pseudomonas*, *Serratia*, *Proteus*, and *Escherichia coli*) *(11, 82, 83)* as well as opportunistic or unusual pathogens *Mycobacteria*, *Aspergillus* spp., *Nocardia*, and others *(11, 83, 84)*. Infections occur at pulmonary and extrapulmonary sites *(11, 85–87)*. This strongly suggests the predisposition to infection in patients with autoimmune PAP may be due to a systemic defect in host defense rather than a consequence of pulmonary surfactant accumulation. Although the frequency of opportunistic infections in published cases was reported not to have changed over time *(11)*, the recent cross-sectional study from Japan reported a very low (5.7%) rate of infection among 223 individuals with autoimmune PAP *(12)*. Further, longitudinal studies are needed to determine if this is due to regional or ethnic differences in PAP or evolution in the care of patients with PAP.

Diagnosis

Since autoimmune PAP usually presents insidiously and with nonspecific symptoms in the context of characteristic but nonspecific radiographic findings *(34, 88, 89)* (Figure 5.1a), an accurate and timely diagnosis requires a high degree of clinical suspicion. The physical exam may be normal or reveal fine mid-inspiratory crackles. Chest radiography typically shows bilateral patchy air-space disease, similar in appearance to pulmonary edema but without other radiographic signs of left heart failure *(34, 88, 90)*. High-resolution computed tomography typically reveals patchy ground-glass opacifications superimposed on interlobular septal and intralobular thickening (Figure 5.1b). The patchy pattern of ground-glass opacification involves secondary lobules differentially

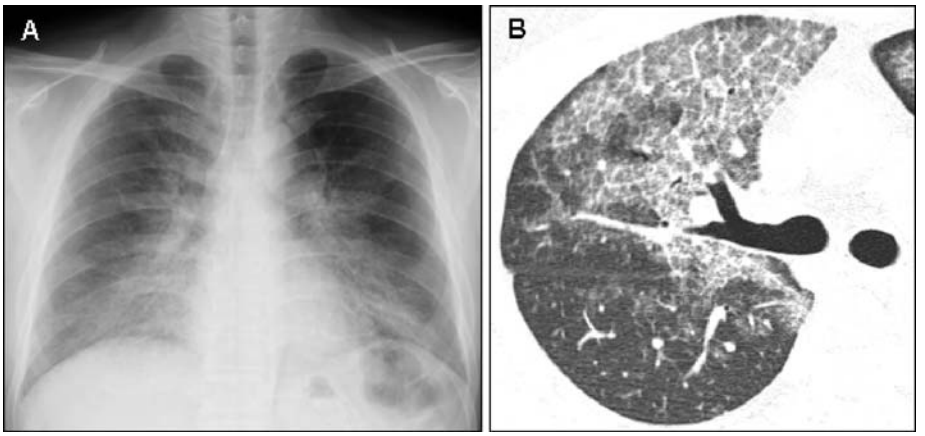

Figure 5.1 Radiographic appearance of the chest in autoimmune PAP. (**a**) Chest radiograph. (**b**) High-resolution computed tomogram of the chest

such that normal and highly abnormal lobules are juxtaposed creating a geographic pattern often described as "crazy paving." This pattern is typical of but not diagnostic of PAP and may spare the subpleural spaces *(91, 92)*. The radiographic abnormalities are frequently disproportionately greater than expected based on the clinical findings, an observation of diagnostic utility that should suggest the presence of PAP. The extent of the radiographic abnormalities correlate well with the degree of impairment quantified by arterial blood gas measurements *(91)*.

Routine hematological indices, blood chemistries, and urinalysis are usually normal *(83, 88, 93)*. Although not diagnostic, in most patients, the serum lactate dehydrogenase is mildly elevated *(11, 94)*. In more severe and untreated patients hemoglobin and hematocrit may be elevated due to chronic hypoxia. Serum biomarkers are elevated in PAP and include KL-6, CEA, SP-A, SP-B, and SP-D, SP-C. However, their utility in the diagnosis of PAP or different clinical forms of PAP remains to be determined.

Pulmonary function tests can be useful and may be normal or may reveal a restrictive defect with mild impairment of the forced vital capacity and total lung capacity and a disproportionate, severe reduction of the diffusing capacity *(11, 12, 95)*. The restrictive defect is reversible with symptomatic resolution either following whole lung lavage or spontaneous resolution *(11, 15, 96)*. Arterial blood gas measurement reveals hypoxemia due to ventilation-perfusion inequality and intrapulmonary shunt that results in a widened alveolar-arterial diffusion gradient in symptomatic patients *(11, 97)*.

Bronchoscopy and bronchoalveolar lavage with or without transbronchial biopsy is useful in establishing a diagnosis of PAP in most clinically suspected cases *(12, 88)*. The BAL fluid in PAP is milky in appearance and forms a waxy sediment upon standing (Figure 5.2a). Microscopically, it is acellular with relatively few inflammatory cells. Alveolar macrophages are morphologically abnormal ranging from small and monocyte-like cells to large foamy cells that are fragile and are destroyed during cytocentrifugation leaving large acellular eosinophilic bodies in a diffuse background of granular basophilic material. The extracellular lipoproteinaceous material and the material within alveolar macrophages stain positively with periodic acid-Schiff (PAS)

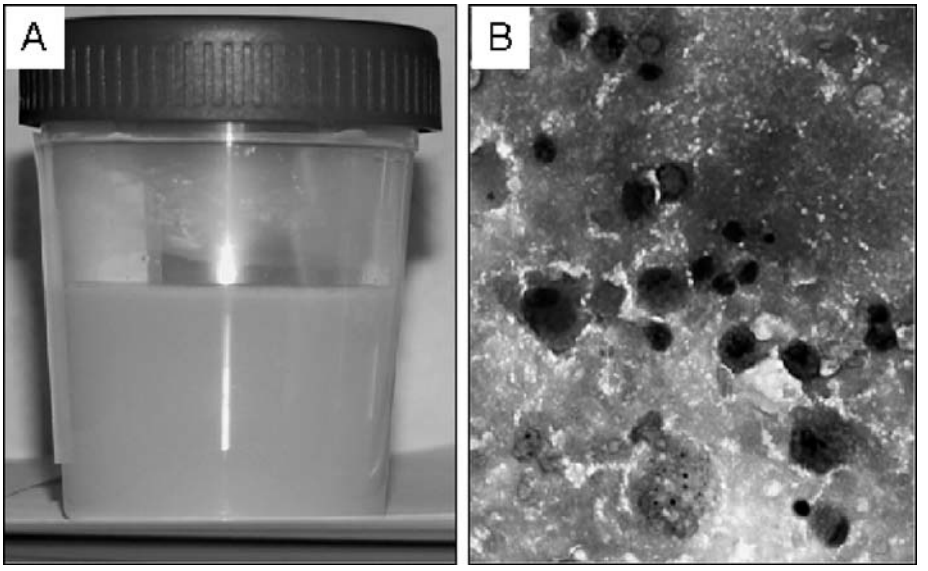

Figure 5.2 Appearance of the bronchoalveolar lavage fluid in autoimmune PAP. (**a**) Gross appearance after settling overnight at 4°C. (**b**) Microscopic appearance after periodic acid-Schiff staining. Histopathological appearance

reagent and surfactant protein immunohistochemical stains *(88, 98)* (Figure 5.2b). Increased numbers of lymphocytes may be present, but relatively few other inflammatory cells typically present *(80, 98)*.

Open lung biopsy remains the gold standard for diagnosis of PAP but is not typically required and can occasionally be complicated by false negatives due to sampling error *(34, 43, 88)*. The lung parenchyma is preserved in autoimmune PAP not complicated by secondary infection. Transitional airways and alveoli are usually normal, but are occasionally thickened by lymphocytic infiltration or less commonly fibrosis. Alveoli are filled with granular eosinophilic material that stains reddish with PAS reagent. Large foamy alveolar macrophages may be seen and degenerating macrophages are usually evident within the granular material. Immunohistochemical staining reveals abnormally abundant accumulation of surfactant protein. Electron microscopy reveals the presence of amorphous debris containing membranous structures resembling lamellar bodies and tubular myelin similar to seen in preparations of normal surfactant.

Neutralizing GM-CSF autoantibodies are present at high levels in individuals with autoimmune PAP, but do not occur at high levels in any other known settings (Figure 5.3) including individuals with secondary PAP, congenital PAP, surfactant metabolic dysfunction disorders, other lung diseases of normal individuals *(75, 76, 78, 99, 100)*.

Although a variety of approaches have been developed to detect and quantify the presence and function of these antibodies *(73, 76, 99)*, an enzyme-linked immunoabsorbent assay (ELISA), utilizing an autoantibody standard, has been shown to be a reliable method for measurement of the GM-CSF autoantibody levels in the serum or lavage fluid of affected individuals. This assay has now been tested on larger numbers of subjects and has a sensitivity and specificity approaching 100% *(15, 73, 99)*.

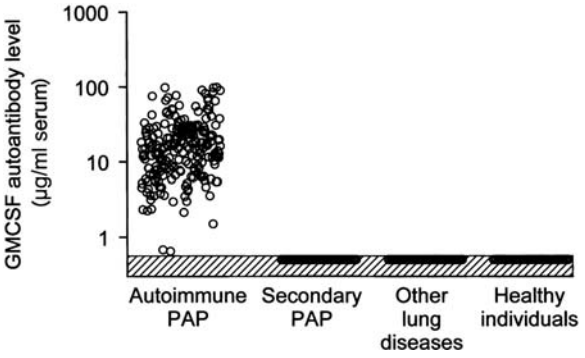

Figure 5.3 Specific association of high serum levels of GM-CSF autoantibodies with autoimmune PAP (Reprinted from reference 12)

Importantly, the serum levels of GM-CSF autoantibody level do not correlate with disease severity as measured by DLCO, which does *(15)*.

A number of biological serum markers have been evaluated for the diagnosis or for monitoring the severity of the lung disease in PAP patients including carcinoembryonic antigen *(15, 101)*, cytokeratin 19 *(125, 102)*, KL-6 *(103)*, monocyte chemotactic protein 1 (MCP-1), SP-A, and SP-D *(15, 104, 105)*. Of these, KL-6, and CEA have the highest specificity and sensitivity while SP-A and SP-D are less specific and sensitive because elevated levels of these proteins occur in a variety of respiratory diseases *(15, 19)*.

In summary, the diagnosis of autoimmune PAP requires a high degree of clinical suspicion and can readily be made based on the basis of a typical history, a characteristic radiograph, high-resolution computed tomogram of the chest, and the presence of high serum level of GM-CSF autoantibodies, performed in a competent testing facility. In the absence of serologic testing, bronchoscopy with bronchoalveolar lavage and pathological and cytological evaluation are frequently helpful.

Conventional Management and Treatment

A wide range of empiric approaches have been evaluated for in the search for effective therapy for PAP over the past decades, including antibiotics, corticosteroids, digestive enzymes (streptokinase, trypsin), heparin, and mucolytics (acetylcysteine, potassium iodide, ambroxol) *(11, 44, 93, 106)*. However, none of these methods were shown to be of any therapeutic value. The first effective therapy for PAP was developed in 1960 and consisted of physically removing the accumulated alveolar material by "segmental flooding" coupled with cough clearance *(107)*. Initially the procedure used a percutaneous transtracheal endobronchial catheter to blindly instill 100 ml of warmed saline drop-wise into the lung, which stimulated violent coughing productive of 30–40 ml of white viscid material. The procedure was repeated up to four times per day for 2–3 weeks with postural positioning to target different lung segments. Although impractical, radical and not particularly well-accepted at the time, with refinements this procedure ultimately led to the development of whole lung lavage.

Whole lung lavage is widely considered today to be the cornerstone of therapy for autoimmune PAP *(14, 107–110)*. Despite wide acceptance, a standardized procedure for whole lung lavage has not been developed and no randomized trial or formal prospective trial has ever been conducted to evaluate its effects on the natural history of PAP. Nor have specific criteria been developed indicating the need for, timing of, or therapeutic response to whole lung lavage. In adults, the procedure typically is done under general anesthesia using a Carlens tube and mechanical ventilation. The patient is supine with the lung to be treated in a dependent position. The lung is filled to functional residual capacity with normal saline at 37°C with or without addition of acetylcysteine or heparin. Then, aliquots of 500–1,000 ml of warmed saline are infused and then aspirated followed by vigorous endobronchial suctioning at the end of the procedure in order to remove as much of the accumulated material as possible. Chest percussion performed manually or mechanically is used by some groups in an effort to maximize the removal of the accumulated material *(111)*.

A number of studies have shown that whole lung lavage improves the clinical, physiologic, and radiographic findings in autoimmune PAP patients *(95, 109, 112–117)*. In one meta-analysis involving 146 cases with adequate documentation, the 5-year survival in PAP patients undergoing whole lung lavage was higher ($95 \pm 2\%$) compared to individuals who did not have the procedure ($85 \pm 5\%$) ($p = 0.04$) *(11)*. This study also showed that the interval between the diagnosis of PAP and the first treatment by whole lung lavage ranged from 0 (immediate) to 210 months with a median of 2 months. Less data are available from which to determine the length of the therapeutic effect. However, among 55 PAP patients for whom sufficient data were available, the median duration of benefit from lavage was 15 months *(11)*. Biochemical evidence also supports the therapeutic efficacy of whole lung lavage *(118, 119)*. Lobar and segmental lavage by fiberoptic bronchoscopy has also been reported for the treatment of PAP, although the practical clinical utility of this approach is unclear *(115, 120, 121)*

Therapy for secondary PAP generally involves treatment of the underlying condition, for example, in PAP associated with hematological malignancies, successful chemotherapy or bone marrow transplantation corrects the associated pulmonary disorder *(69)*. Efficacy of lung lavage for secondary PAP has not been well established, but has been successful in some cases *(24)*.

Current therapy for surfactant metabolic dysfunction disorders is supportive *(122)*, although successful lung transplantation has been reported *(123)*.

Future Therapeutic Targets and Directions

GM-CSF Therapy

The first use of GM-CSF for the treatment of autoimmune PAP followed rapidly after the identification of PAP in GM-CSF-deficient mice *(71)* and before the discovery of GM-CSF autoantibodies *(76)*. Daily subcutaneous injection of GM-CSF (up to 6 μg/kg/day) resulted in significant improvement in exercise tolerance and reduced the alveolar-arterial oxygen gradient ([A-a]DO_2). Improvement was not lasting upon GM-CSF withdrawal but could be restored by re-institution of GM-CSF administration. Seymour et al. then led a multinational trial to test the effectiveness of subcutaneous GM-CSF administration *(104)*. Fourteen patients received 5 μg/kg/day GM-CSF for 6–12 week with serial monitoring of the alveolar-arterial oxygen gradient

([A-a]DO2), diffusing capacity of carbon monoxide, computed tomographic scans, and exercise testing. Patients not responding to 5 μg/kg/day GM-CSF underwent stepwise dose escalation, and responding patients were retreated at disease recurrence. Stored pretreatment sera were assayed for GM-CSF-neutralizing autoantibodies. According to prospective criteria, 5 of 14 patients responded to 5 μg/kg/day GM-CSF, and 1 of 4 patients responded after dose escalation (20 μg/kg/day). The overall response rate was 43% (mean improvement in [A-a]DO2 = 23.2 mmHg). Responses lasted a median of 39 weeks and were reproducible with retreatment. GM-CSF was well-tolerated, with no late toxicity seen. The only treatment-related factor predictive of response was GM-CSF-induced eosinophilia. In another study conducted in the United States *(124)*, patients with autoimmune PAP received daily subcutaneous GM-CSF injections in escalating doses over 12 weeks. Results showed that administration of GM-CSF improved oxygenation as assessed by a 10 mmHg decrease in alveolar-arterial oxygen gradient, as well as improvement in other clinical and quality of life parameters in 12 of 25 patients (48%) with moderate symptomatic disease who completed the trial. In addition, the serum anti-GM-CSF antibody titer correlated with lung disease activity and was a predictor for responsiveness to therapy. In Japan, a network of collaborating investigators is evaluating inhalation therapy of aerosolized GM-CSF in autoimmune PAP. While early results from these trials are encouraging, no firm conclusions can yet be drawn regarding the effectiveness of GM-CSF therapy for acquired PAP. Interestingly, however, a decrease in apparent pulmonary anti-GM-CSF antibody levels in association with clinical improvement has suggested that "desensitization" to GM-CSF may be involved *(98)*. Further, in one study, the neutralizing capacity was reduced by GM-CSF therapy and correlated with clinical improvement *(125)*.

Other Approaches

Recognition of the common form of acquired PAP as an autoimmune disease mediated by the presence of high levels of neutralizing GM-CSF autoantibodies has suggested several alternative immunological therapeutic approaches. One such potential approach is plasmapheresis, which can be employed to remove the autoantibody from the blood *(126)*. The potential beneficial effects of this approach include improvement in both surfactant clearance and host defense functions of macrophages due to improved GM-CSF signaling. Another immunological approach is the use of anti-B-lymphocyte antibodies to decrease the number of anti-GM-CSF antibody-producing cells. At the time of writing, a trial is ongoing to evaluate this approach in 10 patients, Although autoimmune PAP is closely associated with the occurrence of GM-CSF autoantibody, there is no evidence that steroid is effective to improve the pulmonary involvement.

Our understanding of PAP pathogenesis has advanced enormously over the past two decades, stimulated in large measure by the observation of PAP in GM-CSF-deficient mice. GM-CSF regulates pulmonary surfactant homeostasis in mice by regulating the ability of alveolar macrophages to catabolize surfactant. This occurs via a GM-CSF-mediated increase in expression of the transcription factor PU.1, which is critical for alveolar macrophages terminal differentiation. It is likely that a similar mechanism occurs in man and that a high level of neutralizing GM-CSF autoantibodies eliminates GM-CSF bioactivity in vivo in PAP patients, thereby causing a arrest of their maturation at a stage in which surfactant catabolism does not occur. Notwithstanding, many questions remain. The precise mechanism(s) by which GM-CSF regulates surfactant homeostasis have not been identified in mice or man. GM-CSF is clearly critical in the

regulation of many immune and other functions of murine alveolar macrophages, but the mechanisms by which these pathways operate have not been defined. Since the total GM-CSF autoantibody level in autoimmune PAP does not correlate with the disease severity, other correlates must be sought. It will also be essential to understand if there is a genetic predisposition to PAP as well as the pharmacogenetic responses to treatment. Finally, recognition of autoimmune PAP as an autoimmune disorder opens the door to a number of testable immunological therapies for treatment of PAP.

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6

Mutations in Surfactant Protein C and Interstitial Lung Disease

Ralph J. Panos and James P. Bridges

Abstract Less than 5% of all cases of idiopathic interstitial lung disease (ILD) are due to familial pulmonary fibrosis. The clinical manifestations of familial pulmonary fibrosis are indistinguishable from the presenting symptoms in sporadic idiopathic pulmonary fibrosis. Mutations in *SFTPC*, the gene encoding surfactant protein C (SP-C), have been identified in kindreds with familial ILD as well as individuals with sporadic IPF. SP-C is a surfactant-associated protein that is essential for the reduction in surface tension at the air–liquid interface within the alveolus and the prevention of end-expiratory alveolar collapse. Because of its hydrophobic properties, SP-C is synthesized as a proprotein that is processed within the secretory pathway of alveolar type II cells as it is conducted to the lamellar body, the intracellular storage site of surfactant. The carboxy terminus of the proprotein appears to function as an intramolecular chaperone that guides posttranslational processing of the SP-C protein and the majority of mutations associated with ILD occur within this domain. Over 50 distinct *SFTPC* mutations have been identified and individuals with SP-C mutations range in age from infants to adults. The clinical manifestations extend from fatal respiratory failure to no clinically apparent respiratory symptoms. The pattern of inheritance appears to be autosomal dominant with variable penetrance. In infants and children, the most common histopathological pattern is nonspecific interstitial pneumonitis with features of pulmonary alveolar proteinosis. In contrast, usual interstitial pneumonitis is the most frequent pattern in adults. These mutations may cause lung fibrosis through protein misprocessing within the endoplasmic reticulum activating the unfolded protein response, proteasome dysfunction, and alveolar epithelial cell death. Alveolar type II cells expressing SP-C mutant proteins may be more susceptible to environmental factors that may trigger epithelial cell injury, death, and the development of parenchymal fibrosis. Understanding the pathogenetic mechanisms by which mutations in SP-C cause pulmonary fibrosis

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provides unique insights into the cellular and molecular pathogenesis of the idiopathic interstitial lung diseases.

Keywords: familial interstitial lung disease, idiopathic interstitial lung disease, surfactant protein, surfactant protein C

Introduction

Interstitial lung diseases (ILDs) are a heterogeneous group of more than 200 disorders in adults and children that derange the alveolar architecture and cause radiographic and physiologic abnormalities *(1)*. Individuals with ILD usually present with progressive breathlessness, tachypnea, and hypoxemia. The worldwide incidence of ILD is estimated to be 10/100,000 for men and 7/100,000 for women *(2)*. While the histopathological manifestations of these disorders are diverse and may include alveolar wall denudation and/or collapse, fibroblast proliferation and inflammatory cell infiltration, the common final pathway found in all ILDs is cellular damage, physiologic dysfunction of the alveolar epithelium, and fibrosis of the lung interstitium.

Idiopathic interstitial pneumonias (IIPs) represent a subset of ILD whose etiology is unknown. Each of these disorders is distinguished by unique histological findings characterized by alveolar inflammation and progressive fibrosis of the lung. According to the most recent consensus classification by the American Thoracic Society/European Respiratory Society, the IIPs comprise seven categories based on histopathologic, clinical, and radiographic features: idiopathic pulmonary fibrosis (IPF), non-specific interstitial pneumonia (NSIP), respiratory bronchiolitis (RB)-associated ILD, desquamative interstitial pneumonia (DIP), cryptogenic organizing pneumonia (COP), acute interstitial pneumonia (DAD), and lymphocytic interstitial pneumonia (LIP) *(3)*. Of the IIPs, IPF is the most prevalent and devastating with a mean survival time of approximately 3–5 years after diagnosis *(3)*. Furthermore, the mortality rate from IPF increased significantly in the United States from 1992 to 2003, demonstrating the lack of efficacious treatment modalities and/or increased recognition of this disease *(4)*. Prior to the most recent classification scheme in 2002, IPF was referred to as Hamman–Rich syndrome, cryptogenic fibrosing alveolitis, usual interstitial pneumonitis, desquamative interstitial pneumonitis, fibrosing alveolitis, diffuse alveolar fibrosis, and honeycomb or end-stage lung disease. The definitive histologic pattern that delineates IPF from the other IIPs is usual interstitial pneumonia (UIP) which is characterized by a heterogeneous appearance of fibroblastic foci and honeycombing of the lung, patchy collagen deposition, and low levels of interstitial inflammation interspersed with normal-appearing lung parenchyma *(5)*. The clinical, physiologic, and radiographic features of IPF have been very well characterized. However, despite several decades of intensive investigation, the precise etiology and pathogenesis of IPF are not known.

One of the earliest and long-standing hypotheses for the pathogenesis of IPF posited that chronic inflammation beget alveolar destruction and fibrosis through alveolar type I cell damage, proliferation of alveolar type II cells, loss of alveolar epithelial integrity, derangement of the basement membrane delineating the alveolar space, and recruitment and proliferation of mesenchymal cells that produce an exuberant extracellular

matrix *(6)*. Consistent with this concept, transgenic mouse models that overexpressed pro-inflammatory cytokines in lung epithelia, such as IL-1β, TGF-β, TNF-α, or IL-13, demonstrated persistent pulmonary inflammation and fibrosis *(7–11)*. Similarly, ablation of genes encoding inflammatory cytokines/mediators in mice associated with IPF in humans, including *Ifn-γ*, *Smad*3, cytoplasmic phospholipase (*Cpla2*), and lipoxygenase (*5-LO*), decreased the severity of inflammation and lung fibrosis induced by the chemotherapeutic drug bleomycin *(12–15)*. More recently, the inciting role of inflammation has been questioned and greater importance placed upon alveolar epithelial injury and deranged alveolar repair mechanisms *(16)*. For example, data from the bleomycin-induced fibrosis model, generated on transgenic and knock-out backgrounds, demonstrate dissociation between inflammation and the development of fibrosis. Mice deficient in the $\alpha_v\beta_6$-integrin display pronounced pulmonary inflammation at baseline, which is enhanced with bleomycin treatment, but are completely protected from fibrosis *(17)*. In a separate transcriptional profiling experiment using $\alpha_v\beta_6^{-/-}$ mice, Kaminski et al. demonstrated that distinct genetic programs regulate lung inflammation and fibrosis in response to bleomycin *(18)*. Furthermore, the relative lack of inflammatory infiltrates found in lung biopsy specimens from patients with IPF combined with the failure of anti-inflammatory therapy to improve significantly the outcome in these patients challenges the theory that fibrosis results from an incessant inflammatory reaction.

Other investigators have suggested that repetitive injury or antigen exposure stimulates a T-helper type 2 response within the alveolar space altering epithelial and endothelial regeneration and creating a cytokine and chemokine milieu that promotes mesenchymal cell recruitment, proliferation, and excessive production of matrix constituents *(19)*. Theories of the potential origin of these mesenchymal cells have progressed from local migration and propagation to epithelial–mesenchymal transition and pulmonary parenchymal localization and differentiation of circulating stem cells *(20)*. More recent evidence suggests that alveolar epithelial cells may also be derived from circulating stem cells *(21)*. Finally, recent evidence suggests that one of the characteristic features of usual interstitial pneumonia, fibroblastic foci (small areas of proliferating myofibroblasts within nascent, myxoid-appearing matrix that generally are located in the border zone between normal and fibrotic lung parenchyma), are not discrete lesions but appear to be the tips of an extensive fibrotic reticulum that is transforming or invading normal lung tissue *(22)*.

Familial linkage analysis provides evidence that genetic factors may also contribute significantly to the development of fibrotic lung diseases *(23)*. While an exact worldwide figure is difficult to calculate, analysis of patient data from 29 Finnish pulmonary clinics estimated the frequency of familial IPF to be between 3.3 and 3.7% of all IPF in Finland *(24)*. In addition, Loyd et al. found that ∼19% of individuals with end-stage IPF undergoing lung transplantation at the Vanderbilt Medical Center had a family history of lung disease *(25)*. Numerous interstitial lung diseases with varying phenotypic presentations are associated with genetic disorders, including familial hypocalciuric hypercalcemia *(26)*, Gaucher disease *(27)*, Niemann–Pick disease *(28)*, tuberous sclerosis *(29)*, neurofibromatosis *(30)*, lymphangioleiomyomatosis (LAM), and Hermansky–Pudlak syndrome. LAM and Hermansky–Pudlak syndrome are reviewed in Chapters 4 and 8 in this text. Mutations in various genes have also been associated with pulmonary fibrosis (reviewed in *(23)*). Recently, mutations in the genes *hTERT* and *hTR*, whose end

products are required for telomere maintenance, have been linked to IPF in multiple families *(31, 32)*. Furthermore, mutations in the gene encoding surfactant protein C, *SFTPC*, are associated with various forms of familial IIP, including IPF and NSIP, in adults and children, respectively *(33–46)*.

In this chapter, we will review the clinical presentation of IPF and familial pulmonary fibrosis and discuss the potential role of mutations in surfactant protein C in the pathogenesis of interstitial lung disease.

Initial Clinical Evaluation of Diffuse Parenchymal Lung Disease

The evaluation of a patient with a suspected interstitial lung disease necessitates an organized, sequential evaluation process. The initial assessment should include a comprehensive history, thorough physical examination, chest imaging studies, pulmonary function testing, and arterial blood gas measurement. The complete history is usually the most important step in this evaluation and often allows discrimination between many of the known causes of interstitial lung disease. Specific historical points elicited during the evaluation should include a thorough occupational and environmental history including home and work environmental exposures, hobbies, pets, heating and cooling systems, prior or concurrent medical illnesses including collagen vascular disorders, cardiac or renal diseases, drug ingestions including prescribed, illicit or over-the-counter medications, and a family history of similar or other inherited pulmonary disorders.

Epidemiology

The prevalence of idiopathic pulmonary fibrosis appears to be increasing. Previous calculations estimated the prevalence to be 3–5 per 100,000 population *(47)*. Subsequently, Coultas and colleagues *(2)* reported an incidence of 9 per 100,000 in 1994 and more recent studies suggest that the incidence has increased to approximately 14.0–42.7 cases per 100,000 population depending upon the criteria for diagnosis *(48)*. Analysis of a general practice registry in the United Kingdom demonstrated an increase in the incidence of IPF from 27.3 per 1,000,000 person-years in 1990 to 67.8 per 1,000,000 person-years in 2003 *(49)*. This increasing prevalence is reflected in a rising mortality rate. Using the US Multiple Cause of Death (MCOD) mortality database (Centers for Disease Control and Prevention, National Center for Health Statistics), Mannino and coworkers *(50)* determined that the male age-adjusted mortality rates for IPF increased from 48.6 per 1,000,000 in 1979 to 50.9 per 1,000,000 in 1991 and the rate among women increased from 21.4 per 1,000,000 in 1979 to 27.2 per 1,000,000 in 1991. A recent analysis of the same database demonstrated further increases in the age-adjusted mortality rates among men from 40.2 per 1,000,000 in 1992 to 61.9 per 1.000,000 in 2003 and from 39.0 per 1,000,000 in 1992 to 55.1 per 1,000,000 in 2003 among women *(4)*. Both groups found that mortality rates increased with time and age and were higher in men than in women *(4, 50)*. The more recent analysis demonstrated that the mortality rate was increasing faster for women than for men *(4)*.

Clinical Manifestations

Most patients with IPF are 40–70 years of age and men are affected more frequently than women *(6, 51)*. Presenting symptoms are usually breathlessness or a dry hacking, usually non-productive, cough *(52, 53)*. As the disease progresses, nearly all patients with IPF develop breathlessness that usually occurs with exertion initially and at rest during the later stages of the disease. Associated symptoms include malaise and weight loss. Infrequently, arthralgias, myalgias, and fevers may also occur.

The physical examination may be normal at disease presentation. The most common physical examination finding is bibasilar, end-inspiratory, dry, Velcro crackles. Clubbing occurs frequently in the latter stages of the disease. Evidence of pulmonary hypertension, cor pulmonale, and right-sided heart failure commonly develop as the disease progresses. Acrocyanosis may be present in the advanced stages of IPF.

Chest Imaging Studies

The chest X-ray may be normal in up to 15% of patients with IPF *(54–58)*. The initial finding may be only a reduction in lung volumes that is most evident when comparison is made with previous chest X-rays. A diffuse reticular pattern in a lower lung zone predominant pattern develops. Both lungs are usually equally involved. Occasionally a reticular nodular pattern may develop with supra-imposition of nodular opacifications on a network of curvilinear densities. As the disease progresses, a honeycomb pattern with coarse reticular opacifications and small (up to 1 cm) superimposed cysts develop *(59, 60)* (Figure 6.1).

High-resolution thin section computed tomography (HRCT) is the preferred imaging modality in IPF. HRCT provides detailed resolution of the lung parenchyma using imaging thicknesses of 1–2 mm with special algorithms to improve spatial resolution

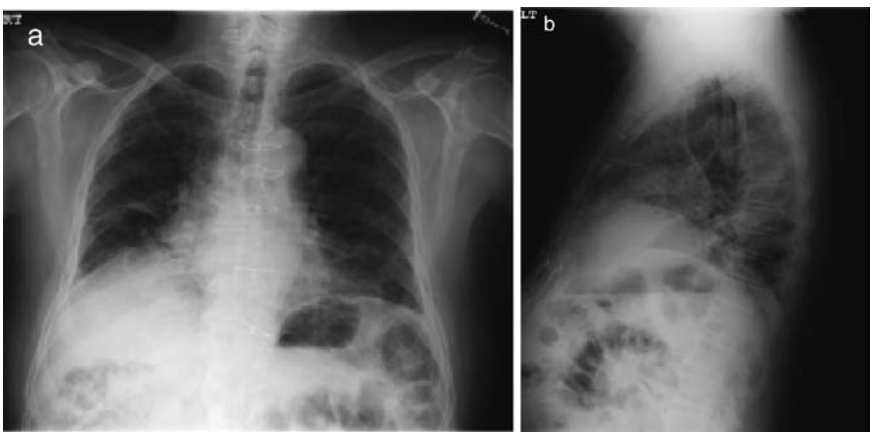

Figure 6.1 **a** and **b**. Chest X-ray [posterior–anterior (**a**) and lateral (**b**)] of a patient with idiopathic pulmonary fibrosis. Reticulonodular opacifications are prominent within the lower lung zones with areas of honeycombing in the right lower lobe. Lung volumes are reduced. Sternal wires were placed during coronary artery revascularization

(61, 62). HRCT scans are useful diagnostically and may obviate the need for an open lung biopsy. In addition, HRCT scans can be used to determine disease progression and may help determine disease course. The most common HRCT findings in IPF include irregular, interlobular septal thickening, intralobar interstitial thickening, honeycombing, and traction bronchiectasis or bronchiolectasis. Ground glass opacifications may also be present. These findings are usually present in a peripheral subpleural location within the lower lung zones. They are often distributed heterogeneously and areas of honeycombing, mild fibrosis, as well as normal lung may be present within the same lung or even the same lobe (Figure 6.2). The HRCT scan may be useful in distinguishing IPF from other diffuse parenchymal lung diseases including sarcoidosis, silicosis, hypersensitivity pneumonitis, pulmonary alveolar proteinosis, and alveolar cell carcinoma, but it is less useful in distinguishing non-specific interstitial pneumonitis *(3, 63, 64)*. In a study of 315 patients with IPF, the overall extent of fibrosis pattern score was a

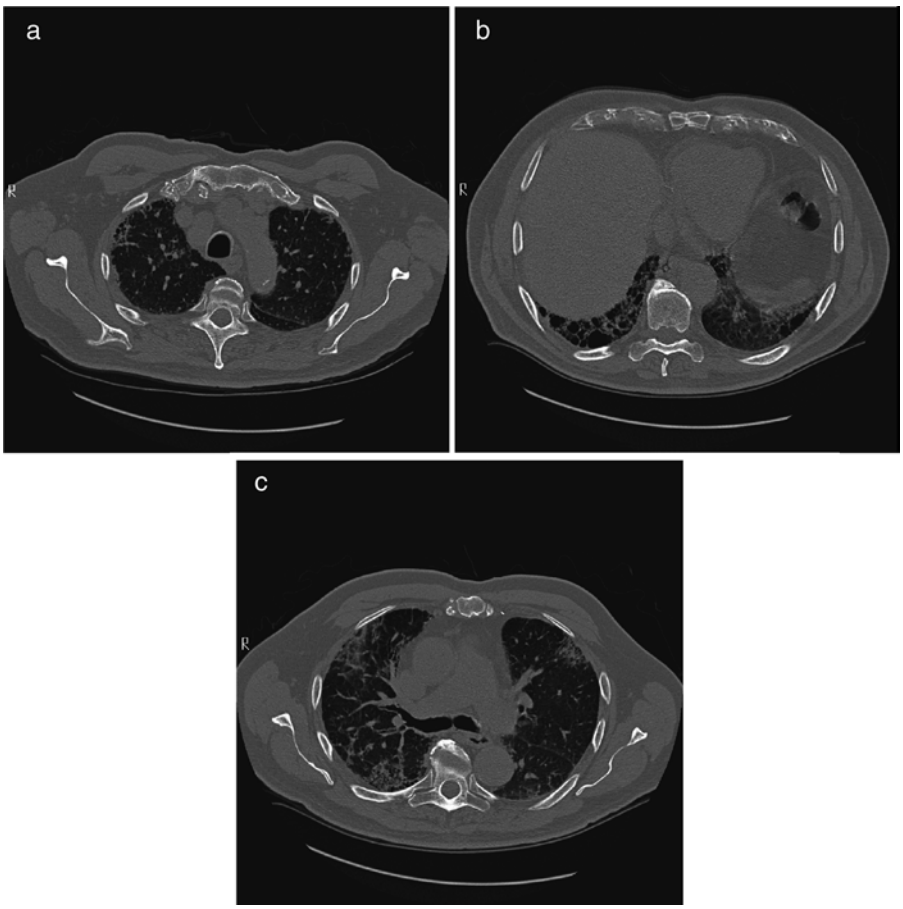

Figure 6.2 High-resolution thin section chest computed tomography of the same patient in Figure 6.1 at upper (**a**), middle (**b**), and lower (**c**) lung zones. Subpleural reticular markings with traction bronchiectasis and bronchiolectasis are present in a lower zone predominant distribution. Honeycomb changes, cytic lesions, are slightly more prominent on the right than on the left side

significant prognostic predictor of mortality (hazard ratio 2.71; 95% confidence interval 1.61–4.55) *(65)*.

Physiological Studies

Pulmonary Function Testing

Although pulmonary function testing in patients with IPF may be normal during very early disease, most patients show evidence of a reduced total lung capacity and decreased vital capacity consistent with restriction. As fibrosis increases, pressure volume studies demonstrate a reduction in lung compliance. The pressure volume curve is shifted down and to the right by the stiff, fibrotic, non-compliant lung parenchyma. Usually, all lung volume compartments, total lung capacity, function residual capacity, and residual volume decrease proportionally as the disease process progresses. Reduced lung compliance increases the work of breathing and patients with IPF are frequently tachypneic and have rapid shallow respiratory patterns *(66, 67)*. Spirometric measures of lung function, forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) are usually decreased in proportion to the lung volumes.

Gas Exchange

Diffusing capacity for carbon monoxide (D_LCO) is decreased and may be one of the earliest physiologic abnormalities in IPF *(68)*. The resting arterial oxygen tension (PaO_2) is often normal in early IPF but inevitably declines. With exercise, the PaO_2 decreases and the alveolar–arterial oxygen gradient widens *(6, 69, 70)*. Hypoxemia in IPF is mainly due to ventilation-perfusion mismatching and only approximately 20% of the widened PaO_2 gradient is due to an impairment in oxygen diffusion *(6, 70 – 72)*. Eaton and colleagues *(73)* studied the reproducibility of various physiologic measures of lung function in individuals with diffuse parenchymal lung disease and showed that the maximal distance walked in 6 min and the maximal oxygen uptake (VO_2 max) demonstrated the least intertest variation. The 6 min walk distance is a better predictor of survival than spirometric measurements in patients with IPF awaiting lung transplantation *(74)*. Flaherty and coworkers *(75)* stratified nearly 200 patients with IPF based upon desaturation ($SaO_2 \leq 88\%$) during a 6 min walk. The best predictor of mortality in the group of patients with desaturation was serial decline in diffusing capacity whereas, in those individuals who did not desaturate during their initial evaluation, increases in the desaturation area (a measure of desaturation severity and duration) and serial decline in FVC heralded the worst outcome.

Pathology

At autopsy, the lungs of individuals with long-standing IPF are contracted and dense, with a nodular pleural surface. Areas of honeycombing are most prominent in the lower lung zones and extend superiorly and medially. Usual interstitial pneumonitis (UIP) is the histopathological pattern that defines IPF. UIP is characterized by a heterogeneous appearance to the pulmonary parenchyma. Areas of normal-appearing lung are interspersed with zones of dense fibrosis and microscopic honeycombing (end-stage lung) and loose, less densely compacted myxoid-appearing matrix. Fibroblastic foci,

localized small aggregates of actively proliferating myofibroblasts with surrounding loose nascent-appearing matrix, are located along the interface between normal and fibrotic lung parenchyma.

Clinical Course

Classically, the natural history of IPF has been considered to be an inexorable, slowly progressive decline in respiratory function with a gradual increase in breathlessness and reduction in lung function. More recent clinical studies suggest that the course of IPF is marked by acute exacerbations with abrupt, marked increases in dyspnea and decrements in pulmonary function *(76–79)*. A recent international consensus has defined an acute IPF exacerbation as subjective worsening over 30 days or less, new bilateral radiographic opacities, and the absence of infection or another identifiable etiology *(80)*. Although the factor(s) precipitating an acute IPF exacerbation are not known, bronchoalveolar lavage and surgical lung biopsy have been shown to worsen pulmonary function abruptly in some individuals with IPF *(81, 82)*. Severe exacerbations requiring mechanical ventilation or admission to an intensive care unit are nearly universally fatal *(83, 84)*. Pirfenidone, prednisone, and anticoagulation or treatment with cyclosporine have been demonstrated to be potentially beneficial in the prevention or treatment of IPF exacerbations *(78, 79, 85, 86)*.

Treatment

Despite several decades of investigation and innumerable studies, no treatment has been demonstrated unequivocally to be beneficial in the management of IPF. As hypotheses of the pathogenesis of IPF have evolved from alveolar inflammation to deranged epithelial repair and mesenchymal-fibroblast proliferation, therapeutic modalities have transitioned from immunosuppressive and cytotoxic agents to biologic modifiers.

Cochrane analyses of the therapeutic effects of corticosteroids or immunosuppressive agents in the treatment of IPF suggest that neither therapeutic class has been shown to improve mortality or physiologic outcomes unequivocally *(87, 88)*. One study demonstrated that prednisone and azathioprine improve age-adjusted mortality compared with prednisone alone *(89)*. However, only a small number of patients were studied. A larger multicenter trial comparing these agents has been proposed within the IPFNet, a NIH-sponsored consortium. A comparison of one group of patients treated with prednisone and cyclophosphamide with another group of untreated patients at another institution showed no significant effect on survival *(90)*.

Although initial studies of interferon-γ_{1b} suggested improvement in clinical outcome and biological markers, a large international placebo-controlled trial demonstrated no effect on progression-free survival (time to disease progression or death), lung function, gas exchange, or quality of life *(91)*. A second study using mortality as the primary outcome was stopped prematurely due to a lack of efficacy. A multicenter trial comparing *N*-acetyl cysteine plus prednisone/azathioprine with placebo plus prednisone/azathioprine showed that the regime containing *N*-acetyl cysteine significantly reduced the rate of decline of FVC and DLCO *(92)*. However, the clinical significance of the measured outcomes, the lack of a placebo treatment group, and the demonstration that NAC reduced side effects of prednisone/azathioprine have limited the acceptance of NAC as a beneficial treatment of IPF *(93)*.

A trial comparing pirfenidone with placebo demonstrated no significant difference in the primary endpoint, the difference in the change in the lowest oxygen saturation during a 6-min exercise test at 6 months *(78)*. However, the study was terminated prematurely due to fewer exacerbations in the pirfenidone-treated group. A larger, international trial of pirfenidone in the treatment of IPF is underway.

Familial Pulmonary Fibrosis

Familial pulmonary fibrosis is defined as a kindred with two or more members who have interstitial lung disease. Although the term familial idiopathic pulmonary fibrosis (IPF) is commonly used synonymously with familial pulmonary fibrosis, familial IPF is misleading and inaccurate. By the most recent ATS guidelines, IPF is the interstitial lung disease identified by a usual interstitial pneumonitis (UIP) pattern; however, UIP is not the only histopathological pattern found in kindreds with inherited pulmonary fibrosis *(94, 95)*. Whether these different histopathologic patterns represent unique pulmonary processes, are distinctive manifestations of the same process at different points in time, or are common, stereotypical histopathologic appearances of different lung diseases is not known. Thus, we use the term familial pulmonary fibrosis and not familial idiopathic pulmonary fibrosis to describe diffuse parenchymal lung disease affecting two or more members of the same kindred.

The prevalence of familial pulmonary fibrosis is estimated to be between 1.34 and 5.9 cases per million population *(24, 96)*. Approximately 0.5–3.7% of all cases of idiopathic pulmonary fibrosis are due to familial pulmonary fibrosis *(15, 24)*. Review of inheritance patterns in kindreds with familial pulmonary fibrosis suggests an autosomal dominant inheritance *(94, 95)*.

Numerous case reports and small series of familial pulmonary fibrosis have been reported (reviewed in the Mendelian Inheritance in Man database, http://www.ncbi.nlm.nih.gov/Omim). The clinical characteristics of patients with familial pulmonary fibrosis from five large series are presented in Table 6.1. There is an approximate 2:1 ratio of males to females. The age at diagnosis is between 55 and 67 years, and the majority of individuals diagnosed with familial pulmonary fibrosis are smokers. In a study of 164 individuals from 18 kindreds affected with familial pulmonary fibrosis, Rosas and colleagues *(97)* found that asymptomatic individuals with significant abnormalities on HRCT were significantly younger than those with known familial pulmonary fibrosis but older than family members with normal findings on

Table 6.1 Clinical characteristics of patients with familial pulmonary fibrosis from five large series.

	Marshall *(95)*	**Hodgson** *(24)*	**Lee** *(10)*	**Steele** *(93)*	**Rosas** *(96)*
Kindreds	21	17	15	111	18
n	57	45	27	309	21
Male/female	1.75:1	No difference	2:1	2.2:1	48:52
Age at diagnosis (years)	55.5	61.9	59.4	66.6	67
Ever smokers	52%	NR	55%	67.2%	67%

NR = Not reported.

HRCT. Approximately 45% of subjects with asymptomatic lung disease and 67% of subjects with familial pulmonary fibrosis had a history of smoking. In contrast, only 23% of family members without evidence of interstitial lung disease were smokers *(97)*.

The clinical manifestations of familial pulmonary fibrosis are indistinguishable from the presenting symptoms in sporadic idiopathic pulmonary fibrosis. The most frequent symptom is breathlessness followed by cough. Clubbing is present in between 1/3 and 1/2 of patients. Bibasilar, Velcro crackles are almost universally present. In 67 cases of familial fibrosis from 25 kindreds, Marshall and coworkers *(96)* found that shortness of breath (94%) and cough (86%) were the most common symptoms and clubbing (53%) and bibasilar crackles (88%) were the most frequent clinical signs (Table 6.1).

Radiographic imaging findings are also similar between familiar pulmonary fibrosis and IPF. High-resolution thin section CT scans reveal subpleural fibrosis with honeycombing in a basilar predominant distribution in individuals with familiar pulmonary fibrosis *(96)*. Family members in kindreds with familial fibrosis may demonstrate increased reticular opacifications, widened septal markings, thickened bronchovascular bundles, and ground glass opacifications or entirely normal scans *(97)*. In 143 asymptomatic individuals from 18 families with familial pulmonary fibrosis, Rosas and colleagues *(97)* demonstrated normal HRCTs in 53 (32%), non-specific changes in 59 (36%), and findings of significant fibrosis in 31 (19%).

Histopathology

In contrast to sporadic idiopathic pulmonary fibrosis, lung biopsies reveal varied histopathologic patterns in individuals with familial pulmonary fibrosis. The most common pattern is usual interstitial pneumonitis which is present in greater than 75% of patients. Other patterns that have been described include non-specific interstitial pneumonitis, cryptogenic organizing pneumonia, central lobular nodules, hypersensitivity pneumonitis, cellular interstitial pneumonitis with organizing pneumonia, and unclassified interstitial lung disease *(94, 97)*. Lung biopsies in six asymptomatic individuals from families with familial pulmonary fibrosis and who demonstrated HRCT evidence of interstitial lung disease revealed usual interstitial pneumonitis in three and hypersensitivity pneumonitis, non-specific interstitial pneumonia, or cellular interstitial and organizing pneumonia in each of the others *(97)*.

In an earlier evaluation of 17 clinically unaffected family members of patients with familial pulmonary fibrosis, Bitterman and coworkers *(98)* identified four individuals with positive gallium-67 scans and eight with increased numbers of neutrophils and activated macrophages in bronchoalveolar lavage fluid (BALF). However, no clinical evidence of pulmonary fibrosis appeared during a follow-up period of 2–4 years in these family members with increased BALF inflammatory cells.

Microarray analysis of lung RNA from individuals with familial pulmonary fibrosis or sporadic idiopathic pulmonary fibrosis compared with normal lung tissue demonstrates differential expression of various categories of genes including chemokines and growth factors and their receptors, complement components, genes associated with cell proliferation and death, and genes in the Wnt pathway *(99)*. The most striking difference between familial and sporadic idiopathic pulmonary fibrosis was an increased intensity of expression rather than differential transcript expression. The genes that distinguish fibrotic lung from normal controls are similar in the sporadic

and familial forms, but transcript expression is more intense in familial pulmonary fibrosis compared with sporadic idiopathic pulmonary fibrosis *(95, 99)*.

Clinical Manifestations of SP-C Mutations Associated with ILD

Mutations in SP-C have been identified in kindreds with familial ILD as well as individuals with sporadic IPF. Individuals with SP-C mutations range in age from infants to adults and the clinical manifestations extend from fatal respiratory failure to no clinically apparent respiratory symptoms. Histopathologically, a broad spectrum of parenchymal lung changes including alveolar proteinosis, UIP, DIP, and NSIP have been described in individuals with SP-C mutations. Over 50 different mutations have been recognized and the majority map to the distal carboxy terminus of proSP-C within a recently described novel BRI domain (BRICHOS) *(46)*. Beers and Mulugeta *(46)* have classified SP-C mutations associated with ILD as group A, within the BRICHOS domain, group B, within the non-BRICHO carboxy terminal domain, and group C, within the cytoplasmic domains.

Nogee and colleagues *(38)* described the initial association between SP-C mutations and ILD in 2001 when they reported a full-term baby girl who developed breathlessness and cyanosis at 6 weeks of age. A chest X-ray showed increased interstitial markings and hyperinflation. Preserved pulmonary parenchymal architecture with type II cell hyperplasia and lymphocytic interstitial infiltrate with scattered myofibroblasts was present on lung biopsy. A maternal grandfather had died of lifelong lung disease and her mother died postpartum of respiratory failure. At autopsy, the maternal lung tissue was diffusely fibrotic with areas of honeycombing, lymphocytic interstitial infiltration, and alveolar damage. SP-C protein was detectable in the patient only after antigen retrieval and was minimally present in the maternal lung tissue. Both SP-A and SP-B protein were readily detectable in tissue from both the patient and her mother. Genetic analysis demonstrated a c.435+1 G>A mutation that eliminated the normal intervening sequence 5′ splice site causing the omission of exon 4 and the deletion of 37 amino acids within the carboxy terminus of the SP-C protein. These investigators subsequently sequenced the SP-C gene (*SFTPC*) in 34 infants with chronic lung diseases of unknown etiology *(39)*. Eleven patients had mutations in one allele of *SFTPC* and six had a family history of lung disease.

Hamvas and coworkers *(36)* described a 3-month-old who developed growth failure, difficulty feeding, and diffuse parenchymal opacifications on chest X-ray. An open lung biopsy at 6 months of age demonstrated alveolar type II cell hyperplasia, distorted lung parenchyma with widened alveolar septae, lymphocytic infiltration, fibroblast, and smooth muscle cell proliferation. Genetic analysis demonstrated a nine base pair deletion in exon 3 of the *SFTPC* gene. There was no parental or family history of respiratory disease.

Two large kindreds with familial ILD and the same mutation in *SFTPC*, a heterozygous exon 5 + 128 T → A mutation, have been described by groups in Nashville, Tennessee, and Saskatoon, Saskatchewan, Canada *(44, 100)*. In the Nashville kindred, 14 of 97 members were affected whereas 11 of 51 were definitely affected and seven possibly affected in the Saskatoon kindred. Individuals with ILD ranged in age from 4 months to 57 years old at the time of diagnosis. Failure to thrive, dyspnea, cyanosis, clubbing, and respiratory failure were the major clinical manifestations in those affected

individuals diagnosed before the age of 2 years. Breathlessness, cough, and clubbing were the presenting symptoms in adults. Fourteen of 25 affected individuals were male. Reported pulmonary function studies suggested restriction and reduced diffusing capacity. Radiographic findings included reticular and reticulonodular opacifications, as well as ground glass opacifications on the chest X-ray. Four of six children had a history of viral illness prior to presentation. Lung histopathology showed a NSIP pattern in all of the children. In the 14 adults, the histopathologic pattern was UIP in 11 and fibrocystic pulmonary dysplasia, interstitial pulmonary fibrosis (Hamman–Rich disease), and NSIP in one each. The *SFTPC* mutation was present on only one allele and the pattern of inheritance was consistent with an autosomal dominant mode of transmission with variable penetrance.

Cameron and colleagues *(34)* screened 116 children with ILD or chronic lung disease of unknown etiology for SP-C mutations and found seven individuals with a heterozygous thymine to cytosine transition at nucleotide 218 that caused a substitution of threonine for isoleucine at codon 73 (I73T). All of the patients were female and developed respiratory symptoms between birth and 24 months. Lung tissue demonstrated DIP in one case and chronic pneumonitis of infancy in four others. Parental genetic analysis was performed for five patients. In two patients, the mother was a carrier for the I73T mutation, and in two patients no I73T mutations were detected. In the other patient, one parent was a carrier of the I73T mutation and the other parent had another SP-C mutation, L110R. All parents with SP-C mutations had no respiratory symptoms but did not undergo formal evaluation. Several other individuals with I73T SP-C mutations have been described *(33, 40, 45)*. All three were male and were between 9 and 13 months of age. Presenting symptoms included dyspnea, hypoxemia, and failure to thrive. NSIP with PAP features, especially significant intra-alveolar accumulation of periodic acid–Schiff-positive material, was present in lung biopsy specimens from all three patients. BAL analyses demonstrated increased SP-A, SP-B precursors, mature SP-B, aberrantly processed proSP-C, and monomeric, and trimeric SP-C *(33, 45)*. Electron micrographs revealed hyperplastic alveolar type II cells containing abnormal vesicular organelles *(33)*.

In a genetic analysis of 22 patients with familial ILD in 13 Japanese kindreds, half had mutations in *SFTPC (101)*. The mean age at diagnosis was 50 years and ranged from 20 to 66 years. Histopathologic examination of lung tissue revealed NSIP in five patients and UIP in nine patients. Males were more frequently affected than females. Two patients had a missense mutation in exon 4, N138T, and nine had a mutation in exon 5, N186S. The N186S mutation occurred more frequently in 30 patients with sporadic IPF and in 11 patients with familial IPF than in 43 healthy individuals. The authors concluded that the N186S mutation, most likely a single-nucleotide polymorphism (SNP), may be a predictor for patients with familial ILD. Markart and colleagues *(102)* performed sequence analysis of *SFTPC* in 25 subjects with IPF, 10 patients with NSIP, and 50 healthy individuals. Symptoms developed at a mean age of 61 years for those with IPF and 50 years for those with NSIP. The same two SNPs within *SFTPC* that were identified by Setoguchi et al. were also found in this patient population, N138T and N186S. However, neither allele nor genotype frequencies were significantly different between the patients with ILD and the healthy subjects. In a study of 158 full-term infants and 245 premature babies, Lahti and coworkers *(103)* demonstrated that the N138T and N186S mutations were independent risk factors for respiratory distress syndrome when gender was considered a confounding factor. An increased, but not

significant, association was noted between these alleles and bronchopulmonary dysplasia.

Thus, over 50 different SP-C mutations have been described in familial and sporadic ILD. The pattern of inheritance appears to be autosomal dominant with variable penetrance. In infants and children the most common histopathological pattern is NSIP with features of PAP. In contrast, UIP is the most frequent pattern in adults. Clinical symptoms range from fulminant respiratory failure to no clinical symptoms, and the age of diagnosis ranges from infancy to adulthood. This broad phenotypic variation with the same or similar SP-C mutations suggests as yet unknown environmental factors may effect the development of respiratory manifestations. Infections have been suggested in children and smoking in adults. The processes that cause the extreme divergence in pulmonary histopathology with the same SP-C mutation are not known. Alternatively, these different histopathological patterns may represent an evolving spectrum of a progressing pulmonary process.

Surfactant Protein C – Structure/Function

Surfactant protein C (SP-C) is a single spanning, transmembrane protein that is synthesized and secreted as a component of pulmonary surfactant by alveolar type II cells of the lung. Pulmonary surfactant is a complex mixture of phospholipids and proteins that reduces surface tension along the air–liquid interface of the alveolus, thereby preventing alveolar collapse at end expiration. The importance of surfactant for normal lung function is underscored by the high prevalence of respiratory distress syndrome (RDS) in premature babies whose immature lungs lack surfactant *(104)*. Native surfactant and synthetic phospholipid preparations containing SP-C are highly effective in treating RDS of immaturity in humans *(105)* and surfactant-depleted animals *(106–108)*.

Four peptide components of surfactant have been identified: surfactant protein (SP)-A, SP-B, SP-C, and SP-D. The hydrophilic proteins SP-A and SP-D are members of the collectin family that bind to and facilitate the clearance of inhaled pathogens from the lung, ensuring a sterile alveolar environment *(109)*. In contrast, SP-B and SP-C are hydrophobic, lipid-associated proteins that are critical for the formation, organization, and function of the surfactant film *(110)*. Due to their hydrophobicity and high affinity for phospholipids, both SP-B and SP-C are synthesized as proprotein precursors and processed to mature forms in the secretory pathway of type II epithelial cells prior to secretion into the alveolus.

SP-C proprotein is highly conserved across all species for which it has been sequenced from frog to man *(110, 111)*. SP-C is synthesized as a 191 or a 197 amino acid proprotein in humans due to alterative splicing of the mRNA transcript *(112)*. The proprotein consists of the mature peptide (residues 24–58) flanked by N-terminal (residues 1–23) and C-terminal (residues 59–191/197) peptides (Figure 6.3). Early in its biogenesis, the proprotein is inserted into the membrane of the endoplasmic reticulum (ER) in a type II orientation with the N-terminal peptide residing in the cytoplasm and the C-terminal peptide in the lumen of the ER *(113–115)*. Trafficking of the proprotein through the regulated secretory pathway to the lamellar body, the major intracellular storage site of surfactant, is dependent upon signals encoded within the N-terminal peptide *(114, 115)* and may be facilitated by oligomerization as the SP-C proprotein has been shown to form dimers and oligomers in transiently transfected A549 cells *(116)*.

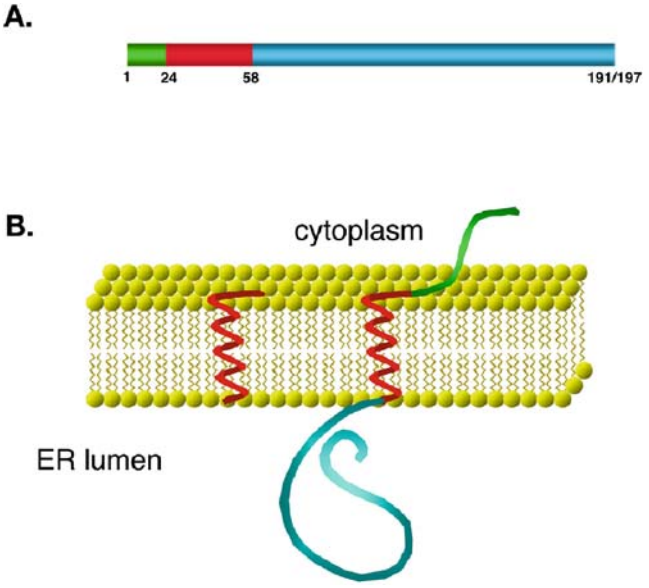

Figure 6.3 Structure of SP-C proprotein. (**a**) Diagram demonstrates SP-C proprotein structure with N-terminal peptide in green (amino acids 1–23), mature peptide in red (amino acids 24–58), and C-terminal peptide in blue (amino acids 59-191/197). (**b**) Diagram demonstrates orientation of SP-C proprotein in the ER membrane with the N-terminus located in the cytosol, mature peptide within the lipid bilayer, and the C-terminal peptide in the lumen of the ER

The N- and C-terminal peptides are cleaved in late endosomes/multivesicular bodies of the distal secretory pathway to generate the mature, bioactive peptide which is comprised predominantly of a hydrophobic, α-helical transmembrane region and a 12 amino acid, N-terminal extramembrane domain *(117, 118)*. The hydrophobic nature of the mature peptide stems from the disproportionate number of valine, leucine, and isoleucine residues in the transmembrane domain and is further increased, in most species, by the presence of palmitoyl groups attached to cysteines 5 and 6 *(119–121)*. Palmitoylation has been shown to stabilize the α-helical confirmation of the mature peptide in vitro *(122–124)* and depalmitoylated SP-C transforms into a β-sheet conformation with subsequent amyloid fibril formation in vitro at a higher rate than native SP-C *(125, 126)*. Furthermore, recent data demonstrate that the C-terminal peptide of wild-type SP-C functions as an intramolecular chaperone for the inherently unstable mature peptide, in both *cis* and *trans (127)*. Interestingly, the majority of the mutations in *SFTPC* associated with IIP map to this domain.

SP-C Mutations and IIP

The index mutation in *SFTPC* was first identified in an infant diagnosed with NSIP at 6 weeks of age *(38)*. The mutation was present on only one allele and was familial in nature as the mother was identified as a carrier of the mutation and both the mother and the grandfather were afflicted with lifelong lung disease. The mutation was a heterozygous base substitution of A for G at the first base of intron 4 (c.435+1 G>A) that

led to the internal deletion of 37 amino acids from the C-terminal peptide, generating a truncated proprotein (SP-C$^{\Delta exon4}$). Levels of wild-type SP-C (SP-C^{wt}) proprotein were significantly decreased and mature SP-C protein was undetectable in lung tissue of the patient, consistent with a dominant negative effect of the mutant allele. The loss of function of wild-type SP-C may indeed play a role in the pathogenesis of disease as ablation of the gene encoding SP-C in 129J mice, *Sftpc*, caused a pulmonary disorder consistent with interstitial pneumonitis *(128)*; similarly, the lack of SP-C in the airways of human patients, in the absence of a mutation in the *SFTPC* coding region, was associated with familial IIP *(129)*.

A separate *SFTPC* mutation associated with familial IIP was reported for two kindreds each spanning five generations *(44, 100)*. Similar to the c.435+1 G>A mutation, the missense mutation was heterozygous and in the region encoding the C-terminal peptide of SP-C, resulting in a substitution of glutamate for lysine at codon 188 of the proprotein (SP-C^{L188Q}). The mutation was found in children diagnosed with NSIP, adults diagnosed with UIP, and in asymptomatic individuals in both kindreds. Lung disease in both kindreds was incompletely penetrant and the age of onset was markedly variable, ranging from 4 months to 57 years. Interestingly, 3/4 children in one kindred *(44)* and 2/3 children from the other *(100)* who carried the mutation were diagnosed with viral infections prior to the onset of disease, suggesting that inflammatory insults may play a role in the pathogenesis of disease.

A third distinct missense mutation in *SFTPC*, SP-C^{I73T}, was detected in association with familial pulmonary fibrosis in a kindred spanning four generations *(34)* and de novo in one infant *(40)* and two children diagnosed with NSIP and alveolar proteinosis *(33, 45)*. To determine if mutations in SFTPC were linked to sporadic ILD in adults, Lawson et al. screened 135 patients with UIP or NSIP for *SFTPC* mutations. The authors found only one coding sequence mutation associated with UIP (SP-C^{I73T}) concluding that *SFTPC* mutations are rare in sporadic IPF in the adult population *(37)*. However, screening of samples from 116 pediatric patients with unexplained chronic lung disease revealed seven additional carriers, making SP-C^{I73T} the most common *SFTPC* mutation to date *(34)*. In fact, the I73T mutation has now been detected in 25 separate families with pulmonary fibrosis (L.M. Nogee, personal communication). Similar to the L188Q kindreds, the penetrance of disease in the I73T kindreds was incomplete. Abundant staining for SP-C proprotein in lung tissue specimens, in contrast to that for the c.435+1 G>A mutation, suggests that mechanisms distinct from loss of function are involved in disease pathogenesis.

Mutations in genes critical for surfactant metabolism are increasingly recognized to cause ILD. UIP and NSIP patterns are reported in adults with SP-C mutations *(44)*. In contrast, patterns of cellular pneumonitis of infancy, alveolar proteinosis, desquamative interstitial pneumonitis (DIP), and NSIP have been observed in young children *(38, 130)*.

In a recent multicenter review of lung biopsies from young children, seven confirmed cases with *SFTPC* mutations were identified. The predominant histologic pattern was chronic pneumonitis of infancy (CPI), though one case had predominant alveolar proteinosis and another had fibrotic NSIP. In contrast, of the six cases with confirmed *ABCA3* mutations in this review, four had a histological diagnosis of alveolar proteinosis, while two had a DIP pattern *(130)*.

As histology of alveolar proteinosis has not been reported in adults with *SFTPC* mutations, it remains unknown whether this differing histologic spectrum represents

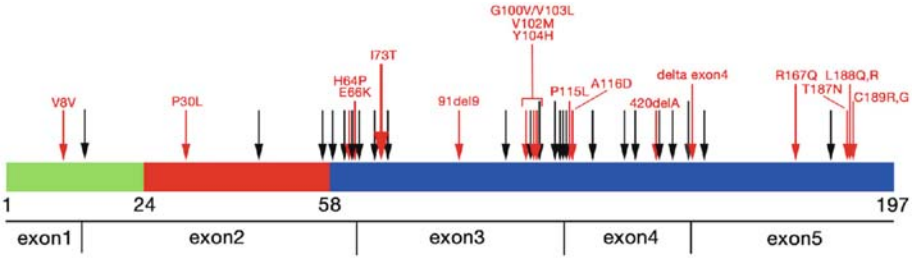

Figure 6.4 Human *SFTPC* mutations. Diagram depicts published (*red arrows*) and unpublished (*black arrows*) mutations that have been identified with respect to location on the SP-C proprotein (*top*) and exonic (*bottom*) sequence. Size of arrow corresponds to number of patients identified with individual mutations. See Table 6.2 for references to published mutations

a developmental or age-dependent manifestation, or whether therapeutic interventions may have modified the observed histology.

Collectively, a total of 56 distinct mutations, 19 of which are published, have been identified in the *SFTPC* locus that are associated with familial and sporadic idiopathic interstitial pneumonia (L.M. Nogee, personal communication) (Figure 6.4 and Table 6.2). The majority of the mutations are heterozygous in nature, consistent with a dominant negative effect of the mutant SP-C proprotein. The penetrance and the age of onset of lung disease are variable in the three reported kindreds with *SFTPC* mutations, suggesting that environmental factors and/or genetic modifiers may modulate disease. The diagnoses of viral infection prior to the onset of disease in patients from two separate kindreds are consistent with this concept. Finally, 51/56 mutations (91%) map to the C-terminal peptide of the SP-C proprotein.

Molecular Pathogenesis of Disease

Protein Folding in the Endoplasmic Reticulum (ER)

The first organelle traversed by secreted proteins is the ER where protein concentration levels approach approximately 100 mg/ml *(131)*. The ER serves as the site for protein folding, phospholipid and sterol biosynthesis, and intracellular Ca^{2+} storage. Folding occurs in a co-translational fashion as the nascent polypeptide is translocated through the ER membrane. While the primary amino acid sequence of a polypeptide contains all of the necessary information for folding, molecular machinery within the ER assists in this process. For example, the ubiquitously expressed chaperone BiP, also known as GRP78, binds to hydrophobic sequences that are intermittently exposed on a nascent protein during the folding process, thereby maintaining the unfolded or misfolded protein in a folding-competent state. Co-chaperones, including members of the ERdj family, assist BiP in this ATP-dependent process. The association and disassociation of chaperones and associated co-chaperones with unfolded/misfolded proteins continue until a mature conformation is reached, at which time the protein exits the ER for its final destination. However, if the protein is recognized as terminally misfolded, it is removed from the ER by a process known as ER-associated degradation (ERAD) *(132)*.

Table 6.2 Published *SFTPC* mutations associated with IIP.

Mutation	Domain	Inheritance	Diagnosis	References
c.435+1 G>A (SP-C$^{\Delta exon4}$)	C-terminal peptide	Familial	Child: NSIP; adult: DIP	*(38)*
c.435+1 G>T (SP-C$^{\Delta exon4}$)	C-terminal peptide	Sporadic	Unknown	*(39)*
L188Q	C-terminal peptide	Familial	Children: NSIP; adults: UIP	*(44, 100)*
I73T	C-terminal peptide	Familial and sporadic	Children: DIP, NSIP, PAP, CPI; adult: UIP	*(33, 34, 37, 39, 40)*
P30L	Mature peptide	Unknown	Unknown	*(39)*
G100V	C-terminal peptide	Familial	Unknown	*(39)*
Y104H	C-terminal peptide	Familial by history	Unknown	*(39)*
P115L	C-terminal peptide	Familial	Unknown	*(39)*
T187N	C-terminal peptide	Familial by history	Unknown	*(39)*
140delA (deletion of adenosine in codon 140) c.420delA	C-terminal peptide	Sporadic	Unknown	*(39)*
del codons 91–93	C-terminal peptide	Sporadic	Child: fibrosis, alveolar proteinosis	*(36)*
E66K	C-terminal peptide	Sporadic	Child: NSIP, PAP	*(43)*
H64P	C-terminal peptide	Unknown	Unknown	*(35)*
C189G	C-terminal peptide	Unknown	Unknown	*(35)*
R167Q	C-terminal peptide	Unknown	Child: PAP	*(45)*
392delT	C-terminal peptide	Unknown	Child: NSIP	*(42)*
A116D	C-terminal peptide	Familial	Child: NSIP	*(41)*

ERAD of terminally misfolded proteins consists of five interconnected processes: (1) recognition of a protein as terminally misfolded by a quality control receptor, (2) unfolding of the misfolded protein, (3) retro-translocation out of the ER into the cytosol, (4) attachment of ubiquitin moieties to the protein, and (5) degradation of the protein by the 26S proteasome. Identification of the molecular machinery that detects terminally misfolded, glycosylated proteins, such as EDEM (see below), has provided insight into the mechanisms underlying the recognition event of ER quality control. Following recognition, the misfolded protein must be unfolded into its primary structure in order to be retro-translocated out of the ER through the Sec61 translocon or an

alternate translocon such as Der1 *(133– 135)*. The attachment of a ubiquitin polyprotein chain, consisting of multimers of the highly conserved 76 amino acid protein, to the misfolded protein occurs while the protein is being retro-translocated into the cytosol. Poly-ubiquitination of the retro-translocated protein by an E3 ubiquitin ligase serves as a degradation signal for the 26S proteasome. The culmination of these events is degradation of the protein by the proteasome, leading to a loss of function at the expense of sparing the cell/organism from the undesired effects of a deployed, aberrantly folded protein.

ER Quality Control and the Unfolded Protein Response (UPR)

The majority of proteins that enter the ER undergo posttranslational modification including disulfide bond formation between cysteine residues and N-linked glycosylation. Glycosylation of proteins serves three primary functions: (1) to increase their overall solubility, (2) to serve as a signal for intracellular trafficking (e.g., mannose-6-phosphate (M-6-P) targets proteins to lysosomes via a M-6-P receptor), and (3) to serve as a reporter of the folding status. Lectin binding proteins, including mannosidase 1 and the chaperones calreticulin and calnexin, transiently interact with and process glycan moieties of immature, glycosylated proteins within the ER. This process, referred to as the calnexin/calreticulin cycle, assists in the maturation process by preventing aggregation of immature proteins and retaining malfolded proteins. EDEM (*E*R *d*egradation-*e*nhancing α-*m*annosidase-like protein) is an ER-resident, transmembrane protein that recognizes terminally misfolded, glycosylated proteins by the composition of their glycan chains and shuttle them for degradation via ERAD *(136, 137)*. Because of its ability to recognize terminally misfolded, glycosylated proteins, EDEM is commonly referred to as an ER quality control receptor. In contrast to ER quality control for glycosylated proteins, virtually nothing is known about the mechanisms underlying ER quality control for non-glycosylated, transmembrane proteins such as SP-C.

Inhibition of global protein glycosylation by the antibiotic tunicamycin or disruption of Ca^{2+} homeostasis in the ER by thapsigargin leads to accumulation of proteins within the ER. Accumulation of misfolded or unfolded proteins in the ER results in a condition known as ER stress and leads to the activation of signaling cascades collectively referred to as the unfolded protein response (UPR) or integrated stress response (ISR). The UPR/ISR signaling pathways are highly conserved from yeast to mammals and serve to alleviate the stress imposed upon the ER and return the cell to a normal, homeostatic state through coordinated translational and transcriptional responses. To date, three proximal sensors of the UPR have been identified, PERK, IRE-1, and ATF6, all of which are ER-resident, transmembrane proteins. Activation of these sensors by ER stress serves two primary purposes: to decrease the protein load in the ER by attenuating translation of newly synthesized proteins (mediated by PERK) and increase the production of chaperones and ERAD machinery to promote productive folding and increase degradation of terminally misfolded proteins, respectively (mediated by ATF6, IRE-1/XBP-1). In the event that the ER stress cannot be alleviated by these responses, apoptosis pathways are activated to rid the organism of the malfunctioning cell (for detailed descriptions of the UPR signaling pathway the reader is referred to *(138–140)*).

In addition to its role in acute ER stress, the UPR is also required for the differentiation of professional secretory cells such as pancreatic beta cells and plasma cells *(141, 142)*. Furthermore, Mimura and colleagues demonstrated that knock-in mice expressing

a mutant form of BiP, the primary ER-resident chaperone, displayed neonatal respiratory failure due to impaired secretion of pulmonary surfactant from alveolar type II cells *(143)*. These data suggest that type II cells undergo physiologic ER stress upon maturation and that a functional UPR plays a significant role in the proper differentiation and function of this cell type. Further investigation is needed to determine which components of the UPR are activated during type II cell differentiation.

SFTPC Mutations: Cell Culture Data

Trafficking of SP-C proprotein through the regulated secretory pathway is completely dependent on a 6 amino acid targeting motif encoded in the cytosolic, N-terminal propeptide *(114, 144)*. While the C-terminal propeptide was shown to be dispensable for trafficking *(114)*, mutations in this ER luminal domain, such as those resulting from *SFTPC* mutations in humans, would be predicted to result in misfolding of SP-C proprotein. The finding that conserved cysteine residues in this domain, predicted to form an intramolecular disulfide bridge, were required for deployment of the proprotein from the ER was consistent with this hypothesis *(145)*.

Data from several studies have demonstrated that heterologous expression of the *SFTPC* index mutation, SP-C$^{\Delta exon4}$, in primary mouse type II cells or in various cell culture lines resulted in (1) incomplete processing of the mutant proprotein, (2) trapping of the mutant proprotein in the ER, (3) a dose-dependent induction of the UPR/ISR (specifically BiP, IRE-1/XBP-1, and HedJ1), (4) rapid degradation via ERAD, (5) aggresome formation, (6) proteasome dysfunction, and (7) activation of apoptosis pathways *(146–149)*. SP-C$^{\Delta exon4}$ was also shown to associate with and re-route wild-type SP-C for proteasomal degradation, demonstrating a dominant negative effect of the mutant proprotein as predicted from patient data *(148)*. Collectively, these data clearly demonstrate that the SP-C$^{\Delta exon4}$ mutation results in a misfolded proprotein, ultimately leading to proteasome dysfunction and cell death.

Current data suggest that the SP-C^{L188Q} mutant behaves similarly to the Δexon4 mutant. Immunohistochemistry performed on biopsy samples from a SP-C^{L188Q} patient showed diffuse, cytoplasmic staining for proSP-C as opposed to the punctate staining pattern observed within normal lung tissue, suggesting that, similar to the Δexon4 mutant, the L188Q mutant is also trapped in proximal compartments of the secretory pathway *(44)*. In the same study, stable expression of the SP-C^{L188Q} mutant in a mouse lung epithelial cell line resulted in cytotoxicity. Microarray experiments performed on HEK293 cells transiently expressing SP-C$^{\Delta exon4}$ or SP-C^{L188Q} invoked nearly identical transcriptional responses, including induction of several known components of the UPR pathways (J.P. Bridges and T.E. Weaver, unpublished observations).

Misrouting of the proprotein in transfected cells has been reported for two separate *SFTPC* mutations, SP-C^{E66K} and SP-C^{I73T}. Distinct from SP-C^{wt}, which trafficked to lysosomes, heterologous expression of these two mutants in A549 cells resulted in co-localization to early endosome antigen (EEA)-1-positive vesicles *(33, 43)*. ProSP-C-positive intracellular aggregates were also seen in lung tissue samples from a patient with the E66K mutation, but not in A549 cells transfected with the E66K mutant *(43)*. Localization of E66K and I73T in post-ER compartments suggests that both of these mutants pass ER quality control and thus are not deemed terminally misfolded. However, it is possible that these mutants fail Golgi quality control mechanisms and are not permitted to traffic to multivesicular/lamellar bodies *(150)*. Whether localization of the

E66K and I73T mutants to EEA-1-positive vesicles is due to Golgi quality control failure, direct targeting or recycling from the plasma membrane is an interesting question that requires further investigation.

Based on the differential localization patterns of the SP-C mutants in transfected cells, Beers and Mulugeta have proposed a classification scheme that groups the *SFTPC* mutations with respect to position and phenotype. The authors propose that mutations in the BRICHOS domain of the proprotein (residues 94–197), such as Δexon4 and L188Q, result in aggresome formation and cytotoxicity, while mutations outside of this domain, such as E66K and I73T, do not from aggresomes and result in a distinct phenotype *(46)*. The mechanisms underlying the apparent heterogeneity of phenotypes caused by distinct mutations in the C-terminal peptide of the SP-C are currently unknown and likely to be clarified with the generation of transgenic mouse models expressing these SP-C mutations.

Adaptation of Type II Cells to Misfolded SP-C?

The incompletely penetrant phenotype combined with the marked variability in severity and age of onset of lung disease in the SP-C^{L188Q} and SP-C^{I73T} pedigrees suggested that genetic modifiers and/or environmental insults may be involved in triggering the onset of IIP *(34, 44, 100)*. Contrary to data obtained from transiently transfected cells, constitutive expression of SP-$C^{\Delta exon4}$ in a clonal cell line did not result in UPR activation, consistent with an adaptive cellular response to the chronic expression of misfolded SP-C *(147)*. These stably expressing SP-$C^{\Delta exon4}$ cells adapted via an NF-κB-dependent manner and demonstrated an increased susceptibility to cell death upon infection with respiratory syncytial virus (RSV). These results suggest that type II cells expressing SP-C mutations may adapt to the chronic ER stress imposed by misfolded SP-C, conferring resistance to interstitial lung disease while environmental insults, such as viral infection, may trigger the onset of disease in patients with mutations in *SFTPC*.

SFTPC Mutations: Transgenic Mouse Data

Although the trafficking patterns of and cellular responses to misfolded SP-C in cultured cells are well documented, to date only two studies have been published reporting the consequences of expressing mutant SP-C in vivo. Constitutive expression of SP-$C^{\Delta exon4}$ in type II cells of transgenic mice was associated with accumulation of SP-$C^{\Delta exon4}$, caspase 3 activation, cytotoxicity, and lung dysmorphogenesis, culminating in neonatal lethality *(146, 147)*. Of note, type II cells expressing the transgene exhibited cell swelling and sloughing from the underlying basement membrane *(146)*, similar to that seen in a patient with the L188Q mutation *(44)*. Since degradation of SP-$C^{\Delta exon4}$ is proteasome-dependent, it is likely that high expression levels of SP-$C^{\Delta exon4}$ in fetal type II cells of transgenic mice saturated the degradative capacity of the proteasome, resulting in apoptosis/necrosis of distal epithelial cells and altered lung morphogenesis. Lower levels of mutant SP-C proprotein may cause a milder phenotype leading to postnatal IIP observed in human patients; this hypothesis remains to be tested.

Role of AEC Dysfunction/Apoptosis in Pathogenesis of Fibrosis

Data implicating type II cell apoptosis in the pathogenesis of pulmonary fibrosis have been reported in both patient samples and rodent models. For instance, ultrastructural evidence of apoptosis has been observed in epithelial cells of both normal alveoli and in those overlying fibroblastic foci of patients with IPF *(151, 152)*. Increased expression of pro-apoptotic/decreased expression of anti-apoptotic markers was also detected in alveolar epithelial cells from IPF patients *(153)* and bleomycin-treated mice *(154)*. Furthermore, direct activation of apoptosis through intratracheal administration of anti-FAS antibody induced fibrosis *(155)* while inhibition of epithelial apoptosis with pharmacologic inhibitors attenuated bleomycin-induced fibrosis in rats *(156)* and mice *(157)*.

Recent data indicate that abnormal protein trafficking and lamellar body dysfunction in type II cells may predispose individuals to pulmonary fibrosis. Hermansky–Pudlak syndrome (HPS) is a rare, autosomal recessive disease characterized by albinism, platelet dysfunction, and pulmonary fibrosis that is highly penetrant (for review see Chapter 8 by Young and Gahl in this textbook). Mutations in two genes associated with HPS, *HPS1* and *HPS2*, lead to protein trafficking abnormalities in a variety of cell types *(158)*. *HPS1* and *HPS2* mice showed an increased susceptibility to bleomycin-induced fibrosis as evidenced by decreased lung compliance and increased collagen deposition and mortality *(159)*. Interestingly, the phenotype was associated with a significant increase in apoptosis of type II cells observed as early as 5 h following bleomycin administration. These data support a causative role for type II cell dysfunction and apoptosis in the generation of fibrosis in patients with HPS.

Familial ILD has also been linked to mutations in *ABCA-3*. ABCA3 is a member of the *A*TP-*b*inding *c*assette family of multi-pass, transmembrane proteins that transport substances across cell membranes in an ATP-dependent manner (for review see Nogee et al. in this textbook). In the lung, ABCA3 is expressed in type II epithelial cells where it serves to transport phospholipids across the limiting membrane into the lumen of lamellar bodies *(160–162)*. Autosomal recessive mutations in *ABCA-3* have been linked to neonates with RDS *(163)* and in older pediatric patients with ILD *(164)*. Ultrastructural analysis of lung tissue from these infants reveals an absence of normal lamellar bodies in type II cells, indicating a role for ABCA3 in lamellar body biogenesis *(163)*. Furthermore, targeted deletion of *Abca-3* in mice causes neonatal lethality due to surfactant deficiency imparted by a complete lack of lamellar bodies and a decrease in surfactant phospholipids *(165–168)*. These data suggest that the pathogenesis of lung disease in these patients results from a loss of function rather than a dominant-negative, gain-of-function phenotype observed with *SFTPC* mutations. However, a subset of disease-linked ABCA3 mutant proteins remain trapped in the ER when ectopically expressed in cultured cells *(162, 169)*. Whether disease-linked mutations in ABCA3 also cause ER stress, proteasome dysfunction and/or type II cytotoxicity remains to be determined.

Animal Models of Pulmonary Fibrosis

The most widely utilized animal model of pulmonary fibrosis is lung injury generated by the anti-neoplastic antibiotic bleomycin. Bleomycin causes reactive oxygen species (ROS)-dependent lesions in genomic DNA, cell cycle arrest, and apoptosis.

Intratracheal administration of bleomycin in rodents induces a biphasic injurious response consisting of an early, acute edematous phase (days 1–5 post administration) and a late fibrotic phase (post-day 14). The acute phase is characterized by a robust influx of neutrophils, lymphocytes, and activated macrophages into the alveolus and disruption of the air–blood barrier, resulting in alveolar flooding *(170)*. Resolution of the acute injury is followed by a fibrotic phase involving peri-bronchial deposition of a provisional extracellular matrix, consisting primarily of type I collagen, fibrin and fibrinogen, and alveolar remodeling. Although the fibrotic component of this model has provided numerous insights into the mechanisms underlying pulmonary fibrosis, controversy exists regarding its validity as a true clinical correlate to UIP seen in humans *(171, 172)*.

Through genetic and pharmacologic approaches, several pathways linked to fibrosis in humans have been implicated in the pathogenesis of bleomycin-induced fibrosis in animal models. These include, but are not limited to, inflammatory mediators including TGF-β1, IL-13, IL-12, TNF-α, TGF-α, IL-1β, and IFN-γ *(7, 8, 12, 173–175)*; components of the fibrinolytic/coagulation system including fibrinogen and urokinase *(176–179)*; matrix metalloproteinases (MMP) MMP-1,7,9; and an inhibitor of MMPs, TIMP-1 *(180–183)*. Of all the mediators of experimental pulmonary fibrosis, the one with the best characterized signaling pathway is TGF-β.

Active TGF-β1 has been detected in BALF of IPF patients *(184, 185)* and is induced by bleomycin in animal models *(186, 187)*. Pulmonary administration of TGF-β1, by adenoviral or transgenic overexpression, results in type II cell apoptosis with a subsequent fibrotic response *(188, 189)*. Mice deficient in the transcription factor early growth response (EGR)-1 are protected from TGF-β1-induced apoptosis of type II cells and the subsequent development of fibrosis *(190)*. Furthermore, TGF-β1-induced fibrosis is dependent upon integrin $\alpha_V\beta_6$ which is primarily expressed in epithelial cells of the lung and is required for activation of latent TGF-β1 *(190)*. It is important to note that while $\alpha_V\beta_6^{-/-}$-deficient mice are protected from TGF-β1-induced fibrosis, pulmonary inflammatory infiltrates are increased 2- to 4-fold, demonstrating a disconnect between excessive inflammation and fibrosis in this experimental model *(17, 191)*. It remains to be determined if active TGF-β1 plays a role in fibrosis associated with *SFTPC* mutations.

Emerging Concepts: Role of Epithelial-to-Mesenchymal Transition (EMT) in Pulmonary Fibrosis

EMT is a process critical to metazoan embryogenesis and has also been implicated in the development of cancer and the fibroses of numerous tissues including heart, liver, kidney, and lung *(192)*. It has recently been demonstrated that type II cells undergo EMT in patients with IPF and in TGF-β1 and bleomycin models *(193–196)*; these cells extinguish expression of the distal epithelial marker SP-C, commence expression of myofibroblast markers including vimentin and α-smooth muscle actin, and increase type I collagen deposition. Furthermore, rodent type II cells are driven to a myofibroblast phenotype when plated on fibrinogen or fibrin, both of which are abundant components of the provisional matrix found in IPF patients *(193)*. Thus, it appears that epithelial cells contribute to the myofibroblast population during fibrosis of the lung. It remains to be seen if EMT is occurring in patients with *SFTPC* mutations and to

what extent this process contributes to disease pathogenesis. The generation of animal models will certainly allow this area to be explored.

Possible Treatment Modalities for Patients with SFTPC Mutations

Processing enzymes for the SP-C proprotein reside in the multivesicular and lamellar bodies of the type II cell. Since the majority of the identified mutations map outside of the bioactive mature peptide, treatments that facilitate trafficking of the misfolded SP-C proprotein to these distal compartments may result in proper processing of the mutant SP-C proprotein, thereby reducing or eliminating the ER stress imposed by particular mutations such as L188Q or Δexon4. Efficacy for this strategy has been shown for the most common misfolded mutant of the cystic fibrosis transmembrane conductance regulator (CFTR), ΔF508 *(197, 198)*. Two pharmacologic agents that may prove useful in treating patients with *SFTPC* mutations include 4-phenylbutyrate (PBA) and hydroxychloroquine.

PBA is a low molecular weight fatty acid that has been shown to function as an ammonia scavenger, glutamine trap, histone deacetylase (HDAC) inhibitor, and a chemical chaperone. PBA is FDA-approved to treat urea cycle disorders in children and has been tested to treat sickle cell disease, thalassemia, cystic fibrosis, and a subset of cancers *(199 – 202)*. Owing to its chaperone function, PBA attenuated misfolding of the PiZ variant of alpha-1 antitrypsin (A-1AT) and CFTR ΔF508 in cultured cells and mice *(197, 203)*. PBA treatment also attenuated aggregation of SP-C$^{\Delta exon4}$ proprotein in cultured cells *(148)*. Furthermore, PBA reduced ER stress induced by misfolded insulin and restored glucose homeostasis in a mouse model of type 2 diabetes *(204)*, supporting the therapeutic potential of this drug for protein misfolding diseases. Indeed, PBA was shown to partially restore CFTR function in a short-term phase I/II study of ΔF508 patients *(200)*.

Hydroxychloroquine is a commonly used anti-malarial drug that is also used to treat autoimmune disorders such as systemic lupus erythematosus and rheumatoid arthritis *(205, 206)*. Hydroxychloroquine is a tertiary amine that accumulates in and increases the pH of acidic subcellular organelles including lysosomes, multivesicular bodies, and lamellar bodies (lysosomal-related organelles) of type II cells *(207)*. Studies performed in isolated type II cells demonstrate that the processing of wild-type SP-C is inhibited in the presence of chloroquine, a derivative of hydroxychloroquine *(208)*. Rosen and Waltz have reported improved lung function in a pediatric patient with a *SFTPC* mutation following administration of hydroxychloroquine *(41)*. The patient was diagnosed with NSIP at 5 months of age and subsequently found to have a missense mutation in *SFTPC*, leading to an alanine-to-aspartate substitution at amino acid 116 (A116D). While the mechanism underlying improved lung function is currently unknown, this study, in addition to those reporting the efficacy of PBA in treating protein misfolding diseases, suggests that PBA and/or hydroxychloroquine may be viable treatments for patients with *SFTPC* mutations.

Conclusion

SP-C is a surfactant-associated protein that is essential for the reduction in surface tension at the air–liquid interface within the alveolus and the prevention of end-expiratory

alveolar collapse. Because of its hydrophobic properties, SP-C is synthesized as a proprotein that is processed within the secretory pathway as it is conducted to the lamellar body, the intracellular storage site for surfactant. The carboxy terminus appears to be an intramolecular chaperone that guides posttranslational processing of the SP-C protein. Mutations within the SP-C gene, especially involving the C-terminus, are associated with diffuse parenchymal lung disease. Over 50 distinct *SFTPC* mutations have been identified and many are associated with sporadic and familial pulmonary fibrosis. Although the precise mechanisms by which these mutations cause lung fibrosis are not known, they may cause protein misprocessing within the endoplasmic reticulum activating the unfolded protein response, proteasome dysfunction, and cell death. Interestingly, constitutive expression of one of the more common SP-C mutations, SP-C$^{\Delta exon4}$, stimulates an adaptive cellular response. However, these cells are significantly more susceptible to viral infection. Thus, alveolar type II cells expressing SP-C mutant proteins may be more susceptible to environmental factors that may trigger epithelial cell injury, death, and the development of parenchymal fibrosis.

The histopathological pattern associated with SP-C mutations varies widely. NSIP with features of PAP occurs commonly in children whereas UIP is the most frequent pattern in adults. SP-C mutations have been associated with sporadic and familial pulmonary fibrosis. In familial fibrosis, the pattern of inheritance appears to be autosomal dominant with variable penetrance. Many children are believed to have an antecedent infection and most adults are smokers. Clinical presentations vary from fulminant respiratory failure to a complete absence of clinical symptoms. Radiographic imaging studies, especially high-resolution thin section computed tomography, may reveal pre-symptomatic abnormalities including ground glass opacifications. Failure to thrive, dyspnea, cyanosis, and respiratory failure is the most common symptoms in children whereas dyspnea, cough, and clubbing occur frequently among adults.

Thus, there is diverse phenotypic variation among individuals with mutations within the SP-C gene. The cause of the broad variation in histopathologic pattern is not known. Other genes or environmental factors may affect the pathogenic processes activated by the production of mutated SP-C proteins or these various histopathologic patterns may represent different stages of an evolving pulmonary process.

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7

Hereditary Haemorrhagic Telangiectasia

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Abstract Hereditary Haemorrhagic Telangiectasia (HHT, Osler–Weber–Rendu syndrome) exemplifies diseases which have catalysed advances in the understanding of fundamental pathophysiological mechanisms. The hallmark of HHT is the development of abnormal blood vessels, involving the lung in approximately 50% of cases. This chapter will focus on the molecular mechanisms that underlie their generation. While not all clinical problems in HHT can be directly attributed to the presence of abnormal vessels, the emergent data suggesting non-vascular sequelae of the underlying gene mutations are beyond the scope of this chapter.

Keywords: Osler-Weber-Rendu, haemoptysis, epistaxis, arteriovenous malformation, endoglin, angiogenesis

Clinical HHT

HHT was first described in the nineteenth century as a familial disease characterised by anaemia, severe recurrent nose bleeds, and gastrointestinal blood loss *(1, 2)*. It was noted that skin lacerations or tooth extractions did not result in significant haemorrhage. This important observation allowed the distinction from haemophilia and proposal that abnormal blood vessels that were visible on mucous membranes (Figure 7.1a) were responsible for the observed bleeding tendency *(1)*. In addition to the originally described complications, in the early twentieth century, reports emerged of HHT-affected individuals developing abnormal vascular structures at other sites, particularly arteriovenous malformations (AVMs) of the pulmonary *(3)*, hepatic *(4)*, and cerebral circulations (Figure 7.1b and Table 7.1). These were recognised to place the affected individual at risk of life-threatening complications such as stroke and liver failure, although for HHT patients who do not present spontaneously to medical

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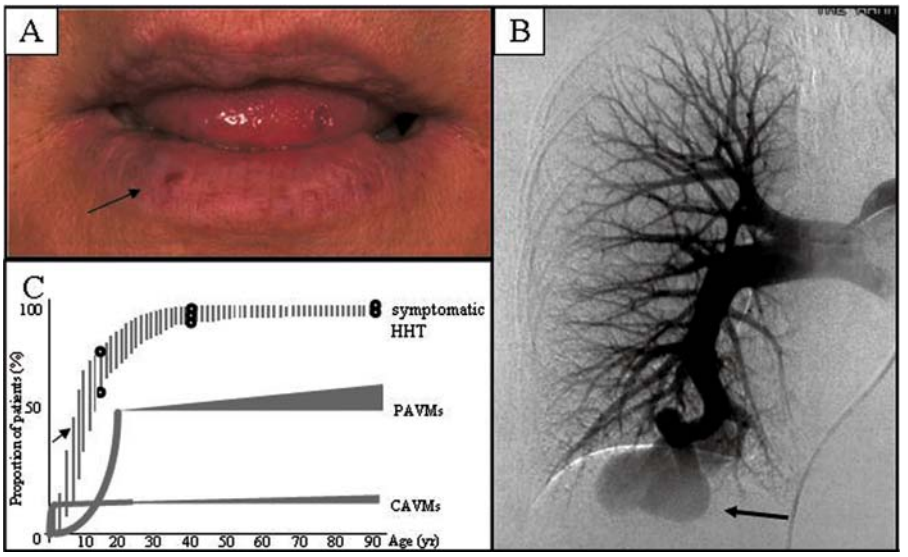

Figure 7.1 Typical manifestations of HHT. (**a**) and (**b**) Mucocutaneous telangiectasia (examples arrowed) on (**a**) the lips and tongue and (**b**) in the large bowel. (**c**) Pulmonary arteriovenous malformation in the right lower zone (angiogram courtesy of Dr James Jackson)

practitioners after the age of 60 years, there is little or no excess mortality *(5–7)*. The opportunity of presymptomatic AVM treatments led to the incorporation of diagnostic screening programmes into the management of HHT families. Such programmes have better delineated the frequency of AVMs; increased the overall frequency of HHT (from 1–2 per 100,000 to 2–4 per 10,000 *(6–9)*) and highlighted that prior to screening, the majority of affected individuals are unaware of their HHT diagnosis *(10)*. Useful recent reviews include *(11–14)*.

Lung Disease in HHT

HHT affects the pulmonary vasculature through the generation of pulmonary AVMs (in approximately 50% of cases *(16)*) and less commonly, pulmonary hypertension (see below). There is no known effect on lung parenchyma or airways, and spirometric tests and lung volumes are normal in the absence of independent pathology *(17, 18)*.

Pulmonaryarteriovenous malformations (PAVMs) range in size from large complex structures consisting of a bulbous aneurysmal sac between dilated feeding arteries and draining veins, to dilated smaller vessels, to communications within the microvasculature (telangiectasia) *(19)*. PAVMs provide a direct capillary-free communication between the pulmonary and the systemic circulations. Pulmonary arterial blood passing through these right-to-left (R–L) shunts cannot be oxygenated leading to hypoxaemia. Unexplained and often profound hypoxaemia is the hallmark of large PAVMs, but recent data confirm that most patients with clinically significant PAVMs do not have respiratory symptoms or profound hypoxaemia *(10)*. In such patients, PAVMs are not benign. The absence of a filtering capillary bed allows particulate matter to reach the systemic circulation where it impacts in other capillary beds, including the cerebral circulation

Table 7.1 Clinical features of HHT.

Feature	Approximate frequency (%)	Main complication(s)	Comments
Nasal telangiectasia	90	Nose bleeds	Usually earliest symptom, developing in childhood. Most are affected by frequent recurrent nosebleeds at some stage in life
Mucocutaneous telangiectasia	80	Cosmetic appearance	Occasionally bleed on lips and tongue
GI telangiectasia	25	Chronic > acute GI bleeds	Complications increase with age
Pulmonary AVMs	50	(a) R–L shunting (b) Haemorrhage (rare)[a]	R–L shunts lead to hypoxaemia and paradoxical embolic strokes.
Cerebral AVMs	10	(a) Haemorrhagic stroke (b) Epilepsy/ headaches[b]	Frequency of haemorrhage may be less than in general population
Hepatic AVMs	30	Left to right shunt[c]	Usually asymptomatic. May develop high output heart failure
Spinal AVMs	<1	Haemorrhage	Unknown

Legend: Current international diagnostic criteria based on (1) spontaneous recurrent nosebleeds, (2) mucocutaneous telangiectasia, (3) visceral involvement (lung, gastrointestinal (GI), liver, brain, and spinal), and (4) an affected first degree relative *(15)*. A definite diagnosis of HHT is made in the presence of three separate manifestations.

[a]Rare outside of pregnancy.

[b]Due to space occupying lesion.

[c]Hepatic artery to hepatic vein.

resulting in embolic cerebrovascular accidents (CVA) and brain abscesses. The incidence of major, usually neurological complications approaches 50% with 10% cerebral abscess and 27% embolic stroke or transient ischaemic attack recorded in all series. Recent data demonstrate that these risks are essentially independent of PAVM size and symptoms *(10)*. Additional complications of PAVMs include haemorrhage (which may be life-threatening, particularly in pregnancy *(20)* and migraine *(21)*).

Pulmonary Hypertension in HHT

Pulmonary hypertension has been recognised in a number of HHT patients, although the overall prevalence is low, as demonstrated in two separate populations: a group of 143 PAVM/HHT patients undergoing PAVM embolisation *(22)* and 68 HHT patients from a separate HHT population examined by echocardiography *(23)*. Two forms of pulmonary hypertension predominate in HHT: a true pulmonary arterial hypertension (PAH) phenotype *(24)* and a postcapillary pulmonary hypertension occurring in the context of high output cardiac failure secondary to hepatic AVMs, a potentially reversible form of

PH *(25)*. For further explanation, the interested reader is referred to recent manuscripts providing primary data *(22)* and circulatory illustrations *(11)* of these phenotypes.

Variable Expression of HHT

The hallmarks of clinical HHT are variability between

- the same individual at different ages due to age-related penetrance *(26)*;
- different vascular beds in the same individual at any one time;
- different affected members of the same HHT family.

Manifestations of HHT are not present generally at birth, but develop with increasing age such that nose bleeds are usually the earliest sign of disease, often occurring in childhood, pulmonary AVMs becoming apparent from puberty, with mucocutaneous and gastrointestinal telangiectasia developing progressively with age (Table 7.1; Figure 7.1c). Data suggests that by the age of 16 years, 71% of individuals will have developed some sign of HHT, rising to over 90% by the age of 40 years *(26–28)*. The intra-individual and intra-familial variation often exceeds inter-familial differences and remind of the need to understand pathology not just at one time point and in one genetic background, but at different time points and in the setting of different genetic and environmental modifiers of disease.

Structural Basis of HHT

Classical Telangiectasia and AVMs

The earliest histological studies demonstrated dilated, thin-walled vessels *(1, 29)* now recognised as pathognomonic for HHT. Dilated feeding arteries and dilated veins are characterised by walls of varying degrees of thickness even over relatively short segments, with disorganised adventitia. Medial thinning is observed, but also prominent are areas of focal thickening with abundant elastin tissue and a varying contribution of smooth muscle cells *(30–32)* (Figure 7.2a). While there are numerous other causes of inherited and acquired telangiectasia *(33)*, a feature relatively unique to HHT telangiectasia is the high frequency of direct arteriovenous communications, even in the smallest pinpoint telangiectasia *(34, 35)*. Unusual stress fibres in the mural pericytes *(34)* suggest increased and turbulent blood flow through the dilated HHT structures. In turn, this is thought to render endothelial cells in the telangiectasia more prone to damage as exhibited by intimal proliferation (Figure 7.2b) and haemorrhage.

Non-criterion Manifestations of HHT

While individuals with HHT can by chance inherit or develop other conditions, two diseases, pulmonary hypertension (discussed above) and juvenile polyposis, appeared more common than expected by chance association. Both primary arterial hypertension and juvenile polyposis can arise as a direct consequence of mutations in different HHT genes, apparently independently to AVMs and/or telangiectasia. Additional HHT phenotypes including prothrombotic states *(36)*, immune system disturbances *(37)*, and potential reduction in ischaemic heart disease frequency *(38)* are being examined to assess if these may be direct consequences of HHT gene mutations. These and other potential disease associations will not be addressed specifically by this chapter.

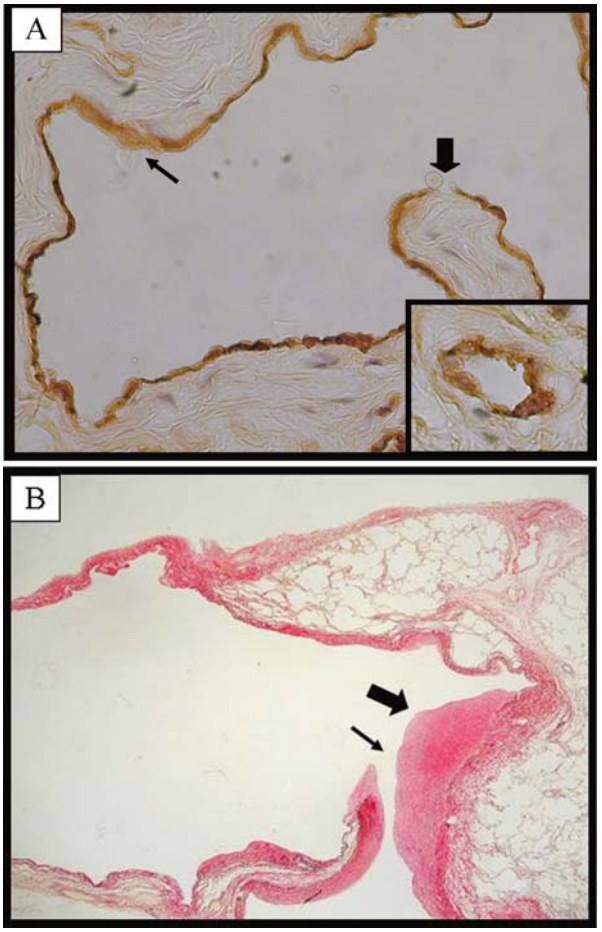

Figure 7.2 Microscopic appearances of HHT vascular lesions in man. (**a**) Telangiectasia in the inner cheek of HHT patient (main image) compared to control at same magnification (*inset*). Note thinned endothelial cell wall (*narrow arrow*) and point of rupture (*black arrow*). (**b**) Pulmonary AVM which ruptured during pregnancy. Note point of rupture (*narrow arrow*) at lower border and region of endothelial intimal fibrous proliferation (*thick arrow*). Reproduced from reference 20, Shovlin et al. , Estimates of maternal risks of pregnancy for women with hereditary haemorrhagic telangiectasia (Osler-Weber-Rendu syndrome): suggested approach for obstetric services, BJOG 2008, Wiley-Blackwell

Genetic Basis of HHT

HHT is inherited as an autosomal dominant trait. Heterozygotes almost exclusively account for the patient population: there are very few reports of probable homozygous cases *(39–41)* and evidence of homozygous lethality *(42)*. Essentially indistinguishable forms of HHT arise from mutations in at least five autosomal genes (Table 7.2). HHT types 1 and 2 have been recognised for more than a decade, and two further loci for pure HHT (*HHT3* and *HHT4)* are awaiting identification of the causative genes. HHT is also caused by mutations in *MADH4* on chromosome 18 *(43)* when it is usually associated with juvenile polyposis.

Table 7.2 HHT genes and loci.

HHT type	OMIM classification	Chromosome	Mutated gene	Mutated protein	Primary reference
HHT1	#187300	9	*ENG*	Endoglin	*(44)*
HHT2	#600376	12	*ACVRL1*	ALK-1[a]	*(45)*
JPHT	#175050	18	*MADH4*	SMAD4	*(43)*
HHT3	%601101	5	?	?	*(46)*
HHT4	%610665	7	?	?	*(47)*

[a]Activin receptor-like kinase-1, ?– to be confirmed

HHT Gene Mutations

All genotyping series indicate that the majority of HHT patients (>80%) will have mutations in either *ENG* or *ACVRL1*. More than 600 different mutations have been found in these two genes in HHT families (see www.hhtmutation.org). Neither gene displays a common mutation; and the majority of mutations have been reported only once (Figure 7.3a). In both *ENG* and *ACVRL1*, the full range of mutational types including in-frame and out-of-frame deletions, insertions, splice site, nonsense, and missense mutations are found.

ENG (Endoglin) Mutations

The prevailing view from human studies is that the primary molecular mechanism of HHT1 development is via the generation of an *ENG*-null allele: First, the predominant mutation type (77%) results in a frameshift or premature codon (Figure 7.3Ai), demonstrated in many cases to result in lack of functional protein either due to absent mRNA (presumed secondary to nonsense mediated decay *(48)*) or aberrant protein trafficking *(49)* and ultimately reduced protein expression compared to controls *(50–53)*. Similar mechanisms were also demonstrated for missense mutations *(49, 51)*. Second, as might be expected for null alleles, there is a relatively even spread of mutations, excepting the transmembrane domain and cytoplasmic tail (Figure 7.3Ai). Third, while there was a debate as to whether there may be occasional examples of mutations acting in a dominant negative manner *(52, 54)*, and soluble/truncated ENG moieties undoubtedly have this potential, dominant negative soluble endoglin generates a non-HHT phenotype, namely pre-eclampsia *(55)*. The fourth line of evidence was the absence of detectable clinical phenotype differences between HHT patients with null (absent mRNA) mutations, in-frame or out-of-frame deletions *(48)*.

Generation of a single null allele in a disease with varying sites of abnormalities immediately raised the possibility that the telangiectasia or AVMs in a particular vascular bed arose due to a second genetic hit *(56)*, analogous to cancerous processes. A second hit in the normal allele of the mutated HHT gene was demonstrated less likely for HHT1, since ENG protein can be detected in the walls of AVMs *(32)*. The possibility of alternative second hits, either in disease-modifying genes or other processes, remains.

There are now also reports of *ENG* mutations causing both pulmonary hypertension *(57, 58)* and juvenile polyposis, phenotypes initially thought to occur in association with *ACRVL1* or *MADH4* mutations, respectively. However, for juvenile polyposis, the

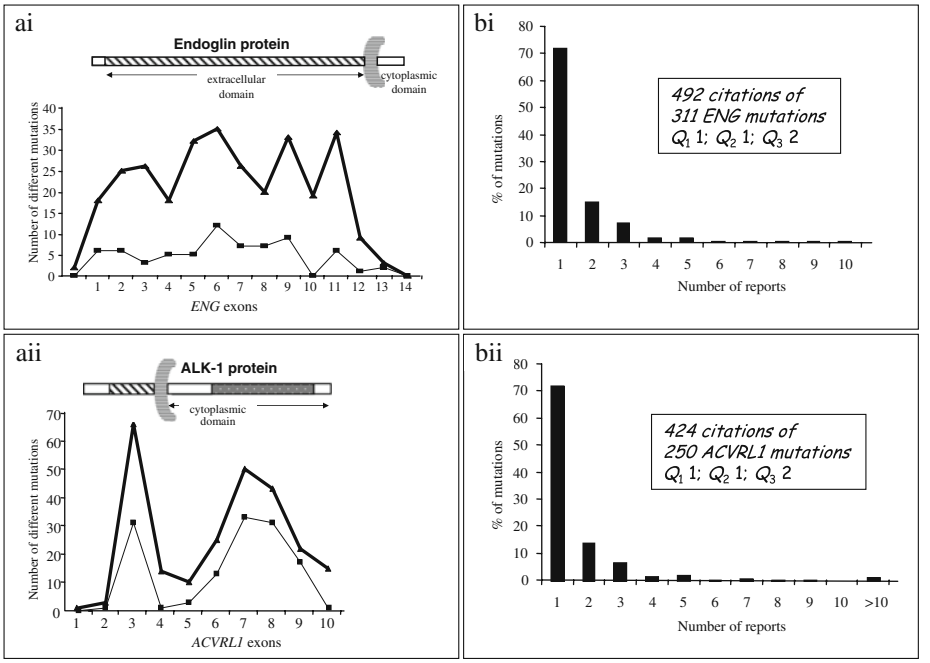

Figure 7.3 *ENG* and *ACVRL1* mutations in HHT. Data for the mutations entered on www.hhtmutation.org by February 2008. (**a**) *Mutation sites*: Representation of the site of all 916 mutations (*triangles/heavy lines*) and subset of frameshift/nonsense mutations (*squares, fine lines*) according to genomic (exons) and protein (cartoon) structures for (*i*) *ENG* /endoglin and (*ii*)ACVRL1/ALK-1. (**b**) *Mutation frequency*: The number of times each individual mutation was reported in (*i*) *ENG* and (*ii*) *ACVRL1*. The overall distribution for both ENG and ACVRL1 conformed to a one phase exponential decay (R^2 0.997)

two reported mutations result in missense substitutions, Arg571Cys and Lys513Arg, neither of which are reported in the HHT mutation or polymorphism databases.

ACVRL1 (ALK1) Mutations

The pattern of mutations reported for *ACVRL1* is very different to *ENG*. First, the proportion of frameshifts and nonsense mutations is lower (47%, Figure 7.3Aii). Second, certain exons demonstrate more mutations, particularly exon 3 (which encodes the extracellular domain), and exons 7–8 encoding parts of the kinase domain (Figure 7.3Aii). Nevertheless, the majority of mutations are thought to operate as null alleles, either due to the generation of premature termination codons and unstable mRNA/proteins resulting in reduced ALK1 protein expression *(12)* as for ENG, or due to missense mutations in residues highly conserved between species in ALK1 and other type I receptors. Such residues include Gly48 and Try50 in the extracellular domain, Ala128 in the transmembrane domain, and multiple conserved residues in the kinase domain, of which Arg374, Arg411, and Arg479 are most commonly mutated. There are no data suggesting that patients with frameshift mutations display different phenotypes to suggest an alternative dominant negative effect of truncated proteins nor of loss of the second normal *ACVRL1* allele in a second hit phenomenon.

ACVRL1 mutations may also result in pulmonary arterial hypertension in a subset of HHT-affected family members *(24, 57)*. The most common genetic cause of primary pulmonary arterial hypertension is a mutation in the *BMPRII* gene but mutations in this gene do not appear to cause HHT. There are no reports of mutations in *ACVRL1* causing juvenile polyposis.

MADH4 (SMAD4) Mutations

Recognition of a clinical association between juvenile polyposis and HHT in some families led to the identification of *MADH4* mutations as HHT-causal *(43)*. The vast majority of HHT-causing mutations are in exons 8–11 of the *MADH4* gene. This encodes the carboxy terminal MH2 domain of the SMAD4 protein which is responsible for cytoplasmic functions of the protein. Juvenile polyposis can also result from mutations in a number of other genes, most commonly *BMPR1A*, but mutations in this gene do not appear to cause HHT *(59)*.

Genotype–Phenotype Correlations

The overall proportions of HHT genotypes vary in different series between predominantly *ACVRL1* to predominantly *ENG*. Both North American and European series have demonstrated either *ACVRL1* predominance (US *(13)*; European *(60, 61)*) or an *ENG* bias (US *(62)*; European *(63, 64)*). While this may reflect genuine geographical differences in mutation distributions within the respective countries, it is important to recognise that substantial ascertainment biases were present in most series, according to the degree to which the initial recruitments included pulmonary AVM screening and treatment programmes.

All series support early observations that pulmonary and cerebral AVMs are more common in HHT1 (*ENG* mutations), and hepatic AVMs are more common in HHT2 (*ACVRL1* mutations). An important finding was that in addition to a numerical excess of AVMs, for both pulmonary and hepatic AVMs, severity as determined by size or symptoms was also more pronounced in the predisposing genotypic group. Although there was an initial suggestion that overall severity of disease is greater in HHT1 than HHT2 *(65)*, this study predated the recognition of pulmonary hypertension, and there was no difference in 90-month mortality in a later series *(64)*. It is difficult to draw conclusions regarding genotypic influence on gastrointestinal bleeding and nosebleeds, since different studies produced conflicting data.

These genotype–phenotype correlation studies suggest there may while normal function of the gene products of *ENG, ACVRL1* and *MADH4* are all required to prevent development of an HHT-like phenotype, there are likely to be differences in the normal requirements for the three proteins in different vascular beds. In addition, it appears that disparate functions of ENG, ALK1, and SMAD4 when compromised can generate the additional phenotypes of pulmonary arterial hypertension (ALK1 more commonly than ENG) or juvenile polyposis (SMAD4 more commonly than ENG).

TGF-β Family Signal Transduction Pathway

General Overview

The TGF-β superfamily consists of more than 40 members of secreted cytokines that can be classified into several groups including TGF-β, activin, growth and

differentiation factor (GDF), and bone morphogenetic protein (BMP) subfamilies *(66)*. TGF-β family proteins are involved in a diverse set of cellular, developmental, physiological, and pathological processes, including proliferation, differentiation, migration, apoptosis, inflammation, extracellular matrix synthesis, and pattern formation. They exert their effects by binding to heteromeric complexes of two types of transmembrane serine/threonine kinase receptors. The type II receptors function primarily as the binding receptors. Upon binding their ligand(s), type II receptors associate with and phosphorylate the type I receptors. Activated type I receptors propagate the signal through phosphorylation of SMAD proteins, which translocate into the nucleus, and regulate downstream target genes by interactions with other nuclear cofactors. It is noteworthy to mention that there are numerous reports describing SMAD-independent signalling pathways for TGF-β family proteins, including PI3 and MAP kinase pathways.

ENDOGLIN

ENG (CD105) is also involved in the TGF-β family signalling, although its precise roles are still elusive (for recent reviews, see *(67, 68)*). ENG is a plasma membrane glycoprotein and functions as a homodimer. As shown in Figure 7.3a, unlike ALK1 and other type I or II receptors, ENG has a relatively short intracellular tail *(69)*. This contains several phosphorylated Ser/Thr residues. Since no HHT-causing mutations have been found in the cytoplasmic domain (Figure 7.3ai), perhaps the primary function of ENG, as far as HHT is concerned, is to modulate interactions between ligands and their corresponding receptors at the cell surface.

ENG is predominantly expressed in endothelial cells (ECs) of all type of blood vessels, but also found in monocytes, mesenchymal cells in the cardiac valves, intestinal stromal cells, placental trophoblasts, and some smooth muscle cells *(70, 71)*. It is unequivocal that ENG can form heteromeric complexes with TGF-β receptors and ligands, but the roles ENG plays in signal transduction remain ambiguous. ENG complexes with TGF-β type I and II receptors *(69, 72)* and binds to TGF-β1 and β3 with high affinity *(69, 72)*. In addition, ENG interacts with other TGF-β family ligands including activin-A, BMP2, BMP7, BMP9, and BMP10 through the corresponding type II or type I receptors *(73–75)*. The *Eng*-null phenotype is morphologically very similar to that of embryos in which TGF-β type I or II receptor is deleted, suggesting that ENG might be necessary for TGF-β signalling in a specific developmental process. However, ENG does not seem to be required for TGF-β signal transduction because TGF-β signals can be transduced in numerous cell types which do not express ENG. Forced overexpression or inhibition of ENG alters cellular responses to TGF-β, indicating that ENG modulates TGF-β signals *(76)*. Data regarding the "modulatory" effect are conflicting: some literatures suggested promotion *(77)*, yet some inhibition *(76, 78)*, of TGF-β effect by ENG owing to the assay systems. To make it more complex, membrane-bound and soluble form of ENG generated by alternative splicing or proteolytic digestion may have opposing function *(79, 80)*. It is also possible that ENG may have other functions beside TGF-β superfamily signalling, such as the cytoskeletal organization affecting migration or adhesion *(81)*.

SMAD4

SMAD proteins are intracellular mediators of TGF-β family signals. Among eight known SMAD proteins in mammals, SMAD1, 2, 3, 5, and 8 are direct substrates

of activated type I receptors and thus called receptor-regulated (R)-SMADs. Depending on which R-SMADs are utilised, TGF-β signalling can be largely separated into two pathways: BMP signals via SMAD1, 5, or 8, whereas activin and TGF-β signal via SMAD2 or 3 (Figure 7.4). The BMP type II (BMPR2) and type I (ALK3 and ALK6) receptors mediate BMP signals, while the TGF-β type II (TGFBR2) and type I (ALK5) receptors mediate TGF-β signals *(82)*. Activin type II receptors (ACVR2 and ACVR2B) utilise ALK2, ALK4, or ALK7, depending on their interacting ligands, which include activins, Nodal, BMP7, GDF8, and GDF11 *(83–85)*. Once phosphorylated, all R-SMADs interact with the Co-mediator SMAD (SMAD4 in mammals) and translocate into the nucleus for regulation of downstream genes. Since binding of R-SMAD with SMAD4 is required for nuclear translocation, SMAD4-deficiency blocks all SMAD-dependent TGF-β family signalling. Inhibitory SMADs including SMAD 6 and 7 inhibit the signalling by interfering interactions of R-SMADs with their receptors or SMAD4 *(86)*.

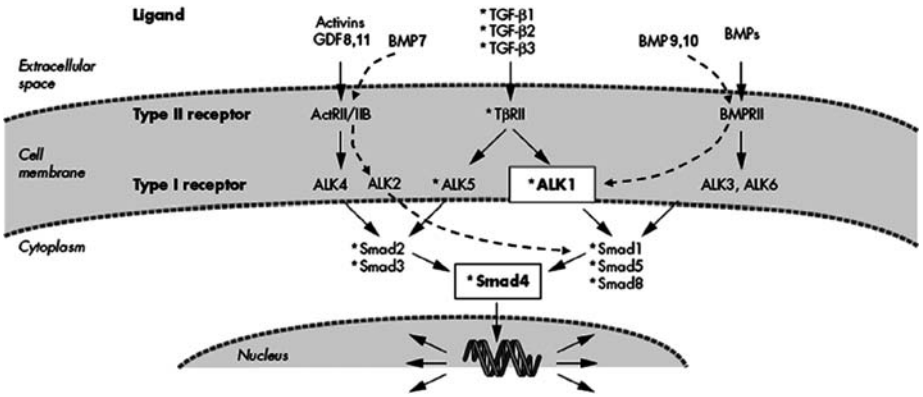

Figure 7.4 Summary of TGF-β superfamily signalling. The known HHT gene products ALK1 and SMAD4 are indicated by *boxes*. ENG is not shown, but it associates with all three groups of transmembrane signalling receptors. Well-established signalling pathways are indicated by arrows with *solid or dotted lines*. BMPs, bone morphogenetic proteins; GDF, growth/differentiation factors. *: gene excluded by linkage analyses of HHT3 family. Modified from reference 46, Cole et al., A new locus for hereditary haemorrhagic telangiactasia (HHT3) maps to chromosome 5. J Med Genet 2005 (BMJ Journals)

ALK1

ALK1 is one of the seven type I receptors *(87, 88)*. Initially, ALK1 was considered to be an orphan receptor, because its binding specificities were obscure and no specific downstream target was identified *(87)*. Although expression in various non-ECs has been reported based on immunohistochemical methods *(89)*, studies using a reporter system in genetically altered mice showed that ALK1 expression was predominantly detected in ECs, especially in the arterial ECs *(90, 91)*. Biochemical studies have shown that ALK1 can bind to a variety of TGF-β ligands, including TGF-β1, TGF-β3, activin-A, BMP-9, and BMP-10 *(74, 75, 92)*. In contrast to the signal transduction of ALK5 which activates SMAD2/3, ALK1 phosphorylates SMAD1, 5, or 8 *(93, 94)*. Since ECs in a tissue culture condition express both ALK1 and ALK5 type I receptors, TGF-β1 treatment

can activate both SMAD pathways. The hypothesis that ALK1 and ALK5 pathways may form a balance for mediating the TGF-β1 signal in ECs and that such a balance plays a crucial role for controlling angiogenesis *(94)* has been investigated by numerous approaches *(95–97)*. However, recent in vivo studies in mice and zebrafish suggested that such a balance mechanism does not play a major role in ALK1 signalling relevant to pathogenesis of HHT *(98)*.

Identity of ENG/ALK1 Ligands Pertinent to HHT: TGF-β or BMP9?

As described above, all three known HHT genes (*ENG, ALK1, and SMAD4*) interact with a diverse range of TGF-β family signals. TGF-β1 has been widely considered to be the most likely ligand relevant to HHT pathogenesis. However, a recent genetic study demonstrated that TGFBR2 (the essential type II receptor for signalling of TGF-β isoforms) is not required for ALK1 signalling, casting doubt on the long-standing presumption that TGF-β isoforms were the ALK1 ligands pertinent to HHT *(98)*. Could impaired signalling through other TGF-β superfamily ligands be associated with HHT pathogenesis? Recent studies showed that BMP9 or BMP10 can specifically bind to and signal through ALK1 and BMPR2 *(74, 75, 99)*. Identification of the physiological ligand of ENG/ALK1 relevant to HHT is crucial for studying detailed molecular mechanism underlying the pathogenesis of HHT.

Animal Models for HHT

Eng- or Alk1-Homozygous Mice

Genetic ablation of *Eng* or *Alk1* in mice resulted in embryonic lethality at embryonic day (E) 9.5–10.5 *(71, 94, 100–102)*. Gross morphological features of $Eng^{-/-}$ and $Alk1^{-/-}$ embryos were very similar: lack of mature yolk sac blood vessels at E9.5 and growth arrest with enlarged pericardium at E10.5. In both *Eng* and *Alk1* mutant embryos, vascular smooth muscle cell (VSMC) failed to completely encase the developing dorsal aorta, indicating that ENG or ALK1 is required for differentiation or migration of VSMC *(94, 101)*. Another common phenotype is the heart defect. Both $Eng^{-/-}$ and $Alk1^{-/-}$ embryos showed markedly simplified trabeculation in the ventricles. Irregularity and hyperdilation of blood vessels and formation of AVMs are cardinal features of $Alk1^{-/-}$ embryos, which seemed to be less apparent, if not absent, in $Eng^{-/-}$ embryos, suggesting a different level of requirement of these proteins for vascular development. Gross morphology and vasculature of these mutant embryos are indistinguishable from their control littermates by E8.5, but the vascular defects become obvious by E9.5. This is a very critical period for the vitality of mouse embryos, because the fetoplacental circulatory system (placenta to embryonic heart) is established in this period. For this reason, knockout mice for a large number of genes involved in the development of heart, placenta, or blood vessels appeared to be lethal at this stage with very similar morphological phenotype. Since impaired placental development alone can lead to embryonic lethality with cardiac defects at this stage *(103)*, it is an intricate issue to determine the primary cause of the embryonic lethal phenotypes of these mutant embryos.

Eng-or Alk1-Heterozygous Mice

Since HHT is a dominantly inherited genetic disorder, and haploinsufficiency is the likely cause of associated vessel malformations, mice heterozygous for a null allele of either Eng or Alk1 were important animal models for HHT. *Eng*-heterozygous mice ($Eng^{+/-}$) exhibited various clinical signs of HHT, such as nose bleeds, telangiectasis-like dilatation of postcapillary venules, and AVMs in subdermal, liver, uterine, and cerebral vessels *(100, 104, 105)*. $Alk1^{+/-}$ mice have also shown to develop HHT-like vascular lesions in subcutaneous vessels and organs such as GI and liver *(106)*. These results confirm that haploinsufficiency of ENG and ALK1 underlies the pathogenesis of the disease.

Similar to clinical symptoms of HHT, age of onset, severity, and location of HHT-like vascular abnormalities in $Eng^{+/-}$ and $Alk1^{+/-}$ mice are highly variable, and only 30–70% of heterozygotes displayed a detectable HHT-like phenotype *(100, 104–106)*. While the heterozygous *Eng* or *Alk1* knockout mice are excellent resources for identifying genetic factors (e.g. genetic modifiers or loss of heterozygosity) or environmental factors (e.g. inflammation, infection, or wound) that influence the disease manifestations, there are practical problems to utilise these heterozygous mice for studying molecular pathogenetic mechanisms for the vascular malformations, owing to a high variability of phenotype, unpredictable onset and location of vascular lesions, and strain dependence (in the case of *Eng*, requiring strain 129/Ola with poor female fecundity).

Conditional Knockout Mice for Alk1, Eng, and SMAD4 Genes

Since *Eng*-, *Alk1*-, and *SMAD4*-null mice are embryonic lethal, and heterozygous mouse models may be too unpredictable for study, the conditional knockout approach using the Cre/LoxP system has been undertaking to determine the precise function of these genes in specific cell types. A prerequisite for this approach is the availability of conditional knockout allele for each gene, in which the LoxP sequences are inserted into a target locus to flank a crucial region of the gene. Such conditional knockout alleles for all three HHT genes have been successfully generated *(107–109)*, and exciting data from these mice are forthcoming. Recently it was demonstrated that endothelial-specific deletion of the *Alk1* gene resulted in vascular malformation in the yolk sac and fetal lungs (Figure 7.5) *(107)*. These mutant vessels displayed the hallmarks of HHT vascular phenotypes – dilation of lumen, thinning of vascular walls, loss of capillaries, development of excessive tortuous vessels, and AVMs. Unlike $Alk1^{+/-}$ or $Eng^{+/-}$ mice, the HHT-like vascular malformations occurred in a consistent and predictable manner with 100% penetrance in this conditional knockout model. Therefore, the endothelial-specific *Alk1*-conditional knockout mice will be a valuable resource for identification of key molecular pathways involved in the initiation and progression of such vascular malformations.

Implicated Roles of ENG and ALK1 in the Pathogenesis of HHT

While pathogenetic mechanism for HHT remains to be determined by models discussed in preceding sections, we would like to speculate on three pathophysiological areas that might be linked to pathogenesis of HHT: dysregulation of angiogenesis, perturbation of arterial/venous identity, and endothelial dysfunction.

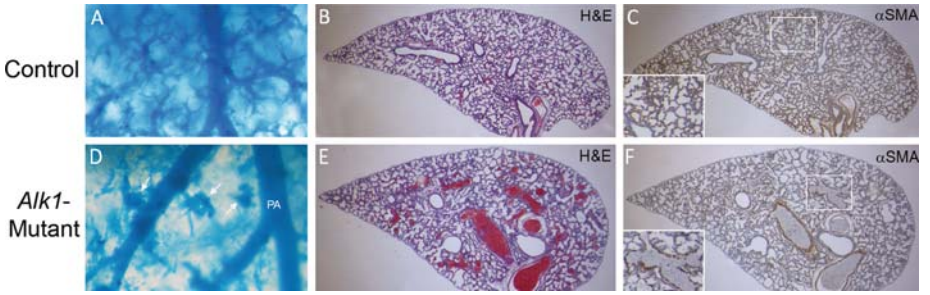

Figure 7.5 Abnormal pulmonary vasculature in *Alk1*-deficient mice. Latex dye injected into the right ventricle displays AVM-like abnormal vascular nodules in mutant postnatal day 3 (PN3) mice (**d**, *arrows*), while it is evenly perfused in control littermates (**a**). H&E staining (**b**, **e**) and immunostaining with alpha-smooth muscle actin (αSMA) (**c**, **f**) demonstrate dilated and disorganised vascular network in the mutant lungs (**e**, **f**). Smooth muscle layers are generally thin, irregular, and discontinuous in AVM-like vascular lesions (*inset*, **f**)

Dysregulation of Angiogenesis

Angiogenesis refers to the process by which new vessels form by sprouting or splitting from pre-existing vessels or from bone marrow endothelial progenitor cells (for recent reviews, see *(110, 111)*). Angiogenesis can be separated into two distinct phases: activation and resolution phases *(112)*. In the activation phase, ECs degrade basement membranes, migrate into extracellular space, proliferate, and form vascular lumens. In the resolution phase, ECs cease migration and proliferation, reconstitute basement membrane, and build up perivascular cell layers. Precisely coordinated regulation between the activation and the resolution phases is essential for development of healthy and functional blood vessels.

It has been speculated that dysregulation of these angiogenic processes is a cause of vascular malformations occurring in HHT. With this regard, numerous attempts have been made to examine whether the signalling through ENG/ALK1 promotes the activation or the resolution phase. In other words, it has been investigated whether under-(or over-)expression of ENG or ALK1 can impact on proliferation, migration, or tube formation of cultured ECs. This seemingly simple and straightforward experimental scheme contains multiple caveats for taking the results from these experiments into consideration as pathogenetic mechanism for HHT. The most concern is the ambiguity of the physiological ligand for these receptors. To overcome this issue, some studies were performed with constitutive active (ligand independent) form of the receptor. Since the constitutive active (ca) receptor activates downstream targets common for several other TGF-β ligands which may be irrelevant for HHT, however, interpretation of the data is complicated. Furthermore, in vitro data often differ from one another depending on culture conditions. For instance, overexpression of caALK1 could inhibit *(97, 113)* or promote *(95)* the proliferation and migration of ECs and that *Eng*-deficient ECs could either enhance *(78)* or inhibit *(77, 114)* EC proliferation upon TGF-β1 treatment.

Several lines of in vivo and clinical data suggest that ENG/ALK1 signalling promotes the resolution phase of angiogenesis, and thus an impaired ENG/ALK1 signalling may result in shifting the balance to the activation phase. First, both *Eng* and *Alk1*-null embryos showed defects in normal development of perivascular layers *(94, 101)*. Second, in zebrafish *alk1* mutants which closely resemble *Alk1*-null mouse embryos, the

dilated blood vessels contain more than twice as many ECs as their wild-type counterparts, suggesting that a blockade of ALK1 signalling results in enhanced proliferation of ECs *(115)*. Third, several marker genes of the activation phase including vascular endothelial growth factor (VEGF) were elevated in *Alk1*-null embryos *(94)*. Fourth, serum VEGF levels were shown to be elevated in HHT patients *(116, 117)*. Lastly, VEGF induces abnormal microvessels in the $Eng^{+/-}$ mouse brain but not in the brains of wild-type animals *(118)*. Recently a clinical case showing that bevacizumab (anti-VEGF antibody) treatment reversed liver AVMs in a HHT1 patient *(119)* received much attention from the HHT community. Further investigation on a larger HHT patient pool would provide insights on whether the anti-angiogenic therapy would be a therapeutic option for some severe vascular malformations.

Perturbation of Arterial and Venous Identity

It has long been believed that the acquisition of arterial or venous identity occurred relatively late in embryonic vessel formation and was largely determined by different physiological parameters, such as the direction of blood flow, blood pressure, blood oxygenation, and/or shear stress. However, recent studies suggest that arterial and venous ECs have distinct molecular identities prior to patent vessel formation. The first report of this phenomenon demonstrated that Ephrin-B2 (Efnb2) was expressed only in arterial ECs, whereas EphB4 (a putative receptor for Ephrin-B2) was expressed almost exclusively in venous ECs prior to the onset of circulation *(120, 121)*. Several other artery-specific genes have been reported in vertebrate embryos, including a Notch ligand *Delta* (Dll4) *(122)* and a Notch–Delta downstream transcription factor (Gridlock) *(123, 124)*. These genes are involved in early lineage distinction between arterial and venous ECs or in segregating two vessel identities at the capillary level.

AVMs, the key feature of HHT, might be due to dysregulation of Notch–delta signalling which result in confused identity of arteries and veins and failure of segregating arteries from veins. Various mutant mice having a dysregulation of the Notch–Delta signalling displayed AVMs *(125–127)*. Alk1 is predominantly expressed in arterial ECs *(90)*. Interestingly, the arterial-specific Alk1 expression pattern becomes apparent after blood flow is established, a relatively late stage in comparison with the vessel type-specific expression patterns of Notch–Delta pathway genes and Efnb2/Ephb4 genes *(91)*. Although no functional interactions between Notch and ALK1 signallings for induction of Efnb2 expression in an assay system was observed *(128)*, decreased Efnb2 expression in *Alk1*-null embryos suggests that ALK1 signalling may play an important role in maintenance of Efnb2 expression *(102)*. If this is the principal mechanism of AVMs in HHT, modulation of Notch signalling would be a therapeutic target. Since either too much or too little Notch signalling results in vascular malformation; however, it would be a challenge to find a drug which can precisely regulate the Notch signalling.

eNOS Uncoupling/Cox2

Several reports suggested that endothelial dysfunction which impacts on vascular tone is associated with HHT pathogenesis. $Eng^{+/-}$ mice showed impaired acetylcholine-dependent vasodilatory function *(129)*. This result correlates with a reduced endothelial nitric oxide synthase (eNOS) expression and impaired NO synthesis in $Eng^{+/-}$ mice *(129)*. Furthermore, ENG has been found in endothelial caveolae, where it associates

with eNOS and modulates its activation by promoting eNOS/Hsp90 association *(130)*. $Eng^{+/-}$ cells also show uncoupled eNOS activity resulting in generation of eNOS-derived superoxide (O_2^-), and treatment with an O_2^- scavenger reverses the vasomotor abnormalities in *Eng* $^{+/-}$ arteries *(130)*. Interestingly *Eng* $^{+/-}$ mice showed elevated COX-2, suggesting that ENG plays a role in the maintenance of vascular homeostasis and the fine balance between eNOS and COX-2 in ECs *(114)*. Recent gene profiling data from HHT1 and HHT2 endothelial precursor cells (EPCs) also showed an elevated Cox-2 level in HHT-EPCs *(131)*. The paradox of reduced NO production and vessel dilatation in HHT can be partially explained by vessel dilatory effect of reactive oxygen species and elevated prostaglandins. However, how the endothelial dysfunction is related to the abnormal vascular formations in HHT remains to be elucidated.

Future Directions of HHT Research

We predict that future directions of HHT research will include identification of new HHT genes, establishment of the true "HHT" ligand for ALK-1, and for patients a move towards establishment of a model system for identifying and validating therapeutic targets and translation of novel findings into preclinical trials.

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8

Hermansky–Pudlak Syndrome

Lisa R. Young and William A. Gahl

Abstract Hermansky–Pudlak syndrome (HPS) is a group of rare autosomal recessive disorders characterized by albinism and platelet dysfunction. A subset of HPS patients also develop highly penetrant pulmonary fibrosis, and some patients have a granulomatous colitis that shares features with Crohn's disease. There are at least eight genetic loci associated with HPS in humans; mutations in each HPS gene result in defects in the biogenesis of lysosomes and lysosome-related intracellular organelles including melanosomes, platelet dense granules, and lamellar bodies. Pulmonary disease manifests as a restrictive disorder with insidious dyspnea on exertion, cough, and interstitial infiltrates and can progress to respiratory insufficiency and death by the fourth or fifth decade. Radiographically, HPS lung disease shares many features with idiopathic pulmonary fibrosis (IPF). Pulmonary fibrosis in HPS has a histologic appearance resembling usual interstitial pneumonia in several respects, but is also accompanied by hyperplastic, hypertrophic alveolar type II cells containing enlarged lamellar bodies, and lipid-filled, activated alveolar macrophages. Pigment deficiencies can be quite subtle. All pulmonary fibrosis patients with albinism and a bruising or bleeding diathesis should be screened for HPS. All patients with HPS should be screened for pulmonary involvement with pulmonary function tests and chest imaging. When indicated, bronchoscopy performed by the oral route should be considered to avoid nasal bleeding. Lung biopsy is frequently contraindicated because of bleeding complications and because diagnosis and prognosis can be determined without the procedure. Currently available approaches to treatment of HPS are limited, but include smoking cessation, vaccination against pulmonary infections, and prevention and management of bleeding complications. Pirfenidone and other targeted anti-inflammatory and antifibrotic agents warrant further study. Lung transplantation is an option for HPS patients with advanced pulmonary disease. The Hermansky–Pudlak Syndrome Network, Inc. (www.hpsnetwork.org), is a support organization available for patients with HPS.

Keywords: pulmonary fibrosis, interstitial lung disease, genetic basis of disease, alveolar macrophage, alveolar type II cell

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Epidemiology

HPS was first described by the Czechoslavakian physicians Hermansky and Pudlak in 1959 *(1)*. While HPS is a rare disorder, it may be the most common single-gene disorder in northwest Puerto Rico, where 1/20 people carry the gene, the disease frequency is 1/1,800, and approximately 600 people are affected *(2)*. Virtually all of them have the same mutation in the *HPS1* gene *(3–6)*. Mutations in *HPS3* have also been identified in Puerto Rico, but are much less common *(7, 8)*.

Outside of Puerto Rico, *HPS1* mutations have been identified in approximately 40 additional patients. Nakatani et al. estimated 65 total cases of HPS in Japan *(9)*, and HPS has now been reported in individuals of almost every nationality. As of 2008, there are 784 affected individuals registered with the Hermansky–Pudlak Syndrome Network, a not-for-profit patient advocacy and support organization (Donna Appell, RN, personal communication).

Genetic Basis and Molecular Pathogenesis

In humans, eight genetic loci are associated with the autosomal recessive disorder HPS *(10)*. The existence of at least 16 genetically distinct mouse models of HPS suggests that there are additional HPS loci to be discovered in humans *(11)*. The most prevalent type of HPS is HPS1, due to mutations in a gene first identified through positional cloning of the genetic lesion shared by HPS patients from northwestern Puerto Rico *(2)*. Other HPS genes were subsequently identified through positional cloning and/or candidate gene approaches. Table 8.1 summarizes the HPS subtypes and genetic etiologies, as well as prominent clinical features. All of the known HPS genes are ubiquitously expressed, and their gene products are involved in biogenesis of lysosomes or specialized intracellular organelles that are related to lysosomes, including melanosomes and platelet dense granules *(11–13)*.

The HPS gene products associate together into one of four stable protein complexes. The best characterized is the adaptor protein-3 (AP-3) complex, which contains the product of the gene mutated in HPS-2 *(14–16)*. Sets of the other HPS gene products interact and form protein complexes termed BLOCs (biogenesis of lysosome-related organelle complexes), numbered 1, 2, or 3. BLOC1 contains the *HPS7* and *HPS8*

Table 8.1 Summary of HPS subtypes, genetic loci, and associated animal models.

Gene symbol	Mouse model	Human disease	Human chromosome locus
HPS1	Pale ear	HPS-1	10q23.1–23.3
AP3B1	Pearl	HPS-2	5q14.1
HPS3	Cocoa	HPS-3	3q24
HPS4	Light ear	HPS-4	22q11.2–q12.2
HPS5	Ruby-eye 2	HPS-5	11p15–p13
HPS6	Ruby-eye	HPS-6	10q24.32
DTNBP1	Sandy	HPS-7	6p22.3
BLOC1S3	Reduced pigmentation	HPS-8	19q13

gene products, as well as several other proteins. BLOC2 contains the products of the genes mutated in *HPS3*, *HPS5*, and *HPS6*, and BLOC3 is composed of the *HPS1* and *HPS4* gene products *(10, 17–19)*. Recent data suggest further interactions between different BLOCs, though the pathways involved may be cell-specific. For example, in melanocytes, BLOC1 and BLOC2 appear to act sequentially in the same pathway, but independent of AP-3 *(20)*. In fibroblast studies, BLOC1 apparently interacts physically and functionally with AP-3 to facilitate trafficking of cargo; it also interacts with BLOC2 in early endosome-associated tubules *(21)*.

The intracellular mechanisms of albinism and platelet dysfunction in HPS have been largely elucidated and provide clues to potential pathogenesis of HPS lung disease. HPS melanocytes are ultrastructurally normal, but contain predominantly early melanosomes or premelanosomes *(22)*. In HPS-1, hypopigmentation results from impaired translocation of tyrosinase and tyrosinase-related protein 1 (TRP1) to large granular complexes rather than melanosomes, thereby compromising melanin synthesis *(23)*. In HPS-2, only tyrosinase, not TRP1, shows an abnormal distribution *(24, 25)*.

The severity of bleeding diathesis in HPS patients varies greatly and is due to an absence of platelet dense granules, as demonstrated on whole-mount electron microscopic analysis of platelets (Figure 8.1). HPS platelets appear unable to form the dense granules or, alternatively, cannot concentrate products within them *(26, 27)*. The absence of platelet dense bodies results in a diminished secondary aggregation response, which is dependent on ATP, calcium, and serotonin stored in dense bodies *(28)*. Recent studies have shown that the contingent of alpha granules remains normal in HPS platelets *(29)*.

All HPS patients exhibit oculocutaneous albinism and the bleeding diathesis associated with absent platelet dense bodies. In addition, approximately 15% of patients have a granulomatous colitis and patients with BLOC3 defects develop pulmonary fibrosis. In the affected lungs, there are histologic cellular abnormalities in both alveolar macrophages and alveolar type II cells. Alveolar macrophages are enlarged and "foamy" in appearance and contain ceroid–lipofuscin *(30–34)*. It is hypothesized that alveolar type II cells are responsible for HPS lung disease, since lamellar bodies, con-

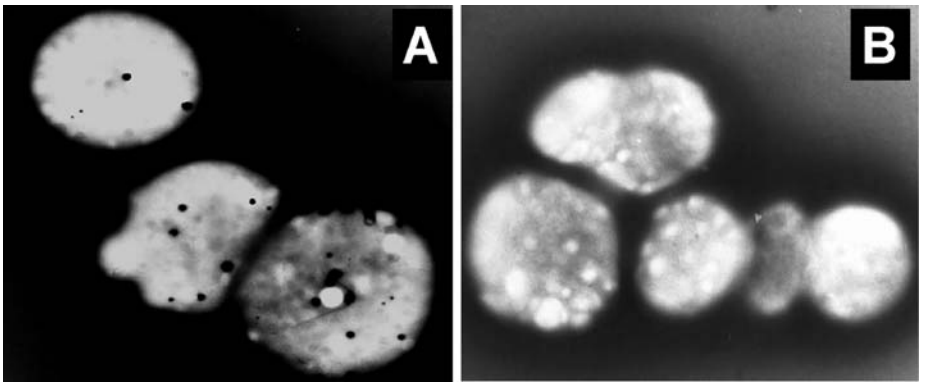

Figure 8.1 Electron micrographs of platelets from a normal subject (**a**) and from a patient with Hermansky–Pudlak syndrome (**b**). Note the absence of dense bodies in the HPS patient. Images provided by James G. White, MD, Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN

sidered to be lysosome-related organelles storing newly produced and recycled surfactant for secretion, show ultrastructural abnormalities *(9, 35)*.

The genetic and molecular bases of the eight known HPS subtypes are summarized below.

HPS-1

First identified in 1996, the *HPS1* gene spans 20 exons on 10q23.1–q23.2 and encodes a ubiquitously expressed, 700 amino acid, 79.3-kDa protein with no known function or informative homologies to other proteins *(5)*. The most common mutation, which is responsible for the Puerto Rican founder effect, is a 16-base pair duplication in exon 15 of *HPS1*; another mutation in *HPS1* has been identified in several families in the Swiss Alps *(8, 36–38)*. *HPS1* gene mutations differ considerably and include a frameshift at codon 322, frequently seen in Europeans, and at least 18 other mutations among non-Puerto Rican individuals with a similar clinical phenotype *(5, 39–41)*. The protein product of *HPS1* is contained in two distinct high molecular weight complexes distributed between uncoated vesicles, early stage melanosomes, and the cytosol.

HPS-2

HPS-2 results from mutations in the *AP3β1* gene, which encodes a protein with important functions in vesicle formation and trafficking. In fact, this protein comprises part of the heterotetrameric complex called adaptor protein-3 complex or AP-3. The function of this complex was elucidated in yeast, the *pearl* mouse, and even in *drosophila* mutants with pigment granule defects. Specifically, the *drosophila* pigmentation gene, *garnet*, encodes the β3A subunit of the AP-3 adaptor complex *(42)*. In 1998, Dell'Angelica et al. identified mutations in the gene for the β3A subunit of AP-3 in two siblings with HPS *(14)*. These and other HPS-2 patients exhibited a neutropenia in childhood, accompanied by an infectious diathesis of variable severity. The neutropenia responded to GCSF.

The cDNA for β3A predicted a protein composed of 1,094 amino acids and a mass of 140 kDa. Fibroblasts from HPS-2 patients contained markedly reduced levels of AP-3 due to enhanced ubiquitin-mediated degradation of mutant β3A and the AP-3 complex. The AP-3 deficiency resulted in inappropriate cell surface expression of the lysosomal membrane proteins CD63, LAMP1, and LAMP2, but not of nonlysosomal proteins, suggesting that HPS2 is required at an early stage of melanosome biogenesis and maturation *(14)*.

Perhaps the pathogenesis of some of the clinical manifestations of HPS-2 can be best understood in the context of known functions of the HPS2 product and the entire adaptor protein-3 complex. The AP-3 complex has been implicated in several potential mechanisms of immune recognition which may explain the increased susceptibility to infections observed in HPS-2 patients. First, AP-3 regulates the trafficking of CD1b (humans) and CD1d (mice), which are transmembrane proteins required for the presentation of mycobacterial lipid antigens to T cells *(43)*. Additionally, the AP-3 complex is involved in the movement of lytic granules of cytotoxic T lymphocytes (CTLs) to the immunological synapse. In the absence of AP-3, CTLs lose their cytotoxicity *(44)*. Finally, AP-3 appears to be critical for directing neutrophil elastase to the neutrophil granule, another lysosome-related organelle. Frameshift mutations in the Ap3b1 subunit

of AP-3 in the gray collie lead to hypopigmentation, but also cyclic hematopoiesis with neutropenia; neutrophil elastase is misdirected to a default destination at the plasma membrane *(45, 46)*. These clues provide powerful insights into the role of AP-3 in intracellular protein trafficking and may provide a foundation for understanding how abnormal intracellular protein trafficking influences type II cells and macrophages in the HPS lung.

HPS-3

Using homozygosity mapping of DNA from families of a cohort of Puerto Rican HPS patients who did not have the 16-bp duplication in *HPS1*, a new HPS susceptibility locus was identified on 3q24 (HPS3) *(7, 47)*. The gene encodes a cytoplasmic 113.7-kDa protein and consists of 1,004 amino acids, including a clathrin-binding motif and signals for targeting to lysosomal vesicles. The exact function of the HPS3 protein is unknown, though it is part of BLOC2; the HPS-3 phenotype includes milder cutaneous and ocular hypopigmentation *(8, 48, 49)*.

HPS-4

Naturally occurring mutations in mice that result in pigment dilution and platelet dysfunction have revealed an additional HPS gene, *HPS4 (50, 51)*. The gene responsible for the phenotype in the "light ear" mouse was mapped to a region of the mouse chromosome that is syntenic with human chromosome 22q11.2–q12.2. The human gene, *HPS4*, encodes a 708 amino acid protein with an apparent MW of 76.9 kDa. Of 21 unrelated HPS patients lacking the *HPS1* mutation, seven were found to have nonsense, frameshift, and in-frame insertion mutations in *HPS4*. The HPS1 and HPS4 proteins interact in BLOC3 *(50)*.

HPS-5

HPS-5 was initially described in a young child and then was further characterized in four additional patients (*52*). *HPS5* is located on chromosome 11p14, consists of 23 exons, and is expressed as at least three splice variants. Immunohistochemical studies in fibroblasts suggest that HPS5 functions in the movement of vesicles from the perinuclear region to the periphery of the cell. Another member of BLOC2, HPS-5, melanocytes resemble those from patients with HPS-3 *(53)*.

HPS-6

HPS-6 has been described in a single family. The HPS5 and HPS6 proteins interact with HPS3 as components of BLOC2 *(54)*.

HPS-7

HPS-7 has been reported in a Portuguese woman and is caused by mutation of the human ortholog of dysbindin (DTNBP1), a component of BLOC1 that interacts with the pallidin protein*55*).

HPS-8

HPS-8 was recently discovered based on identification of a homozygous frameshift mutation in *BLOC1* (subunit 3) (p.Gln150ArgfsX75) in a large family. Affected individuals displayed features of incomplete oculocutaneous albinism and platelet dysfunction *(10)*.

Heterozygotes

No clinical features have been reported with the HPS carrier state, though a recent report of a Spanish family with HPS-1 found in vitro platelet dysfunction in two asymptomatic relatives carrying only one *HPS1* mutation (insC974). Gonzalez-Conejero et al. report that these carriers had a decreased content of platelet dense granules and showed significant reductions in platelet aggregation, expression of CD63 after platelet activation, and serotonin uptake *(56)*.

Clinical Presentation and Natural History

The numerous clinical manifestations of HPS highlight the importance of HPS genes in the genesis of lysosome-related organelles.

Albinism

Albinism is generally the first recognized clinical feature of HPS. Individuals with HPS have tyrosinase-positive oculocutaneous albinism with varying degrees of hypopigmentation of the skin, hair, and irides; dark hair color and relatively normal skin pigmentation may be preserved in some cases (Figure 8.2). In addition, visual acuity can range from mildly decreased to legally blind, and ophthalmologic findings include transillumination of the iris, congenital horizontal nystagmus, strabismus, and impaired dark adaptation *(37, 57–60)*.

Bleeding Diathesis

The bleeding diathesis in HPS varies from mild to severe and may include easy bruising, epistaxis, or prolonged or heavy bleeding with menses, dental procedures, and surgeries. Hemorrhage is a common cause of morbidity in HPS patients, and serious cases have been reported with dental extractions and parturition *(61, 62)*.

Inflammatory Bowel Disease

Some individuals with HPS-1 and HPS-4 develop a granulomatous colitis that is similar to Crohn's disease *(63–70)*. The prevalence of colitis was 7% among a group of 122 HPS patients evaluated at the NIH. Of those HPS patients referred specifically for gastrointestinal symptoms, colitis was found in 33% (8/24). Colitis was found only in patients with HPS-1 and HPS-4 in the NIH cohort, but has also been reported in patients with HPS-3 *(70)*.

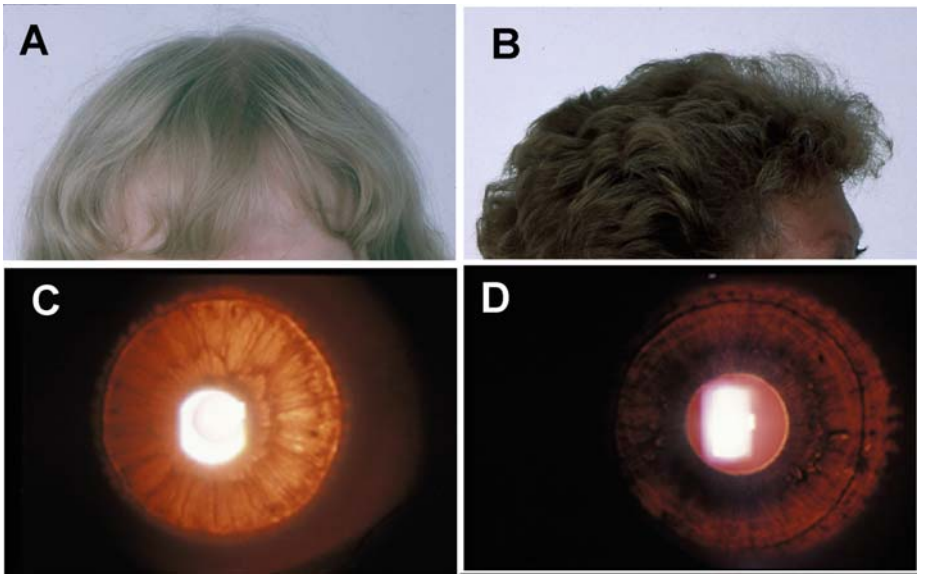

Figure 8.2 Pigmentation in patients with different HPS subtypes. In general, BLOC3 patients have more severe hypopigmentation than BLOC2 patients. (**a**) Hair of a Puerto Rican HPS-1 patient, with a BLOC3 defect. (**b**) Hair of a Puerto Rican HPS-3 patient, with a BLOC2 defect. (**c**) Severe iris transillumination in an HPS-1 patient. Images provided by Ekaternini Tsilou, MD, National Eye Institute, National Institutes of Health, Bethesda, Maryland. (**d**) Mild–moderate transillumination in an HPS-3 patient. Images provided by Ekaternini Tsilou, MD, National Eye Institute, National Institutes of Health, Bethesda, Maryland.

Interstitial Lung Disease (ILD)

ILD develops in most adults with HPS-1 or HPS-4, but has not occurred in HPS-3, HPS-5, or HPS-6 *(37, 71–73)*. Nonproductive cough and progressive dyspnea on exertion are the most common presenting pulmonary symptoms, with a mean age of onset of pulmonary symptoms of about 35 years. There is no known gender predominance. Pulmonary function tests reveal a restrictive defect, although superimposed obstructive defects have been reported in smokers. Chest radiograph patterns vary from normal to fine reticular changes to end-stage honeycombing (Figure 8.3) and bullae and bronchiectasis have been reported *(71, 72)*. Screening with high-resolution computed tomography (HRCT) scans identifies ground-glass and fibrotic changes in a majority of HPS patients, though in early cases chest radiographs may be normal *(71)*.

Because of risk associated with lung biopsy in patients with a bleeding diathesis, only limited lung tissue is available for analysis. As shown in Figure 8.4, the available lung histology suggests that HPS shares some features of usual interstitial pneumonitis (UIP), but large hyperplastic alveolar type II cells, with characteristic swelling and foamy degeneration, and lymphocytic and histiocytic infiltration of respiratory bronchioles are also present *(9, 33, 35)*. At the ultrastructural level, HPS alveolar type II cells also contain bizarre enlarged lamellar bodies *(9)*, which resemble those seen in other forms of inherited pulmonary fibrosis, such as ILD associated with autosomal recessive mutations in the gene encoding the ATP-binding cassette A-3 (ABCA3) *(74)*.

There is significant interindividual variability in the severity of pulmonary disease which is not absolutely related to age or specific mutations. Of the HPS-1 patients followed at the National Institutes of Health, some individuals had pulmonary fibrosis

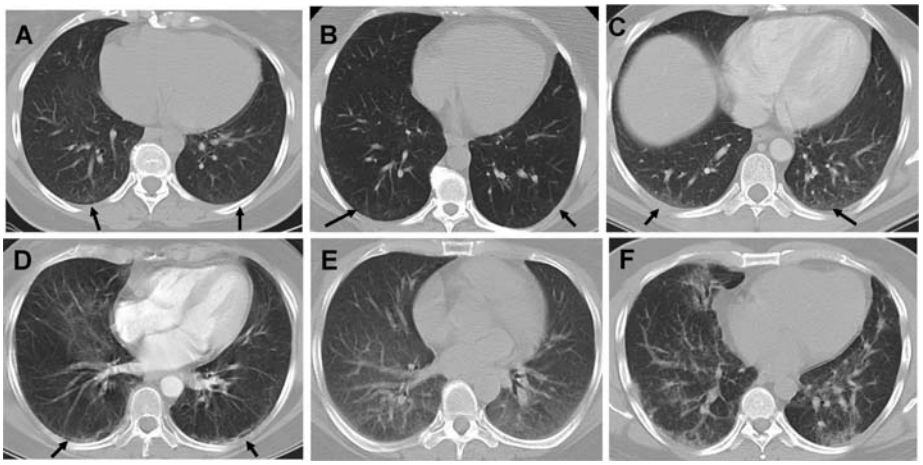

Figure 8.3 HRCT from patients with HPS, ranging from mild (**a**) to severe pulmonary disease (**f**). Findings include reticulonodular infiltrates, subpleural predominant honeycombing (*arrows*), and ground-glass opacities

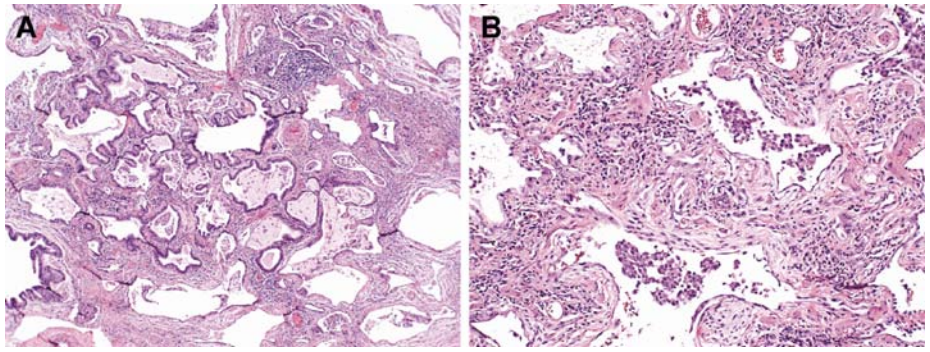

Figure 8.4 Lung histopathololology in HPS. Findings include fibrotic changes with microscopic honeycombing (**a**) and fibroblastic foci (**b**). The accumulations of macrophages in the airspaces (**b**) are a feature which distinguishes HPS lung histopathology from usual interstitial pneumonia (UIP)

which progressed to death by approximately age 40, while others had a later onset of disease, but a subsequent rapid rate of decline in pulmonary function after disease onset. This variability may derive from environmental factors or may be due to epigenetic phenomena *(71–73)*.

Lysosomal accumulation of a poorly characterized lipoproteinaceous material, called ceroid–lipofuscin, in macrophages has been associated with HPS pulmonary fibrosis and granulomatous colitis *(30, 31, 37, 75)*. HPS patients have large foamy alveolar macrophages, although airspace accumulation of lipoproteinaceous material, as occurs in pulmonary alveolar proteinosis, has not been reported. Although frequently implicated in the pathogenesis of lung disease in HPS, these cellular accumulations play an uncertain role in the development of pulmonary fibrosis.

Alveolar inflammation precedes pulmonary fibrosis in HPS, but the direct role of inflammation in the pathogenesis of HPS lung disease has not been determined.

Studies performed at the National Institutes of Health have characterized early pulmonary features in a cohort of HPS-1 patients with relatively preserved lung function (mean forced expiratory volume in one second (FEV1) 70% of predicted) and minimal radiographic evidence of fibrosis. When bronchoalveolar lavage (BAL) was electively performed, HPS patients were found to have significantly elevated numbers of alveolar macrophages (AMs) and high levels of cytokines and chemokines in their airways, including M-CSF and MCP-1 *(76)*. In addition, cultured AMs from HPS-1 patients expressed significantly more MCP-1, RANTES, M-CSF, and MIP1α than controls *(76)*. Recent observations of inflammatory colitis and hemophagocytic lymphohistiocytosis in HPS suggest that constitutive inflammation may play a role in HPS pathogenesis. Greater understanding of the potential relationship between lung inflammation and fibrosis in HPS is needed to improve therapeutic strategies.

Other Manifestations

Other clinical features of HPS may be directly related to the specific HPS subtype. Patients with HPS-2 have immune defects, with neutropenia, recurrent childhood infections, and, in one case, hemophagocytic lymphohistiocytosis (HLH) *(77–79)*. In contrast, a study of 15 Puerto Rican, presumably HPS-1 patients found no evidence of defects in peripheral blood lymphocyte or neutrophil function *(80)*. Renal and cardiac failure has also been rarely reported.

Genotype–Phenotype Correlations

The 16-bp duplication in *HPS1*, prevalent in Puerto Rico, is associated with an increased risk of interstitial lung disease. Gahl et al. reported that 9 of 16 HPS patients with the duplication, but none of the 10 HPS patients without it, had a diffusing capacity for carbon monoxide (DLCO) less than 80% of predicted. HRCT analysis of the patients with the duplication showed a greater incidence and severity of pulmonary fibrosis than in patients with other HPS mutations, i.e., other subtypes *(37)*. Patients with HPS-1 also have a significant incidence of granulomatous colitis (up to 15%) *(37, 70)*, and poor visual acuity has been associated with HPS-1 *(58)*. Remarkably, Swiss HPS-1 patients have been reported to have a normal life expectancy without pulmonary manifestations *(38)*. Although descriptions of HPS-4 patients are limited, this subtype is reported to have a phenotype similar to Puerto Rican patients with HPS-1 *(51)*. Mutations in *HPS3* have been described to result in a milder disorder *(47)*. The rare HPS-2 subtype results in a distinct phenotype with neutropenia and increased infections, but two brothers with HPS-2 also had radiographic evidence of mild lung disease in their third decade of life *(81)*. Pulmonary fibrosis has not been reported in patients with HPS-5, HPS-6, HPS-7, or HPS-8, though many are of younger age than the age at which HPS pulmonary manifestations are typical.

Diagnostic Approach

Individuals with HPS may have a history of prolonged bleeding, but platelet counts and general coagulation cascade parameters will be normal. All patients with a bleeding diathesis and/or any degree of ocular or cutaneous albinism should be tested for HPS.

The sine qua non of the diagnosis of HPS is the absence of platelet dense granules on whole mount electron microscopic analysis of platelets (Figure 8.2), but this testing is currently available only in selected laboratories.

In patients of Puerto Rican descent, the molecular diagnosis can be based on PCR amplification analysis of two founder mutations. However, for HPS patients of non-Puerto Rican origin, full sequencing of all candidate genes is required. Genetic testing for *HPS1*, *HPS3*, and *HPS4* is now available in selected clinical genetics laboratories (www.genetests.org), with other molecular testing performed on research protocols at the National Human Genome Research Institute (NHGRI). Recently, a biochemical assay has been developed to reduce the candidate gene sequencing burden. An immunoblotting assay on extracts from skin fibroblasts is used to determine which of the trafficking complexes (BLOC1, BLOC2, BLOC3, or AP-3) is deficient, so that more focused gene sequencing can be performed *(82)*. Such testing is performed on patients enrolled in an NHGRI research protocol when there is high clinical suspicion for HPS, but when no mutations have been identified through mutation analysis of *HPS1*, *HPS3*, *HPS4*, *HPS5*, or *HPS6*.

Patients known to have HPS should be evaluated for evidence of restrictive lung disease. There are no established guidelines regarding frequency of screening and monitoring with chest imaging and pulmonary function tests (PFTs). However, clinical practice has consisted of obtaining PFTs and a chest HRCT in early adulthood, with subsequent monitoring using PFTs and infrequent imaging, unless clinical symptoms or physiologic progression occurs. Individuals with HPS-1 are at the highest risk for pulmonary fibrosis, and therefore are monitored most aggressively. Because of the considerable risks of bleeding complications, surgical lung biopsy is rarely indicated for the diagnosis of HPS lung disease. Evaluation for colitis should also be considered in HPS patients with lower gastrointestinal symptoms *(70)*.

The differential diagnosis of HPS includes Chediak–Higashi syndrome (CHS), which shares the features of mild albinism and bleeding. However, CHS is also associated with innate immunodeficiency, often with recurrent infections and an accelerated lymphoproliferative phase. In the absence of overt oculocutaneous albinism, HPS could be confused with idiopathic pulmonary fibrosis (IPF), nonspecific interstitial pneumonia (NSIP), or pulmonary fibrosis due to a variety of other causes.

Management and Treatment

Limited therapy exists for individuals with HPS. Prevention and management of bleeding complications is a priority. Medications such as aspirin, ibuprofen, and warfarin are generally avoided. Platelet transfusions may be required in the setting of trauma, bleeding episodes, or surgical procedures and are an effective and necessary therapy despite the presence of normal platelet counts in HPS patients. Patients with HPS are counseled to wear medical alert bracelets. Desmopressin (DDAVP) has been administered to many patients, but with inconsistent efficacy. Cordova et al. reported that DDAVP had no effect on the bleeding times of 19 pediatric Puerto Rican patients *(83)*. Preventative dental care and gynecologic care for women with HPS are also particularly important due to bleeding considerations. Additional components of management include ophthalmology consultation for low-vision aids, use of sunscreen, and psychosocial support.

No clinical treatment trials have been performed for the granulomatous colitis that occurs in HPS patients. Case reports and series suggest a possible benefit of Infliximab *(63–65)*.

No definitive treatment exists for the pulmonary fibrosis associated with HPS, and respiratory failure is the most common cause of death. Steroids and other immunomodulating agents have been used, but there are no controlled studies to guide therapy and no definite benefits have been reported. A trial of pirfenidone in HPS patients was stopped for efficacy, as pirfenidone-treated patients lost lung function as assessed by forced vital capacity (FVC) at a rate that was 5% of predicted (approximately 400 ml) per year slower than placebo-treated patients, with post-hoc analysis showing a greater benefit to patients with an initial FVC at least 50% of predicted. Dizziness was reported in three patients receiving pirfenidone, but no other significant adverse events occurred *(73)*. Pirfenidone is a drug with anti-inflammatory, antioxidant, and antifibrotic effects including inhibition of TGF-β expression. While the molecular targets of this therapy have not been fully elucidated, further investigations into the role of targeted anti-inflammatory therapy in HPS are clearly warranted.

Vigilance and early intervention for respiratory infections, in addition to prophylaxis with influenza and pneumococcal vaccinations, may be beneficial in patients with HPS. Smoking cessation and avoidance of secondhand tobacco smoke should be recommended for all patients with HPS. Since bleeding complications can be mitigated by platelet transfusion, lung transplantation is an option for some HPS patients with advanced pulmonary fibrosis and has been performed successfully *(35, 84)*.

Disease Models

Many proven and potential HPS genes have been identified through study of numerous model systems including yeast and *drosophila*. Mice are the species of choice for the study of HPS. At least 16 different murine models of HPS exist, with most of their causative genes identified by positional cloning *(11, 13)*. Ten of the HPS models are maintained on the C57BL/6 J inbred strain. Table 8.1 summarizes the known HPS genotypes and corresponding mouse models. HPS mouse models share many of the physiological and cellular effects of HPS mutations seen in humans with HPS. Varying degrees of hypopigmentation are observed in all models, and all have a marked deficiency of platelet dense granules in comparison to wild-type mice *(11, 85)*.

Six HPS genes encode known vesicle trafficking proteins. The *pale ear* mouse is the murine analogue of HPS-1 in humans *(86)*, and the *light ear* mouse is the HPS-4 model *(50)*. The *pearl* mouse is the model for HPS-2 with mutation in *AP3β1 (16)*, and the gene mutated in the *cocoa* mouse is homologous to the human *HPS3* locus *(87, 88)*. The HPS-5 and HPS-6 models are the *ruby-eye 2* and *ruby-eye*, respectively *(89)*. In addition, genes have been identified for an additional eight mouse models of HPS that have not yet been described in humans (*mocha*, *pallid*, *gunmetal*, *ashen*, *muted*, *buff*, *subtle gray*, and *cappuccino*) *(10)*. These HPS genes encode members of BLOCs 1, 2, or 3. A mutation in one subunit of a BLOC often results in secondary degradation of other components of the complex, and mutations affecting any component in a given BLOC tend to produce similar coat phenotypes in mice *(13)*. For example, the *pallid*, *cappuccino*, *muted*, and *sandy* mice all have mutations that affect BLOC1 proteins, and all four have very light gray coats and ears. In contrast, the *pale*

ear HPS-1 and *light ear* HPS-4 mice have mutations that affect BLOC3 proteins, and both have relatively preserved and naturally dark tones in their coats but their ears and tails are hypopigmented *(13)*.

HPS mouse models have been largely employed for the study of abnormal vesicular trafficking with respect to melanocyte function and, to a lesser extent, platelet dysfunction. Lysosome-related organelles occur in many cell types, and abnormalities have been demonstrated in many HPS mouse models. Examples include decreased secretion of cytotoxic T-lymphocyte lytic granules in *ashen*, *gunmetal*, and *pearl* mice *(44, 90)* and abnormal secretion of mast cell granules in *ruby-eye (89)*.

None of the known naturally occurring mouse models of HPS develop pulmonary fibrosis spontaneously, but several exhibit progressive airspace enlargement *(91, 92)*. *Pearl* and *pale ear* mice have structural abnormalities in the alveolar compartment that are similar to those observed in humans with HPS, including foamy AMs and enlarged type II cells containing irregular dense inclusions, and expansion of interstitial septae by excessive collagen fibrils at the ultrastructural level. Guttentag et al. have shown that double-mutant HPS-1/HPS-2 (*ep/pe*) mice have impaired lamellar body secretion from type II cells *(93)*, and Lyerla and colleagues found that lung hydroxyproline content is significantly increased compared with controls *(92)*. Additionally, double and triple mutant mice, with combinatorial mutations of BLOCs 1, 2, 3, and/or AP-3, have provided insights into the combinatorial effects of HPS mutations, but again do not spontaneously develop pulmonary fibrosis *(94)*.

Recent studies have elucidated further alveolar cellular dysfunction and fibrotic susceptibility in HPS mouse models that suggest promise in using these models to study HPS lung disease. *Pearl* and *pale ear* mice exhibit pulmonary inflammatory dysregulation, with constitutive AM activation that parallels abnormalities reported in HPS patients *(76, 95)*. Furthermore, *pearl* (HPS-2) and *pale ear* (HPS-1) mice have marked fibrotic susceptibility to bleomycin challenge, including increased mortality, histologic evidence of fibrosis, collagen deposition, and TGF-β expression. HPS mice also exhibited accelerated and increased alveolar type II cell apoptosis in response to bleomycin challenge, suggesting that environmental insults, which overwhelm the homeostatic activities of marginally compensated type II cells, may provide a "second hit" that leads to fibrosis *(96)*. Additionally, Yoshioka et al. have reported that silica-challenged *pale ear* HPS-1 mice develop a persistent accumulation of activated macrophages and increased collagen fibers in alveolar tissues *(97)*.

Other Potential Model Systems

Although Rab38 mutations have not been identified in cohorts of patients with oculocutaneous albinism *(98)*, *Rab38* has been proposed as a potential HPS gene. Like all rabs, Rab38 is involved in vesicular trafficking. The *ruby* rat (*red-eyed dilution*, R) has mutations in Rab38 resulting in absence of Rab38 protein production, hypopigmentation, and bleeding. The fawn-hooded rat also has mutations in *Rab38* and has a phenotype which includes hypertension, hypopigmentation, and a platelet storage pool defect. The *chocolate* mouse has mutations in Rab38, with a mild coat color but normal blood clotting times. Recent studies suggest that *chocolate* mice also have abnormal lamellar bodies and surfactant homeostasis *(99)*.

A potential novel model for HPS is the zebrafish mutant *lbk,* which displays hypopigmentation of skin melanocytes and the retinal pigment epithelium, an absence of

iridophore reflections, defects in internal organs (liver, intestine), and functional defects in vision and in macrophages. This *lbk* mutation has been identified to be an ortholog of the *vam6*/*vps39* gene; Vam6p is part of the HOPS complex, which is essential for vesicle tethering and fusion *(100–102)*.

Future Directions

Significant advances in knowledge about the clinical manifestations of this rare disorder have occurred in recent years. A grass roots patient organization, The HPS Network, has led patient advocacy efforts and has supported the establishment of an intramural research protocol for HPS at the NIH. A natural history protocol and therapeutic trials of pirfenidone for HPS pulmonary fibrosis continue to be conducted at the NHGRI. Additionally, numerous extramural investigators have long-standing research programs focused on vesicular trafficking defects, and their work has been critical for discovering HPS genes and understanding protein functions.

Nonetheless, most aspects of HPS warrant and require further study, with respect to both clinical and basic research. Genetic discovery is one area of great opportunity, as the locus heterogeneity of HPS phenotypes in mice suggests that there may be several additional HPS genes to be discovered in humans. Further, it is unknown whether HPS loci will function as modifier genes in other fibrotic lung disorders. The clinical features of HPS have been described for HPS-1, but knowledge about the natural history, including incidence of pulmonary fibrosis, in other HPS subtypes remains incomplete. Furthermore, HPS patients have a macrophage-mediated alveolar inflammation that precedes the onset of pulmonary fibrosis, and further studies are needed to define the possible relationship between inflammation and onset of lung disease in HPS.

Colitis is a major cause of morbidity in a subset of HPS patients, and studies of the etiology of colitis may provide insights into the pathogenetic mechanisms relevant for HPS lung disease as well. Biomarkers to identify onset of pulmonary disease and disease progression are needed for this at-risk patient population.

There are several ongoing therapeutic trials for HPS at the NIH, all currently enrolling patients: (1) a phase 3 trial of pirfenidone (NCT00001596), (2) a pilot study of a multi-drug regimen for severe pulmonary fibrosis in HPS (NCT00467831), and (3) medical treatment of colitis in patients with HPS (NCT00514982).

Ultimately, the best opportunity for therapeutic success in HPS rests with developing strategies to correct or compensate for the underlying protein trafficking defects. While the molecular underpinnings have been relatively well elucidated with respect to albinism, the relationship between HPS trafficking defects and pulmonary fibrosis remains poorly understood. In this respect, mouse models of HPS may provide insight into mechanisms of vesicle trafficking and ultimately the pathogenesis of the pulmonary fibrosis associated with HPS. Discoveries about the pathogenesis of Hermansky–Pudlak syndrome may also shed light on disease mechanisms of other more common scarring lung diseases.

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9

Alpha-1 Antitrypsin Deficiency

Charlie Strange and Sabina Janciauskiene

Abstract Alpha-1 antitrypsin (AAT), also referred to as α_1-proteinase inhibitor or SERPINA1, is the most abundant serine proteinase inhibitor in human plasma. Genetically determined deficiency of AAT is associated with early-onset emphysema, particularly in individuals who smoke or are exposed to other inhaled environmental toxins. In addition, cirrhosis occurs in some infants, young children, and older adults due to accumulated AAT in hepatocytes. This chapter will review the clinical phenotype of AAT deficiency, the genetics and inheritance of the condition, and the biochemistry of AAT that leads to the common as well as the unusual clinical manifestations of AAT deficiency (AATD).

Keywords: antiprotease, protein misfolding, ER stress, unfolded protein response

AAT Synthesis and Regulation

AAT is a glycoprotein mainly produced in hepatocytes *(1, 2)*. AAT may also be synthesized by blood monocytes, macrophages, pulmonary alveolar cells, and by intestinal and corneal epithelium *(3–8)*. The AAT gene is also expressed in the kidney, stomach, intestine, pancreas, spleen, thymus, adrenal glands, ovaries, and testes *(9, 10)*. De novo synthesis of AAT has also been demonstrated in human cancer cell lines. These observations indicate that AAT gene transcription is not limited to a single tissue *(11, 12)*. In fact, tissue-specific promoter activity for AAT has been reported in the liver, the major source of AAT, and other tissues that synthesize the protein *(13)*.

The normal daily rate of synthesis of AAT is approximately 34 mg/kg body weight and the protein is cleared with a half-life of 3–5 days. This results in high plasma concentrations ranging from 90 to 175 mg/dl when measured by nephelometry. In addition to high circulating levels, AAT is also present in various biological fluids, including saliva *(14)*, tears, milk, semen *(15)*, urine *(16)*, and bile *(17)*. The concentration of the protein in the tissues is not uniform, for example, it is reduced to approximately 10% of the plasma levels in the fluid of the lower respiratory tract *(18, 19)*. AAT also diffuses

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through endothelial and epithelial cell walls and is present in the epithelial lining fluid at levels that are 10–15% of serum concentrations.

As an acute-phase reactant, circulating AAT levels increase rapidly (3–4-fold) in response to inflammation or infection *(20)*. The concentration of AAT in plasma also increases during oral contraceptive therapy and pregnancy *(21)*. During an inflammatory response, tissue concentrations of AAT may also surge increasing as much as 11-fold as a result of local synthesis by resident cells or invading inflammatory cells. For example, human monocytes and alveolar macrophages can contribute to tissue AAT levels in response to inflammatory cytokines (IL-6, IL-1, and TNFα) and endotoxins *(20, 22)*. Recent data demonstrate that AAT expression by α- and δ- cells of human islets *(23)* and intestinal epithelial cells *(24)* is also enhanced by pro-inflammatory cytokines. AAT synthesis by corneal epithelium, on the other hand, appears to be under the influence of retinol, interleukin-2, fibroblast growth factor-2, and insulin-like growth factor-I *(25)*. Interestingly, AAT expression also shows some degree of substrate and/or autoregulation with enhanced synthesis following exposure to neutrophil and pancreatic elastases either alone or complexed to AAT *(26)*. The usual serum concentrations are also determined by the genetic alleles depicted in Figure 9.1. Because of the variability of serum concentrations, associations with human disease are best correlated with AAT gene mutations.

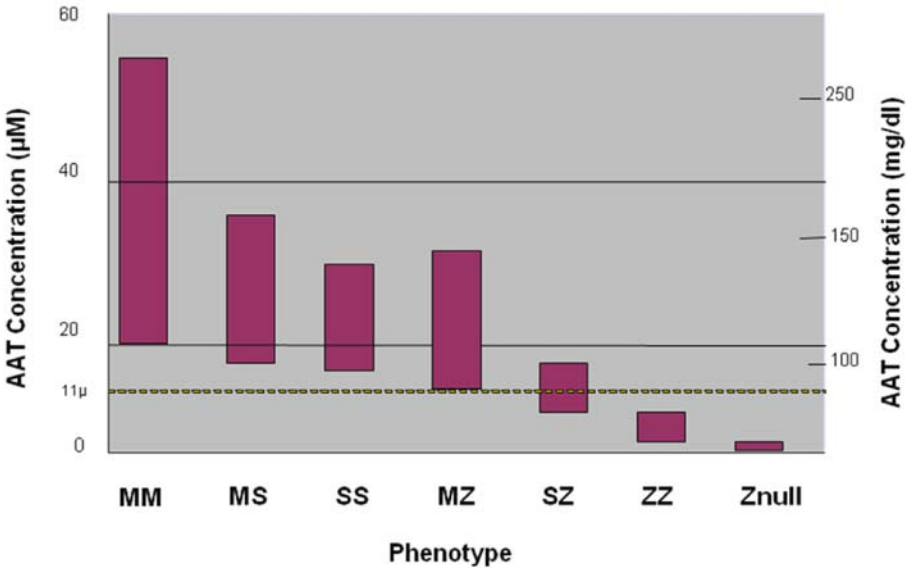

Figure 9.1 Range of serum levels associated with common genotypes of α-1 antitrypsin. One micromole approximately equals 5.2 mg/dl.

AAT Structure and Mechanisms of Protease Inhibitory Activity

Human AAT, a protein of molecular weight 52,000 Da, consists of a single polypeptide chain of 394 amino acid residues containing one free cysteine residue and three asparagine-linked carbohydrate side chains *(27)*. Like other serpins, the structure of

AAT consists of three β-sheets (A, B, C) and nine α-helices (A–I). The amino acid at position P1 in the reactive site center of AAT and other serpins plays an important role in determining the specificity of SERPIN inhibition. In terms of its SERPIN activity, AAT has an exposed polypeptide segment and reactive site loop, which is susceptible to protease attack *(2)*. Cleavage of the scissile bond in the loop results in a large conformational change in which the reactive site loop migrates and inserts into the pre-existing β-sheet A (Figure 9.2) to form a very stable complex between the inhibitor and the proteinase *(28)*. Various biochemical *(29)* and structural *(30)* studies suggest that the loop insertion is necessary for the formation of a stable complex and is thought to be critical for inhibitory function. Complexes of AAT and its most common protease, neutrophil elastase (NE), can be measured in the lower airway.

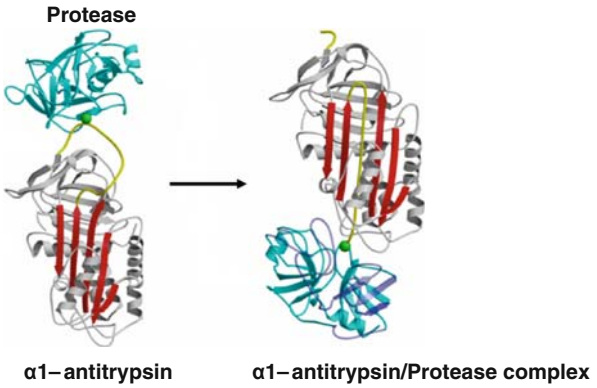

Figure 9.2 Mechanistically, α-1-antitrypsin has an exposed polypeptide segment, the reactive site loop (*yellow*), which is susceptible to protease attack. Complex formation between α-1-antitrypsin and its target protease results in a large conformational change in which the reactive site loop (*yellow*) migrates and inserts into a pre-existing β-sheet. Reproduced with permission from the Huntington Laboratory, University of Cambridge

Until recently it was thought that neutralization of NE, proteinase 3, and other serine proteases released from activated human neutrophils during the inflammatory response was the primary function of AAT. In fact, the rate of formation of the AAT/NE inhibitory complex is one of the fastest known for serpins (6.5×10^7/M/s) *(2)*. However, Petrache et al. have shown that AAT also directly inhibits active caspase-3, a cysteine proteinase, suggesting a broader proteinase inhibitor role with impacts on biological processes including apoptosis and oxidative stress as well as inflammation *(31, 32)*. AAT, like other serpins, can be inactivated by proteases, which are not inhibitor targets. These recognize the reactive site loop of AAT and rapidly hydrolyze it, but are unable to form stable AAT–protease complexes. Several nonserine proteases such as cathepsin L *(33)*, stromelysin-1 (MMP-3) *(34)*, neutrophil collagenase (MMP-8) *(35)*, and gelatinase B (MMP-9) are known to inactivate AAT by cleavage. Inactivation of the reactive site loop in AAT is viewed as a potential pathological mechanism resulting in an imbalance of antiproteinase activity that favors proteolysis and lung destruction.

Genetic Modifications of the AAT Molecule

The AAT molecule is produced on the SERPINA1 gene (OMIM: 107,400) and about 100 human variations in gene structure have been defined *(36)*. The gene is located across three noncoding (Ia, Ib, and Ic) and four coding (II, III, IV, V) exons. Some difficulty has arisen with AAT nomenclature since the capability to sequence the deficiency genes has been of recent vintage. The clinical disease states were originally defined by plasma isoelectric focusing of protein and characterized by the protease inhibitor (Pi) system. Since the AAT concentration is a product of each gene in a codominant fashion and the protein level produced clinical disease, the AATD-deficiency states were called phenotypes. For instance, when a single band of protein migrated to the Z region on the electrophoresis, it is impossible to define whether one or two copies of the Z gene are producing that protein if no other protein bands are seen. This is because some individuals have deficiency genes, which produce no protein (the so-called Null genes). Therefore, the PiZ phenotype defined both PiZZ and PiZNull genotypes. Recently, PCR has been used to probe blood DNA to define these phenotypes by specific gene presence. Therefore, comprehensive genetic diagnosis can be tedious using combinations of blood levels, nephelometry, PCR probes, and gene sequencing for rare deficiency alleles (Table 9.1). Specialty laboratories are available to define unusual genetic variants *(37)*.

There are a few very rare genetic variants that produce dysfunctional AAT protein. AAT Pittsburgh is a thrombin inhibitor rather than an elastase inhibitor *(38)*. PiF produces normal AAT concentrations, but the association with elastase is markedly reduced *(39)*.

There appears to be some racial and regional variation to the common genetic variants. The most common deficiency genetic variants include PiZ and PiSZ. Humans inherit one gene from each parent at the Pi locus that equally contributes to the amount of AAT produced. Homozygous individuals at the PiZ locus (PiZZ) produce a misfolded protein that cannot get out of the hepatic endoplasmic reticulum (ER). As a result, serum concentrations of AAT are approximately 10–15% of normal, and AAT accumulates in hepatocytes where it can cause cirrhosis. A variety of rare Null genes produce no appreciable AAT at the cellular level. These genes do not produce clinical liver disease. Gene distribution studies suggest that the PiZ gene was of Scandinavian origin, while the PiS gene has highest gene frequencies on the Iberian peninsula and is more prominent in Hispanic populations *(40)*.

Epidemiology of PiZ AATD suggests a gene frequency of 2–24 cases per 1,000 population in Europe and 1–2 per 1,000 individuals in the United States. PiS gene frequency is estimated at 1–9 per 1,000 population in Europe and 2–4 per 1,000 in the United States *(36)*. Applied to the US population, these gene frequencies would suggest between 47,000 and 100,000 PiZ-affected individuals *(41)*. Unfortunately, in the United States approximately 5–8% of the estimated deficient population has been identified *(42)*. This leaves the majority of genetically deficient individuals unidentified. It has been suggested that many deficient individuals can be found in COPD clinics where the PiZ and PiSZ gene frequencies are estimated to be 0.5–3%. However, the method of ascertainment profoundly biases the clinical manifestations associated with deficiency states of AAT.

The simplistic theory of COPD pathogenesis suggests that the low total serum concentration of AAT is inadequate to protect against injurious proteases in the lower airways. Cigarette smoking markedly increases the number of lung neutrophils and their

Table 9.1 The most common normal and deficiency alleles.

Normal alleles		Exon	Comments
	M1 (Ala213)	III	Most common M allele
	M1 (Val213)	III	
	M2	II	
	M3	V	
	M4	II	
	M5	II	Two alleles $M5_{berlin}$ and $M5_{karlsruhe}$
	M6	II	
	L	II, III, V	Two alleles $L_{frankfurt}$ and $L_{offenbach}$
	V	II, V	Three alleles V, $V_{donauworth}$, and V_{munich}
	X	III, V	Two alleles X and $X_{christchurch}$
	B	Unknown	$B_{Alhambra}$
	P	III, V	Two alleles $P_{st.\ louis}$ and P_{Albans}
Deficiency alleles			
	Z	V	Most common severe deficiency gene
	S	III	Lesser degrees of deficiency than Z gene but more common
	M alleles	II, V	$M_{herleen}$, M_{malton}, $M_{mineral}$ $M_{springs}$, $M_{procida}$, $M_{bethesda}$, $M_{palermo}$, $M_{nichinan}$
	W	V	
	I	II	
	P	III	Two alleles P_{lowell} and P_{Duarte}
Dysfunctional allele			
	F	III	Will have low normal serum level with dysfunctional protein
	Pittsburg	V	

NE secretory capacity *(18)*. In deficiency states of AAT, unopposed NE cleaves elastin, one of the supporting structures of the lung airway and parenchyma, leading to emphysema and the collapse of airways characteristic of COPD. Low serum levels of AAT also may contribute to other clinical manifestations through low circulating levels of their posttranslational modifications.

Posttranslational-Modified Molecular Forms of AAT

The structural properties of AAT that confer protease inhibitor activity render the molecule extremely sensitive to mutations and posttranslational modifications including the formation of complexes with other proteins, oxidation, nitration, polymerization, and inter-molecular cleavage. These modified forms of AAT have been detected in tissues and fluids at sites of inflammation. The important questions are whether and how these have impact on the inflammatory/disease process.

AAT is known to form complexes with other molecules. For example, complexes between AAT and the kappa light chain of immunoglobulins have been found in serum from patients with myeloma and Bence–Jones proteinemia *(43)*, AAT-XIa factor *(44)*, and AAT–glucose complexes are common in the plasma from diabetic subjects *(45)*. A recent study looking at the plasma of type 1 diabetic subjects demonstrated that AAT can also form complexes with heat-shock protein-70 (HSP70) *(46)*. Disulfide-linked complexes between immunoglobulin A and AAT have been detected at low levels in the sera of healthy volunteers but are significantly increased in the sera and synovial fluid of patients with rheumatoid arthritis, systemic lupus erythematosus, and ankylosing spondylitis *(47)*, diseases possibly associated with AATD. Human tissue kallikrein 3, a serine proteinase commonly known as a prostate-specific antigen (PSA) which correlates with prostate hypertrophy and malignancy, is also known to bind to AAT in sera of subjects with high PSA concentrations *(48)*. Moreover, recent studies demonstrate that AAT is an irreversible inhibitor for kallikrein 7 and 14 *(49)*. Clearly, a considerable amount of work is required to understand the biological implications of protein complex formation with AAT and relate deficiency states to human disease.

Oxidized AAT is a modified form of AAT found in inflammatory exudates at levels of about 5–10% that of total AAT *(50)*. The methionine residues in AAT are highly susceptible to attack by various oxidants produced in the inflammatory response. These include hydrogen peroxide, hydroxyl radicals, hypochloride, chloramines, and peroxynitrite *(51)*. Evidence that AAT undergoes oxidative modifications in vivo comes from the discovery that AAT purified from inflammatory synovial fluid contains methionine sulfoxide residues and is inactive as a serine protease inhibitor *(52, 53)*. In vitro, oxidative inactivation of the AAT can be induced by incubating AAT with purified myeloperoxidase or stimulated phagocytes *(54)*. Of clinical importance, oxidation of AAT is caused by cigarette smoke, suggesting that current smokers should not be given augmentation therapy as a treatment for COPD. Oxidative inactivation of AAT in association with enhanced neutrophil-mediated tissue proteolysis has been implicated in the pathogenesis of pulmonary emphysema *(55)*. Scott and coworkers have demonstrated that oxidation of AAT promotes AAT-immunoglobulin A complex formation in vitro. IgA-oxidized AAT complexes isolated from synovial fluid of rheumatoid disease patients were suggested to protect the oxidized AAT molecule from proteolytic cleavage by free elastase *(56)*.

It has been reported that AAT from human plasma is readily *S*-nitrated under physiological conditions and that its nitrosylation is 10 times more efficient than nitrosylation of bovine serum albumin and glutathione *(57)*. More importantly, *S*-NO-AAT has been shown to have multiple biological functions, including potent antimicrobial activity and inhibition of cysteine protease. In a recent study by Ikebe and coworkers *(58)* it was suggested that *S*-NO-AAT exerted a potent cytoprotective effect on ischemia–reperfusion liver injury by maintaining tissue blood flow, inducing heme oxygenase 1, and suppressing neutrophil-induced liver damage and apoptosis. It was also verified that *S*-NO-AAT had potent serine protease inhibitory activity similar to that of native AAT. Interestingly, the inhibitory action of AAT against porcine pancreatic trypsin and pancreatic and neutrophil elastase was not affected by S-nitration *(59)*. Therefore, *S*-NO-AAT may function not only as a simple NO (nitroso) donor but also as a protease inhibitor with a broad inhibitory spectrum.

Cleavage of AAT may occur when native AAT forms an inhibitor complex with target proteases (e.g., neutrophil elastase) and subsequently undergoes proteolytic degradation by nontarget proteases. Nontarget proteases reported to cleave AAT in

vitro, include cathepsin L, collagenases, macrophage elastase, matrilysin, stromelysin-1 and -3, and bacterial proteinases from *Staphylococcus aureus*, *Serratia marcescens* metalloproteinase, and *Pseudomonas aeruginosa* elastase *(60, 61)*. In addition, gelatinase B (MMP-9) has been proposed as an important nontarget proteinase capable of cleaving native AAT in vivo. The nonspecific cleavage of AAT generates a C-terminal fragment, which may remain noncovalently bound or may dissociate from the parent protein. The hydrophobic C-terminal peptides liberated during proteolytical cleavage of AAT have been isolated from the phospholipid fraction of human bile and spleen *(62)*. The C-terminal fragment of AAT is present in atherosclerotic plaques, particularly within the fibrous cap at the base of the lipid core *(63)*. Similar AAT fragments are found in the lungs *(64)* and in urine from chronic obstructive lung patients with and without AAT-inherited deficiency. Recently, AAT and its peptide degradation products were found to be associated with high-density lipoproteins.

Several studies suggest that cleaved forms of AAT might exhibit novel biological activities in vivo. For example, the C-terminal fragment of AAT, C-36 peptide, corresponding to residues 359–394 was shown to suppress bile acid synthesis in vitro and in vivo via inhibition of 7α-hydroxylase *(65)*. Subsequent studies have demonstrated significant pro-inflammatory activity of C-36 peptide in vitro including the stimulation of cytokine and chemokine release by human monocytes and protease release and chemotaxis in neutrophils *(66)*. The C-terminal 26-residue peptide of AAT appears to inhibit HIV long terminal repeat-driven transcription in epithelial cells transfected with HIV-1 LTR promoter-driven genes *(67)*. Several other studies suggest that AAT peptides may represent a novel class of antiviral agents *(68)*. In addition, the hydrophobic A1-C26 peptide which significantly increases the production of collagen I in skin fibroblasts has been suggested for skin care applications *(69)*.

In addition to cleavage, AAT is also vulnerable to conformational changes that allow inter-molecular linkage leading to formation of polymers *(70)*. AAT polymer formation may involve the generation of an unstable intermediate, which can form polymers or generate latent protein *(71)*. Recently, Zhou and Carrell have proposed that AAT dimers initiate and propagate polymerization by having one exposed loop with an optimal conformation as a β-strand donor and a readily opened β-sheet as an acceptor. The sequential reformation of these activated β-interfaces as the oligomer extends, molecule by molecule, provides a model for the fibril and amyloid formation of conformational diseases in general *(72)*. Polymerized forms of tissue and circulating AAT are found in individuals with and without inherited AAT deficiency *(73) (74)*. Like other modified forms, AAT polymers lack proteinase inhibitor activity but are chemotactic for neutrophils and may participate in the pathogenesis of COPD *(75, 76)*.

Clinical Lung Disease

In 1963, Laurell and Erickson identified five individuals with missing α-bands on serum protein electrophoresis and noted the association of clinical lung disease with absence of AAT *(77)*. In the years since this discovery, AATD has been associated with a variety of clinical lung diseases including COPD with both emphysematous and chronic bronchitis phenotypes *(78)*, asthma, and bronchiectasis *(79)*. Considerable work has attempted to establish the epidemiology of AATD COPD, define whether there are

meaningful differences between AATD and "usual" COPD without AATD, and refine the natural history of the condition.

Most of the work describing the natural history of AATD is derived from a study of 200,000 live births in Sweden between 1972 and 1974 in which 127 PiZ infants were diagnosed. This birth cohort now is >30 years of age with lung function tests that are normal *(80)*. There may be an excess prevalence of asthma in childhood and teenage years. In other populations, advanced emphysema has been described in the fourth decade of life and is interactive with cigarette smoking, although with significant variability. Nonsmoking individuals with AATD may get COPD, but do so at an older age than smoking individuals. Nonsmokers, however, may live a normal life span.

Asthma is described in excess frequency in AATD *(81)*. Asthma prevalence appears to be also increased in PiMZ carriers and in PiZ severely deficient individuals. Some studies have suggested that airway inflammation and clinical asthma diagnoses are simply the beginning symptoms of lung inflammation that will advance to COPD. However, the excess incidence of allergic rhinitis in populations of MZ carriers and in PiZ severely deficient individuals *(82)* suggests that asthma incidence is independently increased in AATD. Since most individuals with AATD present with wheezing and dyspnea as a first symptom, AAT testing is recommended for all individuals with asthma whose spirometry fails to return to normal on appropriate treatment for asthma *(36)*.

COPD is common in AATD. Most individuals with AATD over the age of 40 have emphysema on chest CT. However, chronic bronchitis or asthma incidence is also common and similar in frequency to usual COPD *(78)*. Therefore, the emphysema prevalence does not sufficiently dominate the clinical disease state to facilitate targeted AAT testing. Chronic bronchitis as the only manifestation of disease is sufficiently common to apply AAT testing to all patients with COPD.

Bronchiectasis, defined as permanent enlargement of one or more central airways, is increased in AATD. Recent CT studies have suggested a prevalence of clinically significant disease at 27% *(79)*. Other studies suggest a high percentage of asymptomatic individuals. Bronchiectasis in AATD is in part due to infection with atypical mycobacteria *(83)*. Published evidence that deficiency states of AAT contribute to increased prevalence of mycobacterial disease, particularly *Mycobacterium avium* complex is sparse. However, recent studies suggest an excess of PiMZ phenotypes in atypical mycobacterial patients.

The extent to which carrier-deficiency states (PiMZ and PiMS) contribute to an increased risk of COPD remains controversial *(84)*. Population-based studies have failed to show an increased prevalence of COPD in PiMZ populations although many of the studies were not done in cigarette-smoking populations. However, the PiMZ gene frequency in COPD populations has been found increased (4–12%) compared with the normal population without lung disease in which PiMZ prevalence is usually 3–4%. In addition, studies evaluating genes associated with progression of COPD have shown the PiMZ state to be independently correlated with disease progression *(85)*.

Treatment of lung disease is not different in most respects from usual COPD, although the frequency of bronchodilator responsiveness in patients with predominant emphysema is sometimes small. The only specific treatment for AATD is intravenous augmentation of plasma-derived AAT, first developed and approved for severe deficiency of AATD in 1989. Because the development of emphysema takes years and the yearly decline of FEV1 is typically small, this medication was not subjected to randomized trials at the time of licensing. Therefore, the efficacy of

the protective effects of augmentation therapy remains controversial throughout the world.

Proof of efficacy has been attempted in several clinical trials. In a prospective non-randomized study, the US National Heart, Lung, and Blood Institute Registry followed 1,129 individuals receiving, sometimes receiving, or not receiving augmentation therapy and recorded the rate of FEV1 decline. The slope of FEV1 decline was less in individuals receiving AAT augmentation in a subgroup analysis when baseline FEV1 was between 30 and 65% predicted. Moreover, mortality was less in individuals who received AAT augmentation, an effect that occurred predominantly in the group with baseline FEV1 <30% *(86)*.

FEV1 decline has also been shown to be less in a group of AATD individuals receiving augmentation in Germany compared to a group not receiving augmentation in Denmark *(87)*. A pilot ($N = 56$) prospective randomized trial of augmentation therapy using CT densitometry as the primary efficacy outcome showed trends in preventing emphysema in the infused group ($p = 0.07$) *(88)*. Larger prospective randomized trials are currently ongoing in Europe.

Augmentation therapy is usually given intravenously at a dose of 60 mg/kg/week *(89)*. Studies administering the drug at 4 times the weekly dose every 4 weeks have shown significant periods of time below the presumed protective threshold of 11 μM *(90)*. Studies administering inhaled AAT to augment the antiprotease activity of the lower airways have been performed but to date have been hampered by the lack of robust outcome markers for COPD necessitating long and expensive trials for a rare disease *(91, 92)*. A recent study in 52 patients with cystic fibrosis has shown that a daily deposition by inhalation of 25 mg AAT for 4 weeks increased AAT levels and decreased the levels of elastase activity, neutrophil counts, pro-inflammatory cytokines (TNFα, IL-1β), and the numbers of *P. aeruginosa (93)*.

Clinical Liver Disease

In 1969, liver disease was first described in 10 children with severe AATD *(94)*. In the interval since, liver disease has been defined both in infancy and in adulthood, although the understanding of the pathobiology of why a minority of AATD-affected individuals have clinical liver disease remains unknown. Periodic acid-Schiff diastase-resistant globules are seen in the liver biopsy of almost all individuals with AATD (Figure 9.3). Cirrhosis is also seen with increasing age although it is often clinically silent *(95)*. Since there is no specific therapy for AATD liver disease, the most important treatment determination is the appropriate timing for liver transplantation. To this end, patients should avoid obesity *(96)* and pathologic use of ethanol that could accelerate liver disease and prevent transplantation.

One area that remains controversial is whether PiMZ phenotypes that have a single deficiency allele cause or increase the incidence of liver disease. Large clinical series have been described in which no other apparent cause of cirrhosis is found *(96)*. However, the incidence of cryptogenic cirrhosis in PiMM individuals is not small and definitive statements about the risk for liver disease in PiMZ individuals cannot be established at this time.

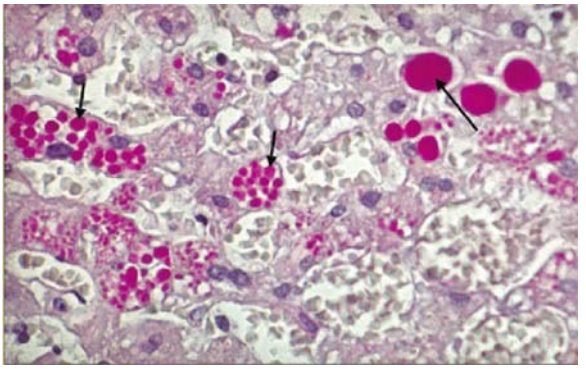

Figure 9.3 AAT polymers accumulate within the endoplasmic reticulum of hepatocytes to form the PAS (periodic acid-Schiff)-positive inclusions that are the hallmark of PiZZ liver disease. *Arrow* indicates AAT accumulation. Picture from C-B Laurell presentation Oak Ridge Conference May 4–5, 2001, Seattle, Washington, with permission

Other Diseases Associated with AATD

Panniculitis is a rare manifestation of AATD that presents with painful raised inflammatory lesions of fat. Rarely, these can be disabling with fat necrosis and draining fistulae. Augmentation therapy via the intravenous route has been reported to be curative in case reports and case series *(97)*.

An excess prevalence of Z alleles has been found in a number of connective tissue diseases and in antineutrophil cytoplasmic antibody (ANCA) positive (particularly antiproteinase-3 associated) vasculitis *(98)*. Deficiency of antiprotease activity as described earlier in the chapter is suspected to allow a more prolific vasculitis to progress *(99)*. Data remain equivocal on whether there is a link between AATD and nephropathies, abdominal aortic aneurysms, intracranial aneurysms, and fibromuscular dysplasia *(36)*.

Clinical Testing

Current recommendations for AAT testing have been approved by professional societies and include the clinical conditions listed in Table 9.2. Screening is performed most efficiently by measuring serum AAT concentration. A serum concentration <58 mg/dl (<11 μM) will be found in all individuals with PiZ AATD. Since this is the group of individuals studied in all trials of augmentation therapy to date, augmentation therapy for other deficiency allele combinations that make more AAT is felt to be ethically unfounded, given the cost of therapy.

Home and office PCR-based testing kits have been developed and are appropriate when establishing family genetics, when testing patients on augmentation therapy, when evaluating individuals with cryptogenic cirrhosis, and for screening when blood levels are difficult to obtain. Because AAT is an acute-phase reactant, PiMZ and PiSZ deficiency states can have highly variable serum levels, which fall into the normal range of PiMM subjects. Therefore, family testing will require phenotyping or genotyping.

Table 9.2 Indications for testing for AAT deficiency *(36)*.

• Absence of an α-1 peak on serum protein electrophoresis
• Early-onset pulmonary emphysema
• Family members of known α-1 antitrypsin-deficient subjects
• Dyspnea and cough in multiple family members
• Liver disease of unknown cause
• All individuals with COPD
• Adults with bronchiectasis of unknown cause
• Adults with asthma whose spirometry fails to return to normal with therapy
• Unexplained panniculitis
• Antiproteinase 3 vasculitis

Summary

In summary, AATD is a rare genetic condition that results in COPD in part due to the deficiency of antiprotease defences in the lung. Recognition of the condition requires an inexpensive blood test for AAT concentration that should be obtained once in a lifetime of all individuals who have COPD. Treatment of the deficiency includes improved environmental control, smoking cessation, and discussion of AAT augmentation therapy for individuals severely deficient in AAT. Family screening is appropriate. Liver disease can be prospectively monitored to allow appropriate and timely interventions. The future holds the promise for more biologic functions of AAT being described. Each biologic function will need to be evaluated for associations with clinical disease in this genetically deficient population.

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10

The Marfan Syndrome

Amaresh Nath and Enid R. Neptune

Abstract Marfan syndrome (MFS), a multisystem disorder of connective tissue, was described more than a century ago. Ground-breaking advances in the understanding of MFS were punctuated by the discovery of fibrillin, the identification of the FBN-1 as the causative gene, and the dissection of molecular pathogenesis through the creative use of animal models. The role of TGFb signaling in fibrillinopathies and related disorders is the most recent mechanistic development. Respiratory system involvement, although not as well characterized as the classic skeletal, ocular, and cardiac manifestations, can be clinically consequential. The lung manifestations of MFS may provide a mechanistic window not only on the pathobiology of the syndrome but also on other lung disorders with similar presentations.

Keywords: Marfan's syndrome, lung, fibrillin, FBN1, TGFBR 1, emphysema

Introduction

In 1876, E. Williams, an ophthalmologist, described ectopia lentis in a sibling pair who were exceptionally tall and loose-jointed since birth *(1)*. The significance of this finding was initially obscure. However, in 1896, Antoine-Bernard Marfan, an eminent French professor of pediatrics described the case of a female child with classical tall stature, spidery fingers with contractures, and a long, narrow skull. She had kyphoscoliosis, chest wall deformity, and pulmonary tuberculosis *(2)*. The relationship between skeletal manifestations and ectopia lentis was described almost four decades later. The first description of aortic dilatation came from the Johns Hopkins Hospital in 1943. An excellent historical perspective has been written by Victor McKusick *(1)*, who did the seminal early work on this disorder and introduced the concept of heritable disorders of connective tissue. The genetic principle of pleiotropism (multiple phenotypic expressions of a single mutant gene) as it applied to the MFS was also appreciated by McKusick. In 1991, Dietz et al. *(3)* discovered that mutations in the *FBN-1* gene cause

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classical MFS. Over the next 15 years, the diagnostic requirements for MFS have been further refined with the current use of the Ghent nosology which mandates requisite combinations of major and minor criteria. Although pulmonary involvement, punctuated by bullous lung disease, chest wall restriction, and reduced exercise tolerance, is only represented as minor criteria, the respiratory phenotype has re-emerged as mechanistically consequential with the examination of murine models of MFS. In this chapter, we detail current understanding of the pulmonary phenotype of the disorder focusing on the clinical presentations, underlying pathogenesis, and implications for other non-syndromic diseases of the lung.

Prevalence

Classical MFS is probably underdiagnosed due to variability in clinical presentation, especially with age. The frequency is estimated at 2–3/10,000 *(4)*. Pleiotropy in MFS adds to difficulties with estimates of prevalence, and as patients are living longer, pleiotropy appears to be steadily increasing *(5)*.

Genetic Basis

Single-gene disorders represent the most facile investigative window into disease pathogenesis in that they (1) permit easy genetic modeling in robust, informative systems and (2) frequently lead to the identification of potential targets via pathway dissection. Unfortunately, very few lung disorders can be attributed to a single gene. However, one can apply the same approach to single-gene multisystem disorders that involve the lung, viewing the lung phenotype as an informative readout of a defined genetic disturbance. In this way, the dissection of the lung phenotype of Marfan syndrome has not only provided a more refined mechanistic model of the disorder but also expanded our understanding of the contribution of the extracellular matrix to lung morphogenesis.

Marfan syndrome (MFS) is a single-gene autosomal dominant disorder affecting ~1 in 5,000 persons. The culprit gene, fibrillin-1, is a 250 kB gene located on chromosome 15 that encodes a large extracellular matrix glycoprotein that functions as a critical structural component of the microfibrillar lattice *(3, 6, 7)*. Although the 350 kDa fibrillin-1 protein has several well-defined functional motifs, affected patients harbor mutations that span the length of the whole gene *(8)*. Most mutations are missense mutations causing cysteine substitutions within functional domains thought critical for microfibrillar function. Greater than 500 distinct mutations have been identified and are registered in the UMD-Fbn1 database and include both de novo and familial mutations *(8)*. Strong genotype–phenotype correlations have not been clearly established for the organ-specific manifestations of MFS.

Animal Models and Molecular Pathogenesis

Genetically defined animal models which replicate features of single-gene disorders and allow mechanistic interrogation have been critical reagents for the molecular and physiologic characterization of various diseases. In the case of Marfan syndrome, such

models not only reinforced the role of fibrillin-1 in organ-specific disease expression but also resolved major inconsistencies in prevailing concepts of disease pathogenesis. The identification of FBN-1 as the causative gene in Marfan syndrome broadly implicated the extracellular matrix as the primary site of impairment. Furthermore, since the matrix has well-known structural functions in the affected sites (aorta, ocular lenses, lung, etc.), a pathogenetic model that invoked a structurally compromised tissue ultimately collapsing under cumulative stressors seemed coherent and plausible. Since microfibrils were thought to function as scaffolds for elastin deposition in many organs, the loss of fibrillin might reduce tissue integrity by impairing elastin lamellar formation. However, some features of the syndrome did not follow from this paradigm, including bone overgrowth, craniofacial abnormalities, reduced adiposity, and apparent muscle hypoplasia. Thus, to account for the mechanistically disparate multisystem phenotypes, a more elaborate conception of microfibrillar function had to be considered. Animal models of Marfan syndrome provided the reagents for this type of interrogation. The genetically targeted murine models of fibrillin deficiency represent an allelic series that largely replicate the full spectrum of the syndrome *(9–12)*. They all display selective aspects of the Marfan phenotype, differing only with respect to the severity of the organ-specific phenotypes and lifespan (Table 10.1). Notably, defects in the aorta and lung exist in all four models.

Table 10.1 Published genetically targeted fibrillin-1-deficient mouse strains.

Genotype	WT fibrillin levels (% of controls)	Mortality	Lung phenotype	References
mg△/mg△	Null; 5% mutant transcript	PD7-10 k	Neonatal airspace enlargement	*(11, 13)*
mgR/mgR	15%	3–6 months	Neonatal airspace enlargement, peribronchiolar inflammation at 4–6 months	*(12, 13)*
C1039/+	<50%	3–6 months	Neonatal airspace enlargement, progressive airspace enlargement in adult	*(10)*
mgN/mgN	Null	PD10-14	Neonatal airspace enlargement	*(9)*

PD – Postnatal

Marfan Mice Implicate Novel Functions for Fibrillin-1

Two remarkable findings in the first Marfan model, which harbored a targeted deletion of exons 19–24 of fibrillin-1, led to a revision of previously held notions of microfibrillar pathobiology. First, the discovery of initial preservation of elastin content and deposition but early and widespread fragmentation in the aortas of mutant mice implicated a critical role for microfibrils in elastin homeostasis, but not elastin deposition. Second, the detection of early airspace enlargement without inflammation, evidence of destruction, or reduced elastin content or deposition strongly invoked a developmental context

to the lung phenotype *(13)*. These two seminal findings prompted the consideration of non-elastin, non-structure-dependent roles for fibrillin-1 and the microfibrillar lattice in the Marfan phenotype. The lung phenotype also availed a quantifiable readout (airspace caliber) which could be used to assess this novel developmental function of fibrillin-1.

How might fibrillin-1 participate in a developmental program in the lung? More importantly, would such a role have implications for the multisystem manifestations of Marfan syndrome? In the vertebrate lung, a stereotyped series of temporospatially defined morphogenic events describe lung maturation *(14)*. In the murine system, the lung anlage is generated from the lateral primordial endoderm at embryonic day (ED) 9.5. Subsequently, the endoderm undergoes a series of dichotomous branching to form the airway structures and the investing vasculature and interstitium by ED16. Airspace formation punctuated not by branching but by alveolar septation, occurs during the late phase of embryonic development (ED18) and proceeds through the first 2 weeks of postnatal life. Given this ontogeny, molecular or signaling disturbances that occur at distinct developmental time points may have predictable morphologic sequelae. Two fibrillins are expressed in the murine lung: fibrillin-1 and fibrillin-2 *(15)*. Fibrillin-1 is expressed primarily in the lung parenchyma (airspaces and microvasculature) during the late phase of embryonic growth. By contrast, fibrillin-2, a highly homologous protein, is expressed in the proximal airway epithelium during the early to mid-phase of embryonic development. Thus, conceding that there might be some degree of functional redundancy between the fibrillins and that fibrillin-1 is involved in lung maturation, one would predict that fibrillin-1 deficiency might have selective effects on the formation of the distal lung and airspaces. The observation of a distinct airspace phenotype in fibrillin-1-deficient mice was fully consistent with the ontogeny of the protein, suggesting that fibrillins, in particular, and microfibrils, in general, participate in the lung developmental program.

Fibrillin-1 Deficiency – Model of an Active Matrix in Lung Morphogenesis

Alterations in lung development, especially as evidenced by findings in genetic model systems, typically reflect cell signaling abnormalities that disrupt the temporospatial cues that are critical to a lung tissue development program *(16, 17)*. Since fibrillin-1 harbors signaling domains that are homologous to latent TGFb binding proteins, LTBPs (latent TGFb binding proteins), TGFb signaling disturbances were explored as a candidate mechanism for the airspace defects in fibrillin-1-deficient mice *(18, 19)*. This concept was novel as microfibrils were thought to primarily function as scaffolds for elastin deposition, effectively connecting elastin to cells and basement membranes *(3, 20)*. However, since genetic and acquired alterations in TGFb signaling can cause marked defects in lung development, reviewed in *(21)*, the lung was a uniquely informative system to explore this candidate mechanism. Assessment of TGFb activation in neonatal fibrillin-1-deficient lungs, using an antibody specific for active TGFb as well as a fluorescent transgenic reporter allele, demonstrated enhanced activation compared with wild-type mice *(13)*. Furthermore, excessive apoptosis accompanied the airspace phenotype suggesting that the pro-apoptotic effects of TGFb might contribute to the airspace enlargement in these mice. Antagonism of TGFb with a neutralizing antibody rescued airspace septation in fibrillin-1-deficient mice, consistent with TGFb dysregulation playing a critical role in the generation of the developmental airspace phenotype. Using a variety of

methods, increased TGFb signaling has also been observed in the lungs of mgR/mgR and C1039/+ mice, two other fibrillin-1-deficient strains that exhibit developmental airspace enlargement *(13, 22)* and unpublished observations (ERN and F. Ramirez). Importantly, increased TGFb signaling seemed to contribute to multiple other manifestations of fibrillin-1 deficiency, including atrioventricular valve pathology and skeletal muscle weakness *(23, 24)*.

Mechanism for TGFb Dysregulation in MFS

How might fibrillin-1 deficiency, which occurs in MFS pts and murine models of MFS, result in increased TGFb signaling? As mentioned above, even though the homology between the fibrillins and the LTBPs is well established, the role of TGFb in fibrillin-1-deficient phenotypes was initially unclear. The TGFb/BMP superfamily members are multifunctional cytokines that initiate various receptor-mediated intracellular cascades resulting in cell-specific morphogenic sequelae *(25, 26)*. TGFb isoforms (1, 2, and 3) are each secreted as a latent complex that is sequestered as an inactive assembly within the extracellular matrix *(27)*. This latent complex is comprised of the mature cytokine associated with a propeptide and frequently a latent TGFb binding protein. Upon activation by thrombospondin, proteases, integrins, or oxidative stress, the mature cytokine is released from the complex and is able to productively engage cell surface receptors. By this scheme, if the microfibrillar lattice, containing fibrillin-1, is the site of TGFb sequestration, then the breakdown of this lattice might alter TGFb abundance and/or state of activation and secondarily result in inappropriate signaling. Since MFS is associated with both a functional and a quantitative deficiency in fibrillin-1, this constellation could reasonably confer increased TGFb signaling.

An unresolved issue concerning the airspace lesion is whether excess TGFb signaling has distinct effects in the developing versus the mature lung. Experimental evidence from our lab and others supports adverse sequelae from dysregulated TGFb in the perinatal lung, primarily manifest in developmental arrest of airspace maturation *(28–30)*. In studies by others using transgenic mice overexpressing TGFb, enhanced signaling induces both matrix production and matrix turnover resulting in a fibrotic phenotype accompanying the airspace simplification *(31)*. Although matrix deposition in the adult fibrillin-1-deficient lung has not been well characterized, the lack of lung fibrosis in developing fibrillin-1-deficient lungs may reflect immaturity in the cellular compartments required for induction of a full fibrotic phenotype or a relatively low level of increased TGFb signaling (i.e., elevated but not meeting a fibrotic threshold).

Airspace Septation Defects and Adult Emphysema

Even though fibrillin-1-deficient mice exhibit neonatal airspace enlargement, a major mechanistic question is whether such a phenotype persists to adulthood. Given that Marfan syndrome associated bullous disease and pneumothorax is seen in children and adults, one would predict that the airspace lesion in the mice, if truly reflective of the human disorder, should be persistent or progressive. Mice that express low levels of fibrillin-1 from a genetically targeted hypomorphic allele but that survive to adulthood allowed a full exploration of the evolution of the lung lesion *(12)*. The hypomorphic allele resulted in a wild-type protein expressed at ~15% of the level of the endogenous fibrillin-1 protein. Despite the fact that the aortic lesion was milder and developed in

a more protracted fashion compared with the mice with greater functional deficiency in fibrillin-1, the neonatal airspaces in these mice were enlarged (unpublished observations, ERN). More importantly, the airspace caliber progressed throughout adulthood and in the late stages manifested inflammation and metalloprotease induction reminiscent of acquired emphysema *(13)*. This finding established an important paradigm that developmental disorders of airspace formation can lead to adult phenotypes that approximate acquired emphysema. Thus, in addition to the known risk factors for adult emphysema, such as cigarette smoking, anti-protease deficiency, toxic environmental exposures, one must also consider childhood disorders of airspace formation as conferring increased risk. The recent discovery of the genetic contribution of fibulin-5 deficiency to cutis laxa associated adult emphysema coupled with the demonstration of developmental airspace enlargement in the Fib5-targeted murine model supports this paradigm *(32–34)*.

Fibrillin-1 Deficiency and Respiratory Muscle Dysfunction

An emerging aspect of the multiorgan phenotypes exhibited by these models is the issue of musculoskeletal function. Careful dissection of skeletal muscle morphology and function in the C1039G/+ model showed a profound defect in muscle regenerative responses *(23)*. This phenotype was evident in axial musculature as well as the diaphragm of adult mice. Since reduced exercise tolerance and low lung capacity is a common feature of the clinical Marfan phenotype, a potential contribution of underdiagnosed diaphragmatic dysfunction is plausible. Murine models which survive to adulthood also have skeletal defects in the thoracic region that may functionally simulate the lung restriction seen in many patients with MFS *(10, 12)*. Consequently, further investigation of the respiratory musculoskeletal phenotype in the Marfan animal models should prove informative about the basis of the reduced lung capacity observed in this disorder.

Mouse Versus Human Lung Phenotype

The early airspace enlargement phenotype, with different degrees of severity, has been observed in all of the Marfan models. Given that infants with the most exaggerated effects of the disorder, neonatal Marfan syndrome, for example, frequently have pulmonary emphysema, one could postulate that the lung is particularly vulnerable to the developmental effects of fibrillin-1 deficiency. The observation that spontaneous pneumothorax, likely from a ruptured airspace bulla, frequently occurs in adolescents with Marfan syndrome is consistent with this concept of a primary disturbance in airspace formation. It remains unclear why the airspace phenotype is so penetrant in murine models but relatively uncommon (<15%) in patients with Marfan syndrome. An attractive possibility is that the human lung may express other fibrillins that typically compensate for fibrillin-1 deficiency. For example, fibrillin-3, a third fibrillin homologue, was recently identified and was found to be expressed in humans (prominently in the lung) but not in mice *(35)*. Studies delineating its role and degree of functional redundancy among the fibrillins will be of great interest. Another explanation for the human-mouse discrepancy is the lack of histological data documenting the presence of airspace enlargement in patients with MFS. Lung biopsies are not typically obtained

in these patients during aortic surgery. Lung function studies in patients with MFS are not the most sensitive screen for mild-to-moderate airspace disorders and may also be confounded by associated lung restriction from musculoskeletal abnormalities. Airspace disease is typically invoked only in patients who present with spontaneous or recurrent pneumothoraces. Accordingly, defects in airspace formation may be vastly underdetected and underreported in the MFS population.

Translation of Findings from the Marfan Lung

Losartan Studies

Once excessive TGFb signaling was shown to be a causative signaling disturbance in fibrillin-1 deficiency and antagonism of TGFb signaling was found to rescue several multisystem pathologies, TGFb-blocking reagents which could potentially be used in a clinical setting were of great interest. The exploration of losartan as a candidate therapy for MFS manifestations followed from compelling experimental evidence in a variety of model systems that angiotensin II antagonism reduces TGFb signaling *(36–40)* and the longstanding use of losartan as an antihypertensive agent without significant side effects or toxicities. The TGFb-blocking effect appears complex involving both ligand and receptor interactions. Losartan treatment of fibrillin-1-deficient C1039G/+ adult mice rescued aortic morphology and prevented aneurysm development *(22)*. Importantly, airspace enlargement was attenuated in mutant mice started on treatment as adults, suggesting that therapeutic intervention was of value even after the airspace lesion was established. Although Losartan reduced TGFb signaling in this model, the full dissection of the tissue-specific mechanisms of rescue and possible cooperative effects between the angiotensin and TGFb cascades are ongoing. An interesting prospect is that therapies targeting both of these pathways may have efficacy not only for MFS-associated lung disease but also for other disorders of airspace formation.

Fibrillin-1 Deficiency as a Paradigm for Emphysema and Disorders of Lung Prematurity

Disturbances in TGFb signaling have been observed in acquired emphysema and in lung simplification of prematurity (bronchopulmonary dysplasia), two common but complex disorders with both genetic and environmental causation *(41–45)*. Translational findings in the fibrillin-1-deficient model, which displays many features of both of these disorders, can potentially be extrapolated to these much more common and clinically burdensome disorders. Accordingly, signaling pathways validated in the fibrillin-1-deficient model should be explored as candidate targets for both emphysema and bronchopulmonary dysplasia. From a clinical perspective, targeting the TGFb cascade has to be approached with great caution in the lung as either excessive or deficient TGFb signaling can impair normal lung morphogenesis. Therefore, genetically defined animal models, such as fibrillin-1-deficient mice, can be used as critical tools in the therapeutic examination of such agents.

Clinical Presentation

The skeletal, ocular, and cardiac manifestations are the most common presenting features in MFS. These have been extensively reported upon and are beyond the scope of this chapter. Pulmonary manifestations are less common but frequently described in case reports or small series. The apparently low prevalence of lung involvement is somewhat surprising since the dry weight of the lung is mostly composed of connective tissue, primarily type I collagen and elastin *(46)*. A classification has been proposed here in an attempt to create a systematic approach, possibly based on pathogenetic mechanisms or structures involved (Table 10.2).

Table 10.2 Pulmonary manifestations and reported associations.

1. Lung and Pleural Abnormalities
   1.1. Spontaneous pneumothorax
   1.2. Subpleural bullae
   1.3. Emphysema
   1.4. Upper lobe fibrosis[a]
   1.5. Cystic lung disease
   1.6. Pulmonary tuberculosis[a]
   1.7. Malignant mesothelioma[a]
2. Airway Manifestations
   2.1. Primary ciliary dyskinesia[a]
   2.2. Cystic bronchiectasis[a]
   2.3. Airway hyperresponsiveness
   2.4. Tracheobronchomegaly[a]
   2.5. Tracheobronchomalacia[a]
   2.6. Tracheal collapse[a]
   2.7. Tracheal stenosis from aortic aneurysm
   2.8. Endotracheal Castleman disease[a]
3. Sleep disorders
   3.1. Obstructive sleep apnea
   3.2. Snoring
4. Musculoskeletal Developmental Abnormalities with Respiratory Consequences
   4.1. Pectus excavatum
   4.2. Kyphoscoliosis
   4.3. Myopathy with respiratory failure
   4.4. Diaphragmatic hernia/eventration
5. Pulmonary Vascular Issues and Respiratory Failure
   5.1. Dilatation of pulmonary artery
   5.2. Compression of right pulmonary artery
   5.3. Cor pulmonale
   5.4. Acute respiratory failure
   5.5. Chronic respiratory failure
6. Congenital malformations
   6.1. Rudimentary right middle lobe
   6.2. Mono- or bi-lobed left lung
   6.3. Pulmonary aplasia[a]
7. Pulmonary Function Abnormalities

[a] Isolated case reports or small case series that do not prove an association with MFS.

Lung and Pleural Abnormalities

Spontaneous Pneumothorax, Subpleural Bullae, and Apical Pulmonary Fibrosis

Spontaneous pneumothorax remains one of the best recognized and commonest pulmonary manifestations in the MFS *(47–50)* (Table 10.2). The mechanism of bullous disease formation is unclear as there appears to be no immunohistochemical difference in collagen type I or elastin abundance or distribution in the lungs of patients with the MFS compared to controls. However, spontaneous pneumothorax has been reported in other connective tissue disorders such as the Ehlers–Danlos syndrome and cutis laxa. In a retrospective study of 249 patients from one center, the frequency of pneumothorax in MFS was 4.4% in patients aged above 12 years *(51)*. More than half the patients had bilateral or recurrent pneumothoraces. Apical bullae were seen on chest films in 9 of 11 patients. In this survey, no case of spontaneous pneumothorax occurred before age 13. Males were more commonly affected. Interestingly, all patients whose pneumothoraces did not resolve with chest tube placement had had to undergo resection. As this study was done before the widespread use of CT scans, these authors recommended getting a chest radiograph in all adolescents and adults with MFS. A more recent study reported four children with MFS and spontaneous pneumothorax *(52)*. Of note, these authors advocated the use of screening CT scans for optimal detection of potentially treatable lesions and recommended surgical intervention if the pneumothorax did not resolve within 5 days after placement of an intercostal catheter. Of four patients taken to surgery, two had bullae while one patient had pulmonary fibrosis with no bullae. Recently VATS resection of a giant bulla occupying almost an entire hemithorax was reported in a patient with Marfan's syndrome, severe kyphoscoliosis, and limited pulmonary reserve *(53)*. The experience gained in VATS LVRS should lead to better surgical outcomes, and make surgery possible in patients with compromised lung function, which is common in patients with MFS-related restrictive lung disease. Interestingly, spontaneous pneumothorax has been described in young males with thin, asthenic body habitus, and long and narrow chest walls in the absence of disproportionately long limbs or other classical features of Marfan syndrome. An autosomal-dominant inheritance has been proposed without the cosegregation of a single *FBN1* allele in three pedigrees of spontaneous pneumothorax *(54)*. The role of concomitant spinal deformities in the genesis of pneumothorax in MFS is unclear, though the two have been associated in some reported cases. An important therapeutic consideration in patients with MFS who develop pneumothoraces is the tailoring of interventions to the possibility of future aortic surgeries (aortic replacement, valvular replacement, etc). Since chemical pleurodesis typically results in widespread pleural adhesions which might complicate future aortic procedures, mechanical pleurodesis should be the procedure of choice. In conclusion, all persons presenting with spontaneous pneumothorax should undergo a detailed clinical history and exam to rule out the possibility of connective tissue disorders, both hereditary and acquired. Furthermore, patients with MFS who develop spontaneous pneumothoraces should have a thorough assessment for pulmonary lesions that might be amenable to surgical intervention. In addition to pneumothoraces, upper lobe fibrosis has been noted both radiologically and pathologically in several studies of MFS patients, albeit in a small number of patients *(49, 51)*.

Cystic Lung Disease

Radiographic and autopsy findings of cystic lung disease have been described in patients with MFS *(55, 56)*. No specific clinical features can be developed from these isolated case reports and no speculation as to mechanism and causality can be made. Since pulmonary emphysema and lung cysts can occasionally be difficult to distinguish, some of these cases may represent the aforementioned bullous lung disease.

Pulmonary Tuberculosis

By report, the first patient described by Marfan had radiological findings of pulmonary tuberculosis. There have been sporadic cases reported since, from tuberculosis endemic regions *(57, 58)*.

Airway Abnormalities

Bronchiectasis

There have been few reports of bronchiectasis in MFS *(49, 59–61)*. It is not clear from these case reports whether bronchiectasis is a part of the constellation of Marfan syndrome and what the mechanism might be. There is, however, a case report of primary ciliary dyskinesia in a case of MFS *(62)*.

Acquired Tracheobronchomegaly (TBM)

A single case of TBM in an adult "marfanoid" patient leading to respiratory failure has been reported *(63)*. Although this patient had a past history of Hodgkin's disease and had received mantle radiation, it is plausible that Marfan syndrome was responsible for the TBM, as cases have been described with other disorders including cutis laxa and Ehlers–Danlos syndrome.

Tracheomalacia, Tracheal Compression, and Tracheal Collapse

Well-documented cases of tracheomalacia and transient tracheal obstruction occurring intraoperatively in cases of MFS undergoing correction of scoliosis have been described *(64)*. These patients were usually in the prone position during surgery, and therefore difficult to oxygenate and ventilate. In one case it was clear that the pressure from surgical instruments caused increased tracheal compression, while another patient with tracheal stenosis due to compression from an ascending aortic aneurysm had a marked difficulty in ventilation after induction of anesthesia and placing in the lateral position. Chronic tracheal compression from ascending aortic aneurysm has been well described in case reports *(65)*. A single case of localized endotracheal Castleman disease in a 50-year-old female with MFS has been described *(66)*. She presented with progressive respiratory distress and an enlarging mass producing bibasilar atelectasis. This was likely a coincidental association and the authors proposed no mechanism explaining the association.

Bronchial Hyperreactivity

In order to assess airway reactivity in a pediatric cohort of MFS patients, pulmonary function tests (PFTs), bronchodilator responsiveness, and methacholine challenge testing were performed in 11 children with MFS and an equal number of normal children. An unexpectedly high prevalence of reduced FEV_1 was noted in children with MFS, with an even higher reduction in $FEF_{25–75\%}$ and $FEF_{50\%}$ detected on exposure to methacholine. A similar frequency of bronchodilator response was seen between the two groups, more so in the small airways parameters *(67)*. Most patients were asymptomatic. Since a family history of asthma was present in five patients with MFS and all patients were receiving atenolol, these factors could obviously confound the significance of bronchial hyperreactivity in this small cohort. The authors do, however, propose the possibility of small airways abnormality related to laxity of connective tissue. Small airway obstruction frequently accompanies lung restriction because of the anatomic constraints of breathing at low lung volumes, and the presence of a restrictive physiology related to musculoskeletal causes in MFS could presumably produce abnormalities on measurement.

Sleep Disturbances

A group from Australia has published considerably on obstructive sleep apnea (OSA) in MFS, beginning with their initial report in 1991 *(68)*. They found the prevalence of OSA was significantly higher in adult patients with MFS compared with age-, height-, and weight-matched controls. BMI was normal in these subjects, and interestingly there were premenopausal women in those with OSA. Most patients have snoring and mild-to-moderate hypersomnolence *(69)*. More recently another group subjected patients with MFS and Ehlers–Danlos syndrome (EDS) to the Epworth Sleepiness Scale and the SF-36 health-related QOL questionnaire *(70)*. Sleep apnea was exclusively reported in MFS patients while periodic limb movements were more frequent in the EDS. This group found that sleep complaints were not uncommon in both studied groups compared to controls and correlated well with QOL items by SF-36. Several mechanisms for the higher than expected prevalence were postulated by Cistulli's group, including easily collapsible upper airways as an airway manifestation of excessively floppy tissues, high arched palate encroaching on the nasal cavity producing increased nasal resistance, and higher prevalence of retrognathia. These authors went on to show that there was indeed increased upper collapsibility as measured by a reduction in upper airway closing pressures (UACP) in slow wave sleep, though the number of subjects in the study was small and there were a few control subjects that showed the same phenomenon *(71)*. Nasal airway resistance was shown to be twice that of controls and was inversely related to two lateral maxillary measurements. There was additionally a modest correlation between various maxillary measurements and the apnea–hypopnea index *(72)*. Based on dental impressions and various measurements derived from them, these authors have suggested the term "high arched palate" is not an accurate descriptor, and it is the relationship of the height of the palate to the constriction of the lateral maxilla that is more relevant. They also found that palatal height did not correlate with nasal resistance and nasal resistance did not correlate with the apnea–hypopnea index. Thus, the best anatomic and functional measure which can be used to gauge risk for OSA in this population is unclear. A case report of a patient with MFS and

retrognathia, who presented with loud snoring, agitated sleep, arousals, daytime hypersomnolence, and fatigue-documented improvement in all these parameters with surgical correction *(73)*. Cistulli's group showed that multiple craniofacial abnormalities were present in 13 of 15 consecutive MFS patients with OSA *(74)*. Of these multiple abnormalities described, univariate analysis showed a correlation between the AHI and total anterior face height, upper anterior and posterior face heights, and the mandibular length.

Two groups have shown attenuation of progressive aortic root dilatation by treating OSA with nasal intermittent positive pressure *(75, 76)*. It is thought that systemic blood pressure rises during the latter part of an apnea, and the marked negative intrathoracic pressure during snoring and apneas could produces aortic dilatation due to elevated systolic aortic pressures and aortic transmural pressure. In summary, given the high prevalence of sleep-disordered breathing in the MFS and the long-term potential vascular sequelae, the clinician should maintain a high index of suspicion, obtain a detailed sleep history, and formal polysomnography in selected patients.

Musculoskeletal and Developmental Abnormalities with Respiratory Consequences

Pectus Excavatum

Pectus excavatum (PE) is one of the most common chest wall deformities in MFS. Although pectus abnormalities constitute one of the minor criteria in the Ghent nosology, if they are severe enough to require surgery, they move up to the list of skeletal abnormalities needed to establish a major diagnostic criterion for MFS. Severe PE can cause ventilatory defects and requires surgical correction. A pectus severity index (PSI) has been established and requires measurements based on CT scans. However, most patients with PE are asymptomatic and the reduced exercise capacity is frequently attributed to cardiovascular factors including deconditioning. In a study involving patients with PE (1 of 15 patients had MFS), pulmonary function improved with minimally invasive repair by the Nuss technique. There was no statistical correlation with the PSI preoperatively, though there was a trend toward greater improvement with a higher PSI. It has been postulated that benefit may be greater from cardiovascular performance than from improvement in ventilatory abnormalities, although few large studies documenting pre- and post-surgical cardiopulmonary function have been published *(77)*.

Kyphoscoliosis

In a retrospective review of 600 patients who had Marfan syndrome, 14 had infantile scoliosis *(78)*. Mean curvature was 38º, and bracing did not prevent progression, unlike idiopathic scoliosis in this age group. The authors recommended surgical correction for severe disease with curvature greater than 40°. It is well established that uncorrected severe scoliosis results in respiratory failure later in life. In fact, older studies have indicated that when the external angle of scoliosis is >100°, kyphosis is >20°, and VC < 1 l, cor pulmonale can result. In this series, there were several deaths, due to known or presumed cardiac comorbidity. Based on a larger subset, Sponseller's group

has recommended evaluation of cardiopulmonary status, preoperative CT to assess bone adequacy for fixation, and MRI to evaluate dural ectasia in these patients prior to surgical correction *(79)*.

Myopathy with Respiratory Failure

Hypotonia and myopathy have been described in MFS. Several histological and structural defects described in the Marfan muscle have been validated using a mouse model with a missense mutation in fibrillin *(80)*. The extent to which the muscle phenotype contributes to physiologic restriction, reduced exercise tolerance, and complaints of dyspnea in MFS patients is unknown.

Diaphragmatic Hernia and Eventration

Congenital diaphragmatic abnormalities are uncommon in MFS. A few reports detail congenital diaphragmatic eventration, especially in the neonatal form, nMFS (MIM 154700). In one case, mutation in exon 25 of the FBN1 gene was found and associated with bilateral ureterohydronephrosis and bladder dilatation *(81)*. Neonatal intrathoracic stomach has been described in three cases *(82, 83)*. It is postulated that fibrillin deficiency during fetal development may be responsible for the diaphragmatic defect. A mechanistic possibility is that since hepatocyte growth factor (HGF) is a critical mediator of diaphragmatic fusion and TGFb is known to antagonize HGF signaling, the enhanced TGFb signaling observed in MFS may participate in the generation of congenital diaphragmatic lesions. Nonetheless, early diagnosis and surgical correction is recommended by one group to avoid ischemic necrosis *(84)*.

Pulmonary Aplasia

Various malformations and developmental abnormalities have been described in the MFS including malformed or absent right middle lobe, mono- or bi-lobed left lung, and pulmonary aplasia. Some have been tabulated in an early paper by Dwyer and Troncale *(47)*.

Pulmonary Vascular Issues and Respiratory Failure

Although aortic enlargement is the most defining vascular lesion in MFS, primary pulmonary artery pathology is quite common but typically less clinically consequential. However, secondary pulmonary vascular compromise from aortic mass effects or from a chronic reduction in ventilatory capacity can occur. Case reports of occlusion of the right pulmonary artery by an ascending thoracic aortic aneurysm, giving rise to absent unilateral perfusion and the false impression of unilateral pulmonary embolism, have been reported *(85, 86)*. Cor pulmonale can occur secondary to severe untreated kyphoscoliosis *(87)*. A single case of a thin saccular aneurysm of the pulmonary artery with virtually absent cuspal tissue in the pulmonic valve has been described *(88)*. Myxomatous medial degeneration of the pulmonary artery has also been described *(89)*.

Pulmonary Function Abnormalities

Studies in very small numbers of patients have described varied abnormalities in lung function-related primarily to musculoskeletal abnormalities or coexisting bronchiectasis, pulmonary fibrosis, or infections *(90, 91)*. In a systematic study involving 79 patients, some with age-matched controls, it was found that patients with MFS had a lower FVC and TLC when referenced to their standing height. These values were normal when sitting height was used in the predicted calculations, in the absence of moderate-to-severe pectus excavatum or scoliosis. The authors point out that sitting height probably more accurately reflects thoracic cage size and ignores the disproportionate contribution of the long legs to the height in subjects with the MFS *(92)*. As expected, a moderately severe ventilatory abnormality was seen in patients with moderately severe pectus excavatum or scoliosis. Unfortunately, normograms based on sitting height may not be easily available in available in all populations.

Pulmonary Considerations in the MFS Patient Undergoing Surgery

In patients with MFS undergoing surgery for scoliosis or kyphosis, careful evaluation of cardiac and pulmonary status is recommended, and an experienced anesthesiologist is desired *(93)*. Occasionally, excessive laxity of the cervical spine occurs, and care must be taken not to injure the spine during intubation. If chest wall deformities such as pectus excavatum or pectus carinatum are severe, these will have to be taken into consideration before cardiovascular surgery. There have been successful repairs to cardiac structures and the chest wall performed successfully in the same sitting *(94)*. Spontaneous pneumothorax may occur after cardiovascular surgery and extreme care should be taken to look for it during mechanical ventilation and in the perioperative period. Prolonged air leaks have been described in this setting. Bilevel noninvasive mechanical ventilation has been utilized successfully in the setting of post-extubation respiratory failure following aortic root replacement in an oxygen- and CPAP-dependent patient with severe chest wall deformity *(95)*.

Diagnostic Approach

The diagnosis of the Marfan syndrome has gone through several refinements to impose greater stringency, given that certain phenotypic characteristics are shared by several disorders. The first attempt to systematize diagnostic criteria in a consistent manner and to help prognosticate were put forward in the Berlin criteria of 1988. These were further revised by the Ghent nosology *(96)*, which has been in use since. This diagnostic algorithm relies on the assignment of major and minor criteria observed in various organ systems. The instrument further incorporated family history and a greater reliance on positive skeletal findings. In the absence of a positive family history, a diagnosis of MFS requires the presence of a major criterion in two systems and involvement in a third system. There are several heritable and nonheritable disorders of connective tissue that share one or more clinical features with MFS (Table 10.3). Some of these include MASS phenotype (familial mitral valve prolapse, myopia, minimal or no aortic dilatation, subtle skeletal changes, and striae atrophicae: OMIM 157700), homocystinuria (OMIM 236200), familial aortic aneurysm (dilatation and dissection of the aortic root: OMIM 132900), familial ectopia lentis (autosomal dominant, may be accom-

Table 10.3 Marfan syndrome related disorders and related genes.[a]

Disorder	Gene
Marfan syndrome	*FBN1, TGBR1, TGBR2*
Neonatal Marfan syndrome	*FBN1*
Familial thoracic aneurysms and dissections	*FBN1, TGFBR1, TGFBR2*
Isolated ectopia lentis	*FBN1*
Shprintzen–Goldberg craniosynostosis syndrome	*FBN1, TGBR2*
Autosomal dominant Weill-Marchesani syndrome	*FBN1*
Loeys–Dietz syndrome	*TGFBR1, TGFBR2*

[a] Mizuguchi and Matsumoto *(99)*.

panied by mitral valve prolapse and skeletal features: OMIM 129600), familial tall stature, Shprintzen–Goldberg syndrome (skeletal, ocular, and cardiovascular features of MFS with craniostenosis and ocular proptosis: OMIM 182212), congenital contractural arachnodactyly (arachnodactyly, malformed ears, contractures of digits, elbows, and ears: OMIM 121050), Weill–Marchesani syndrome (OMIM 277600). From a conservative viewpoint, these disorders represent a continuum of disease plausibly reflective of differences in microfibrillar function and abundance. Efforts to model these disorders using genetically targeted mice should provide greater clarity on the molecular basis for these varied phenotypes.

Conventional Management and Treatment

The management of MFS is best dealt with by a physician who is experienced in the management of this disorder. A team approach is necessary, often involving closely coordinating care with geneticists, ophthalmologists, cardiologists, cardiothoracic surgeons, and spine surgeons who have special expertise in dealing with medical problems in this cohort. Given that the pulmonary manifestations are uncommon, pulmonologists that have experience with connective tissue disorders should be sought when such issues arise. With careful attention to medical issues and appropriate prophylactic therapy, some patients can expect and achieve normal life expectancy. For many years, beta-blocker therapy to prevent the progression of aortic enlargement was considered the standard of care for MFS patients with aortic involvement. However, the use of beta-blockers was based on limited objective clinical data establishing its efficacy. Recently, there has been interest in the use of losartan, an AT1 antagonist, as an alternative to beta-blockers. Angiotensin receptor blockers not only reduce aortic shear stress but also antagonize TGFb signaling, a pathway thought to be dysregulated in MFS. A seminal paper recently demonstrated that losartan treatment prevents aortic aneurysm formation in the mouse model of Marfan syndrome *(97)*. Although small studies have supported the use of agents which antagonize angiotensin signaling in MFS, an NIH-sponsored randomized clinical trial of ARBs versus beta-blockers is currently ongoing. Given that many patients with MFS are intolerant of beta-blockers, the potential incorporation of angiotensin receptor blockade or ACE inhibition into the therapeutic armamentarium would be of value.

Future Directions and Therapeutic Targets

In summary, a variety of pulmonary abnormalities occur in patients with Marfan syndrome. Clinicians who care for these patients need to have a heightened awareness of these manifestations in order to diagnose them early and potentially initiate preventative or ameliorative treatments. From a molecular standpoint, the examination of the Marfan lung phenotype has provided a mechanistic window into a more sophisticated understanding of the multisystem manifestations of the disorder. The initial characterization of the lung phenotype in murine models led directly to the identification of TGFb as a candidate therapeutic target. Importantly, even though the extracellular matrix is known to play a critical role in airspace homeostasis, findings from examination of the Marfan lung in murine models suggest a delicate interplay between matrix elements and well-conserved signaling pathways. These interactions likely serve as a prototype for many multiorgan genetic disorders of connective tissue with lung manifestations. Thus, the Marfan lung story underscores the value of detailed examination of minor phenotypes in multisystem single-gene disorders. The study of MFS and its structural and genetic abnormalities has given a new insight into the pathogenesis of emphysema. The stage is probably set for further work based on current knowledge of the structure and function of fibrillin and the mutations in the FBN-1 and FBN-2 genes. In a recent publication by Robbesom et al., the expression of fibrillin-1 was studied in 69 human lung specimens from patients with early-onset emphysema *(98)*. Aberrant fibrillin-1 staining was strongly correlated with the degree of destruction of the parenchyma, with no correlation with age and smoking.

Marfan Syndrome Patient Organizations

The first and second international Marfan symposia were held in Baltimore and San Francisco in 1988 and 1992, respectively. During the second symposium an International Federation of Marfan Syndrome Organizations (IMSFO) was formed. The IMSFO facilitates dissipation of knowledge, updates diagnostic methods and therapies, and supports research in to the MFS. The development of the Berlin and Ghent nosologies was supported by the IMFSO. The current web site www.marfanworld.org was launched by the IMSFO and has links to several local organizations around the world, research opportunities, and grant application tools. Communication among medical professionals and the general public, research centers, and researchers is an important goal.

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11

Surfactant Deficiency Disorders: SP-B and ABCA3

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Abstract Single gene disorders disrupting surfactant metabolism and resulting in acute and chronic lung disease have been identified in recent years. This review focuses on lung disease resulting from mutations in the genes encoding surfactant protein B (SP-B) and member A3 of the ATP-binding cassette (ABCA3) family of membrane transporters. The roles of these proteins in surfactant metabolism is reviewed, along with the epidemiology, molecular genetics, clinical features, natural history, lung pathology findings, approach to diagnosis, and treatment of the disorders resulting from mutations in these genes. Irreversible neonatal hypoxemic respiratory failure due to diffuse parenchymal lung disease is a common presentation of both disorders. Children with ABCA3 mutations may also present with relatively milder disease and findings of interstitial lung disease. While rare, these disorders result in significant respiratory morbidity and mortality, have provided insights into normal surfactant metabolism, and implicate these genes as having a role in more common lung diseases.

Keywords: newborn, respiratory distress syndrome, persistent pulmonary hypertension of the newborn, interstitial lung disease, surfactant protein, genetic basis of disease, alveolar proteinosis, lung transplantation

Introduction

Pulmonary surfactant is the mixture of lipids and specific proteins needed to reduce surface tension at the air–liquid interface and prevent end-expiratory atelectasis. A common disease related to surfactant deficiency is respiratory distress syndrome (RDS) in prematurely born infants, where pulmonary immaturity results in inadequate production of surfactant components *(1)*. Surfactant inactivation due the presence of other proteins or lipids in the airspaces can also contribute to the pathophysiology of RDS in

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premature infants, as well as acute respiratory distress syndrome (ARDS) in older children and adults *(2)*. Surfactant deficiency in premature infants can be very effectively treated with modern ventilators and replacement therapy with mammalian-derived exogenous replacement surfactant preparations, such that mortality from RDS has declined dramatically in the past two decades *(3)*. However, in the past 10 years, it has been recognized that full-term infants with genetically determined deficiencies of specific proteins important in surfactant metabolism may develop severe lung disease that is refractory to treatment or results in chronic interstitial lung disease *(4–7)*. It is important for clinicians to have an understanding of these rare disorders as the morbidity and mortality due to these conditions is very high, and to be able to counsel families appropriately regarding prognosis and recurrence risk. In addition, these disorders are of interest to scientists interested in lung development and lung cell metabolism, for an understanding of their pathophysiology has provided insights into normal surfactant metabolism.

Pulmonary surfactant is synthesized, stored, and secreted by alveolar type II epithelial cells (AEC2) in the lung *(8–11)*. Within the type II cell, surfactant is stored in a lysosomally derived organelle called the lamellar body and is secreted by exocytosis after fusion of the lamellar body with the apical plasma membrane. Newly secreted surfactant appears in the thin layer coating the surface of the alveolus as a highly ordered structure called tubular myelin. Tubular myelin is thought to be the precursor from which the surfactant material then adsorbs to the air–liquid interface to form a monolayer *(12, 13)*.

As isolated from lung lavage, surfactant contains about 90% lipid by weight. These lipids are critical for its ability to lower surface tension, particularly dipalmitoylated (or disaturated) phosphatidylcholine (DPPC or DSPC), although other phospholipids, including phosphatidylglycerol (PG) and neutral lipids such as cholesterol, also have roles in augmenting the surface tension lowering properties of surfactant *(14)*. While surfactant is greatly enriched in DSPC, the enzymes responsible for DSPC synthesis are not lung or type II cell specific, and precisely how DSPC becomes concentrated in surfactant remains unknown. About 10% of surfactant by weight is comprised of protein, and while much of the protein is serum derived, specific proteins that are primarily expressed in the lung have important roles in surfactant function and metabolism. Four specific surfactant proteins (SP-) have been identified, SP-A, SP-B, SP-C, and SP-D *(15, 16)*.

SP-A and SP-D are structurally related hydrophilic proteins that are members of the collectin family, in that both contain a collagen-like domain and a carbohydrate-binding (lectin) domain. Both proteins form large multimers in the airspaces, and the genes for both are located on human chromosome 10, with two genes (*SFTPA1*, *SFTPA2*) contributing to SP-A and a single gene (*SFTPD*) encoding SP-D *(17)*. The primary roles for SP-A and SP-D appear to be in innate immunity rather than in surfactant surface tension lowering properties, although each may also have a limited role in surfactant metabolism *(16, 18)*. Both SP-A and SP-D bind to a wide array of microorganisms and facilitate their uptake by alveolar macrophages, and immunomodulatory roles for these proteins have also been recently recognized. Lung diseases due to genetic deficiencies of SP-A or SP-D have not been reported to date.

SP-B and SP-C are low molecular weight, extremely hydrophobic proteins that have essential roles in augmenting the surface tension lowering properties of surfactant lipids. Both SP-B and SP-C are derived from proteolytic processing of much larger

precursor proteins (proSP-B, proSP-C) and are encoded by single genes (*SFTPB*, *SFTPC*) on chromosomes 2 and 8, respectively *(15, 19)*. Addition of either SP-B or SP-C to purified or synthetic surfactant lipids yields a surfactant preparation that has good surface tension lowering properties in vitro and is effective in treating animals with experimental RDS, and SP-B and SP-C in varying amounts are important components of the animal-derived exogenous surfactant preparations used to treat infants with RDS *(20)*. Mutations in both *SFTPB* and *SFTPC* result in human lung disease *(7)*; SP-C related interstitial lung disease is covered in a separate chapter.

An important role in surfactant production for member A3 of the ATP-binding cassette (ABCA3) family of transporters has recently been recognized. The ABC transporters are transmembrane proteins that hydrolyze ATP to mobilize a wide variety of substrates across biological membranes, with the ABCA subfamily often involved in lipid transport *(21, 22)*. ABCA3 mRNA is expressed in multiple tissues, however, expression is particularly high in type II cells within the lung, where ABCA3 protein has been localized to the limiting membrane of lamellar bodies *(23, 24)*. ABCA3 is encoded by a single gene (*ABCA3*) on the short arm of chromosome 16 *(25, 26)*. While the exact function of ABCA3 remains unknown, observations both in human infants and experimental animals, as well as in in vitro models, are consistent with it having a role in transporting lipids critical for surfactant function into lamellar bodies *(27–29)*. Mutations in *ABCA3* have now been associated with the phenotypes of severe RDS in full-term infants *(30)*, as well as interstitial lung disease (ILD) in older children *(31)*, and are described in more detail below.

Epidemiology

The precise incidence and prevalence of lung disease due to mutations in the SP-B and ABCA3 genes are unknown. Population-based studies examining the incidence of these disorders have not been performed and would be difficult owing to their rarity, the similarity in clinical presentations to more common disorders, and difficulties in achieving a specific diagnosis. As they are relatively newly described disorders, the basis for lung disease in children who died from these disorders may not have been recognized. Death from respiratory failure in more mature newborns is fortunately now uncommon in the United States. Approximately 1,000 deaths were attributed to RDS in the United States in 2002, with the vast majority (94.8%) of these being in premature infants *(32)*. In a study of approximately 1,000 infants $\geq$ 34 weeks gestation with respiratory failure, there were 11 deaths not attributable to congenital anomalies *(33)*. Of 29,000 children treated with extracorporeal membrane oxygenation (ECMO) over an 18-year period, 219 died with a diagnosis of RDS *(34)*. As there are approximately 3,800,000 live births in the United States per year, even if one makes the assumption that all of the deaths from RDS in full-term or near-term infants in the above studies were due to a genetic cause of surfactant deficiency, these are very rare disorders. This, however, assumes that the outcome for these disorders is death in the newborn period, which is not always the case, particularly for ABCA3 deficiency. In a recent study of group of children < 2 years of age who underwent open lung biopsy for diffuse lung disease of unknown etiology, the cause of lung disease was attributed to surfactant dysfunction disorders in 10%, with one-third of these due to ABCA3 deficiency *(35)*. While the contribution of

these disorders to both severe neonatal lung disease and ILD in older children may have been underappreciated in the past, it remains likely that they are rare disorders.

While data from phenotype-based population studies are limited, a rough estimate of the incidence of these disorders may be obtained from population-based studies of mutational frequency. One mutation in the SP-B gene, termed 121ins2, has accounted for approximately 60–70% of the disease-causing mutations reported in *SFTPB* to date *(7, 36, 37)*. Several studies have now examined the frequency of this particular mutation in population-based studies utilizing blood samples from neonatal screening programs for metabolic disorders and have yielded a carrier frequency of 1 in 1,000 individuals of Northern European descent *(38–40)*. Extrapolating from the relative contribution of this single mutation to all *SFTPB* mutations, this translates to a carrier rate of 1 in 625 for any *SFTPB* mutation. As SP-B deficiency is inherited as an autosomal recessive disorder, the predicted disease incidence would be roughly 1 in 1.5 million ($625 \times 625 \times 4$) births. The mechanism for a common disease-causing allele is due to a common ancestral origin (founder effect) *(41)*, and this very rough estimate thus would not be applicable to other populations of different ancestry. Other relatively common mutations have been found in other ethnic groups; however, the allele frequencies of these mutations in these subpopulations have not been examined, so it is possible that the incidence of SP-B deficiency could be much higher (or lower) in other subpopulations.

An estimate of the population frequency of *ABCA3* mutations is not yet available. The population frequency of one *ABCA3* mutation identified mainly in older children with ILD was estimated at 1 in 275 individuals; however, the relative contribution of this mutation to all *ABCA3* mutations is unknown at this time *(40)*. The relative incidence of *ABCA3* deficiency is likely to be greater than that of SP-B deficiency. In a study of 17 near-term or full-term infants with the phenotype of fatal surfactant deficiency, 12 were ABCA3 deficient, and only 2 were SP-B deficient *(42)*. This study also only focused on children with fatal disease, and thus the relative contribution of *ABCA3* mutations to pediatric lung disease is likely to be much greater than that of *SFTPB* mutations.

Genetic Basis and Molecular Pathogenesis

The gene encoding SP-B spans approximately 10,000 bases and contains 11 exons, the last of which is untranslated *(19, 43)*. The gene is transcribed into an approximately 2,000 base mRNA, which is translated into a 381 amino acid preproprotein. After co-translational removal of a 23 amino acid signal peptide, the resulting SP-B proprotein undergoes further processing first at the amino-terminal and subsequently at the carboxy-terminal ends. The latter processing steps occur only in type II cells in a distal cellular compartment, likely the lamellar body *(44–47)*. The proteases napsin A and cathepsin H have both implicated as having roles in the processing of proSP-B to mature SP-B. The final mature SP-B peptide contains 79 amino acids, corresponding to residues 201 (phenylalanine) to 279 (methionine) in the proprotein and is encoded in exons 6 and 7 of the SP-B gene. A small percentage of SP-B transcripts utilize an alternative splice site at the beginning of exon 8 that would result in the deletion of four amino acids from the proprotein *(48)*. The functional significance of this alternative splicing is unknown. The proprotein has two potential sites for N-linked glycosylation, one in the carboxy-terminal domain and one in the amino-terminal domain that is dependent on a single-nucleotide polymorphism (SNP) in the last codon in exon 4

(49). A large number of both common and rare genetic variants have been identified in the SP-B gene, including a promoter polymorphism that affects SP-B gene transcription, and a complex variable nucleotide tandem repeat that has been used in association studies with RDS and other lung diseases *(50–54)*.

Over 40 different disease-causing mutations have now been identified in the SP-B gene *(7, 36, 37, 55–63)*. The first mutation identified involves a substitution of three bases (GAA) for one (C) in codon 121 of the SP-B mRNA, which results in a net 2 base insertion, and is termed 121ins2. The mutation causes a frameshift and results in premature codon for the termination of translation in exon 6. The transcript resulting from the 121ins2 mutation is unstable, likely as the result of nonsense-mediated decay, and the net result is to preclude any SP-B production from an allele with this mutation *(64)*. This first reported mutation remains the most frequently identified, found principally in individuals of Northern European descent *(40)*, although other mutations have been identified in unrelated individuals of different ethnic backgrounds (R295X in Mexican Americans, 122delT in Middle Eastern individuals *(7)*). All SP-B mutations identified to date are loss-of-function mutations, in that they result in a severe reduction or complete absence of mature SP-B in lung tissue and fluid from affected children. Missense mutations, in-frame small insertions or deletions, or splicing mutations may allow for the production of proSP-B, but the mutated proSP-B is not processed to mature SP-B *(55, 59, 61)* (Figure 11.1).

While the loss of functional SP-B is the primary cause of lung disease, secondary changes resulting from SP-B deficiency contribute to the pathophysiology of lung disease in affected infants. Ultrastructural analysis of lung tissue from affected infants reveals a lack of normally formed lamellar bodies, with the type II cells instead containing disorganized, poorly lamellated structures with multiple vesicular inclusions *(65)*. These findings indicate a fundamental intracellular role for SP-B in lamellar body biogenesis. In addition, the lung tissue and fluid of SP-B-deficient infants contain large amounts of partially processed proSP-C peptides with retained epitopes from the amino-terminal portion of proSP-C *(66)*. These peptides contain some hydrophilic domains and are secreted, but are not very surface active, and thus likely inhibit surfactant function contributing to the surfactant deficiency state *(67)*. The block in processing of proSP-C to mature SP-C also results in SP-C deficiency, such that SP-B deficiency is in effect a double knock-out. The precise mechanisms underlying the impaired processing of SP-C are not known, but as the final processing steps for SP-C take place in lamellar bodies, the inability to properly form these organelles likely plays a role. Secondary changes in phospholipid profiles have been observed, most notably a marked reduction in the amount or absence of PG *(64)*. The net result of the lack of mature SP-B and SP-C along with aberrant SP-C and altered phospholipid profile is that the surfactant from these children is ineffective in its ability to lower surface tension.

The gene encoding ABCA3 spans over 60,000 bases and contains 33 exons, the first three of which are untranslated. The gene encodes a 1,704 amino acid full transporter with 12 membrane-spanning domains and 2 nucleotide-binding domains *(21, 24)*. ABCA3 protein expression is highest in lung tissue, although it is also found in lower levels in liver, stomach, kidney, adrenal, pancreas, trachea, and brain *(68)*. While the gene for ABCA3 was isolated in 1996, it was not until 2002 that its potential role in the lung was explored, when a 180,000 Da protein isolated from lamellar body membranes (LBM180) was identified as ABCA3 *(69)*. This localization of ABCA3 in conjunction with the role of other ABCA proteins in transporting lipids, and the

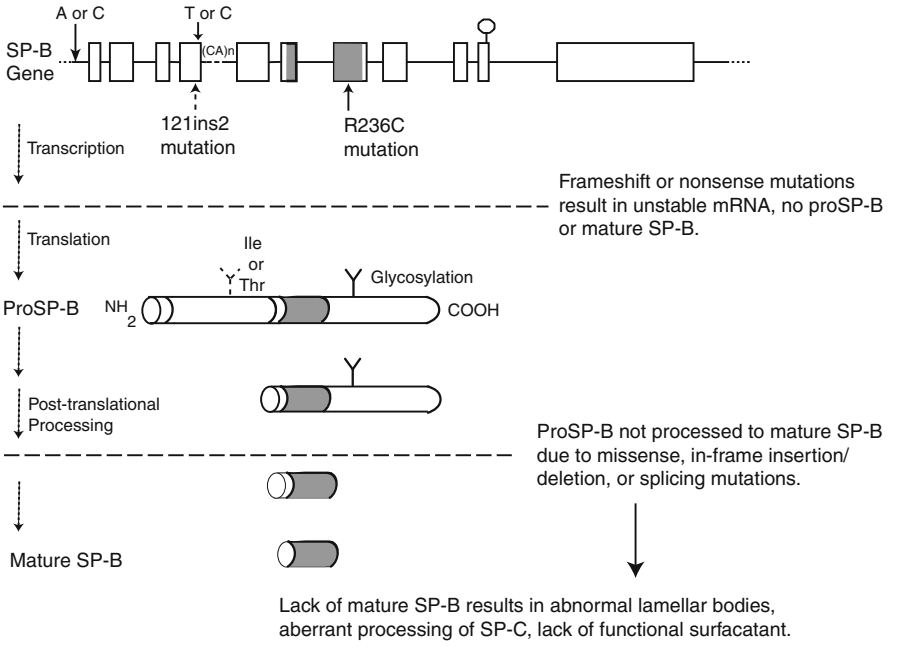

Figure 11.1 SP-B gene, protein processing, and functional results of mutations. The 11 exon SP-B gene (*SFTPB*) is shown at the *top* with exons represented by *rectangles* and introns by *lines*. The positions of the most commonly encountered *SFTPB* mutation, 121ins2, and a missense mutation (R236C) that results in partial deficiency are shown. The locations of single-nucleotide polymorphisms that can affect SP-B gene transcription (–18 C or A in the 5′ untranslated region) *(54)* and protein processing (end of exon 4) *(49)* and a variable nucleotide tandem repeat in intron 4 *(53)* are also shown. The hexagon in exon 10 indicates the location of the codon for the termination of translation. *Shaded* regions in the gene and proprotein correspond to the regions encoding mature SP-B

frequent involvement of other ABC proteins in human genetic diseases led to the consideration of *ABCA3* as a candidate gene for surfactant deficiency in full-term newborns. A clear role for ABCA3 in human lung disease was established with the finding that 16 of 21 infants with the phenotype of severe surfactant deficiency and no known cause for their lung disease had *ABCA3* mutations *(30)*.

Over 90 mutations in *ABCA3* have now been reported *(28, 30, 31, 70–78)*. The mutations are scattered throughout the coding exons and include single base changes resulting in nonsense, missense, and spice site mutations as well as both in-frame insertions and deletions and frameshift mutations. The effects on protein expression have been investigated for a minority of *ABCA3* mutations associated with lung disease *(27, 79)*. *ABCA3* protein expression was absent or markedly decreased in human lung tissue from infants with *ABCA3* mutations compared to control tissues in one study *(76)*. Different effects of mutations have been identified based on studies where constructs expressing disease-causing *ABCA3* mutations were transfected into cells in culture. Some mutants were retained in the endoplasmic reticulum (ER), which would result in a lack of functional ABCA3 at the lamellar body. It is not known whether these ER-retained mutants are degraded or accumulate. A second class of mutants resulted in impaired binding of ATP, impaired ATP hydrolysis, or impaired lipid uptake into vesicles *(27, 79)*. It

seems likely that some mutations might thus result in partial deficiency, either through some residual function or reduced trafficking to lamellar bodies as opposed to complete retention and degradation in the ER (Figure 11.2).

Not unexpectedly given its localization, ABCA3 deficiency is associated with abnormal lamellar body formation. Ultrastructural examination of type II cells in ABCA3-deficient infants reveals numerous small dense bodies with eccentrically placed dense inclusions often giving them a "fried-egg" appearance, although higher power examination can show very densely packed membranes *(30, 77, 78, 80, 81)*. These small dense bodies may represent incompletely developed lamellar bodies, but this has not yet been directly demonstrated. These abnormal lamellar bodies have only been reported in association with ABCA3 deficiency to date; however, the sensitivity and specificity of these bodies for ABCA3 deficiency are unknown at this time.

While the exact function of ABCA3 is unknown, analysis of lung lavage fluid obtained from ABCA3-deficient infants undergoing lung transplantation revealed a marked impairment of surface tension lowering ability, and an abnormal phospholipids profile, with particularly marked decreases in PC, DSPC, and PG compared to control samples obtained from infants transplanted for pulmonary vascular disease or SP-B deficiency *(28)*. These findings are consistent with a role for ABCA3 in transporting these lipids into lamellar bodies. In addition, processing of both SP-B and SP-C was

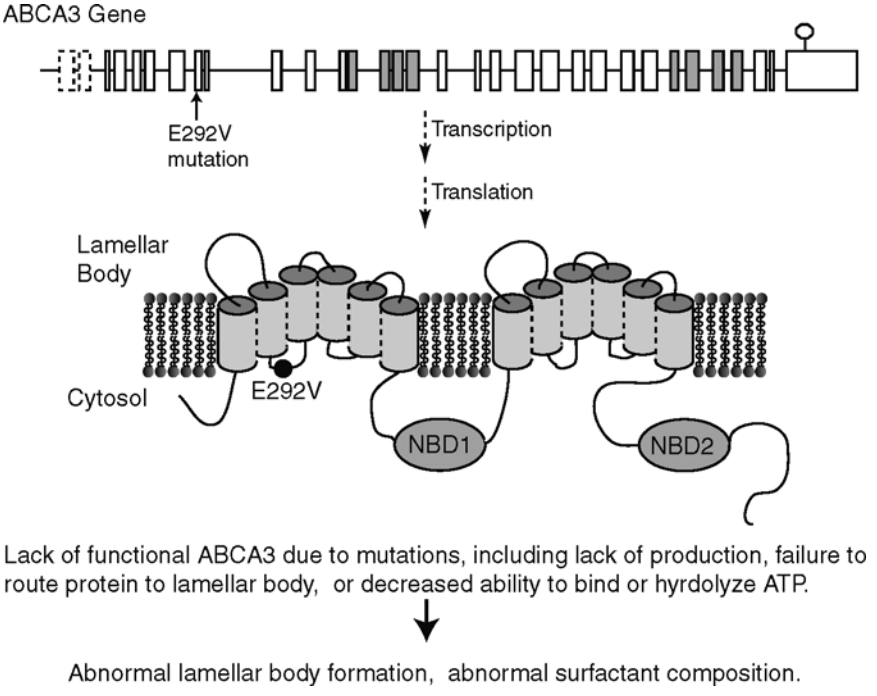

Figure 11.2 ABCA3 gene, protein, and functional results of mutations. The ABCA3 gene is shown at *top*, and the putative structure of the protein embedded in the lamellar body membrane is shown. The position of a mutation (E292V) frequently observed in older individuals with interstitial lung disease is shown *(31)*. The *hexagon* in the last exon indicates the location of the codon for the termination of translation. *Darkly shaded regions* correspond to nucleotide (ATP)-binding domains (NBD)

impaired in lung tissue of ABCA3-deficient infants, likely related to abnormal lamellar body formation *(31, 76)*. While not completely deficient in either SP-B or SP-C, the impaired production of these proteins also likely contributes to the severity of the lung disease observed in these infants.

Common lung histopathology findings in SP-B- and ABCA3-deficient infants include hyperplasia of type II cells, interstitial thickening with variable degrees of inflammation, prominent macrophage accumulation in the airspaces, and variable amounts of granular eosinophilic material in the distal airspaces *(35, 42, 82, 83)*. This latter finding is similar to the appearance of pulmonary alveolar proteinosis (PAP) in adults, and the term "congenital alveolar proteinosis" has been applied to these disorders *(37, 83)*. However, the amount of proteinosis material is highly variable in affected infants, with some airspaces filled with proteinaceous material, but scant amounts found in others. The proteinosis material may be less uniform in appearance than what is observed in older patients with PAP and contain more macrophages. The mechanism for PAP in older patients is very different, reflecting an auto-immune disorder due to antibodies directed against GM-CSF (reviewed in Chapter 16). The histopathology findings are similar in lung tissue from SP-B- and ABCA3-deficient infants, and one cannot distinguish between the two disorders on the basis of routine lung pathology; electron microscopy findings on properly fixed specimens may distinguish between SP-B and ABCA3 deficiency *(77)*. Other diagnostic terms that have been applied to children with these conditions include desquamative interstitial pneumonitis, non-specific interstitial pneumonia, and chronic pneumonitis of infancy *(30, 31, 35, 76)*. The term surfactant dysfunction has recently been used to denote the likely etiology as an inborn error of surfactant metabolism *(35)*.

Animal Models

Genetically engineered mice unable to express either SP-B or ABCA3 have neonatal lethal phenotypes due to respiratory insufficiency, thus mirroring the human diseases *(84–88)*. These animals also have the same secondary changes in surfactant metabolism observed in both disorders. SP-B null mice have abnormally formed lamellar bodies similar to those observed in human infants and also have incompletely processed proSP-C with accumulation of incompletely processed proSP-C peptides *(84, 89)*. The profile of phospholipids extracted from ABCA3 null mice is abnormal, with marked decreases in PC, DSPC, and PG content *(86, 88)*. ABCA3 null mice have small, dense bodies observed in type II cells by electron microscopy, similar to those observed in human ABCA3-deficient infants *(85–88)*. Impaired processing of proSP-B to mature SP-B has been observed in ABCA3 null mice *(86)*.

The routine lung histology findings in these animals are different from what has usually been reported in human infants. Specifically, changes of alveolar proteinosis were not observed in lungs from either SP-B or ABCA3 null mice *(84–88)*. The likely reason for this discrepancy is that SP-B and ABCA3 null mice die very shortly after birth, whereas most human infants with these conditions receive aggressive medical support and have usually survived for weeks to months before lung tissue is obtained for microscopic examination. These observations indicate that many of the histological changes observed in human infants develop postnatally and could also be partly due to the therapies used to sustain them.

In addition to mice completely unable to produce SP-B, mice conditionally able to express SP-B under the control of a tetracycline responsive promoter have been generated. While maintained on the antibiotic, these animals survive the newborn period and do not develop lung disease. Withdrawal of the antibiotic results in a gradual decrease of SP-B levels over 5–7 days with the development of respiratory symptoms, with abnormal pulmonary compliance observed when SP-B levels fell to 20–30% of those of control animals *(90)*. These studies clearly demonstrate that there is a critical level of SP-B needed for normal lung function and support the observation that mutations resulting in partial deficiency can result in a non-lethal phenotype. In addition, mice heterozygous for an SP-B null allele were more susceptible to pulmonary oxygen toxicity than their wild-type littermates *(91)*. These findings support the hypothesis that haploinsufficiency for SP-B could be a risk factor for the development of lung disease in situations where expression of SP-B is delayed, such as prematurity, or reduced due to extrinsic factors, such as inflammation *(92–95)*.

Clinical Presentations and Natural History

The typical presentation for a child with SP-B deficiency is that of a full-term infant with respiratory distress and diffuse lung disease. Symptoms and signs include cyanosis in room air, tachypnea, grunting, and retractions. Chest radiographs demonstrate diffuse alveolar disease, most often with a homogenous ground glass appearance typical of RDS in premature infants *(96)*. Airleak (pneumothorax, pneumomediastinum) is common. Affected infants may also have signs of pulmonary hypertension. The onset of disease is usually shortly after birth, but in some cases symptoms may not be appreciated for hours to days. The disease is often quite severe with rapid progression to hypoxemic respiratory failure requiring intubation and mechanical ventilation, as well as high frequency ventilation, inhaled nitric oxide, and ECMO. Some children may have initially mild respiratory symptoms, but the disease is relentlessly progressive, with increasing difficulty in maintaining oxygenation and persistent alveolar and interstitial infiltrates on chest radiographs. Chest CT imaging shows diffuse ground-glass opacities progressing to fibrotic changes with prominent interlobular septal thickening *(97)*. Death from hypoxemic respiratory failure usually occurs by 3 months of age, even with maximal medical therapy. Very rarely children with mutations that allow for some SP-B production may survive past the first year of life with variable need for respiratory support *(55, 61)*.

Children with ABCA3 deficiency may present in the identical manner as SP-B-deficient infants with the early onset of hypoxemic respiratory failure and radiographically diffuse lung disease, and the two disorders cannot be differentiated on clinical or radiographic grounds *(30, 42, 76)*. The initial severity of lung disease and subsequent clinical courses may differ, however. While ABCA3 deficiency may cause severe hypoxemic respiratory failure, much milder early disease may also be seen. Sufficient improvement in the respiratory status can occur, and affected infants may be discharged from the hospital and felt to be free of respiratory disease at the time of discharge *(31, 71, 73)*. These children present later with non-specific symptoms and signs including poor feeding, failure to thrive, tachypnea, digital clubbing, and pectus excavatum. Chest radiographs demonstrate diffuse alveolar and interstitial disease, with ground-glass opacities and parenchymal cysts *(73)*. Generally an extensive

diagnostic evaluation has been unrevealing in terms of providing an etiologic diagnosis. Due to the failure to thrive, diffuse lung disease and presence of fat-laden macrophages in bronchoalveolar lavage fluid (BALF) samples, surgical treatment for gastroesophageal reflux is common. While reflux can complicate the course of these children, lipid-laden macrophages may reflect the underlying disturbance in endogenous pulmonary lipid (surfactant) metabolism, as opposed to aspirated fat.

The age of onset of symptoms for some ABCA3-deficient children may extend well into early childhood, with no history of lung disease in the neonatal period or infancy *(73)*. Whether these children have unrecognized pulmonary symptoms and pathology or truly do not develop disease until later in life is not known. These observations indicate that some *ABCA3* mutations may allow for sufficient surfactant production for appropriate perinatal adaptation, and indicate that the mechanisms of lung disease in older children involve more than just simple surfactant deficiency, such as secondary injury to type II cells resulting from altered surfactant metabolism, or effects on alveolar macrophages from the altered nature of secreted surfactant components. Survival from ABCA3 deficiency is possible for decades. Many older ABCA3-deficient patients are heterozygous for the same mutation, a substitution of valine for glutamic acid in codon 292 (E292V). This observation supports the hypothesis that genotype may be an important determinant of disease severity, and that mutations associated with less severe disease result in reduced rather than absent ABCA3 function *(31, 73)*. The E292V mutation was also over-represented in a group of relatively mature (28–34 weeks gestation) premature infants with severe RDS *(40)*. No second ABCA3 mutation was identified in these infants, suggesting that *ABCA3* variants may also influence the risk for RDS in premature infants, a hypothesis also supported by another study which noted an association of a specific *ABCA3* haplotype with RDS risk in a preterm population *(98)*.

Diagnostic Approach

Other conditions including infection, respiratory distress syndrome, transient tachypnea of the newborn (TTN), and developmental lung abnormalities such as pulmonary hypoplasia and alveolar capillary dysplasia (ACD) also present with neonatal respiratory distress and diffuse lung disease. Infants with ACD often have other anomalies and may have less impressive clinical and radiographic findings of pulmonary parenchymal disease and more findings of severe pulmonary hypertension *(99)*. Risk factors for pulmonary hypoplasia, such as renal disease and prolonged oligohydramnios, are usually absent in SP-B- or ABCA3-deficient infants. Children with TTN and RDS should show improvement with time and appropriate therapy, although initially one cannot distinguish whether near-term or full-term infants with severe RDS have a transient condition from which they will recover, or mutations in *SFTPB* or *ABCA3* that will result in persistent surfactant deficiency. The longer the signs of RDS persist, the greater the index of suspicion for a genetic mechanism. A family history of neonatal lung disease or unexplained neonatal death due to lung disease should prompt earlier investigation.

Analysis of BAL or tracheal aspirate fluid for levels of specific surfactant components is currently confined to research laboratories only. Specific diagnosis is dependent on genetic testing, and testing for both SP-B and ABCA3 mutations is now available in certified diagnostic labs in the United States and Europe. Labs offering such testing are listed at www.genetests.org. As such testing is non-invasive and may yield a definitive

diagnosis, genetic testing should ideally be pursued before more invasive approaches. Limitations of genetic testing include cost, the length of time needed to obtain results in a critically ill child, and the sensitivity of testing. Analyses are confined to coding exons and their intron–exon boundaries, and thus mutations in untranslated regions that affect gene expression or mRNA splicing or stability will not be detected. Current methods for genetic analysis are based on PCR amplification of relatively small portions of the genes, and thus large deletions, insertions, and gene rearrangements may be missed. A major deletion encompassing two exons in *SFTPB* has been reported *(100)*. The affected child was homozygous for the deletion which facilitated its discovery; had the infant been heterozygous for this deletion, it could have been missed.

While a finding of clear loss-of-function mutations (nonsense or frameshift) on both alleles strongly supports the diagnosis, the interpretation of genetic findings may also be problematic. Missense variants that alter a single amino acid are often identified. If a mutation has been previously found in other unrelated children with lung disease in conjunction with other known disease-causing mutations, this supports that the variant is likely to be disease-causing. If the variant is novel, it may not be possible to determine whether it is functionally significant or a rare, yet benign variant. The finding of a nonconservative amino acid substitution in a highly evolutionarily conserved region of the gene is consistent with the variant being deleterious, but not definitive. Finally, it may not be possible to determine whether a symptomatic individual found to be heterozygous for a single mutation is affected with an unidentified mutation on the second allele or is simply a carrier with a functionally normal second allele with the cause of the lung disease unrelated to the finding of the sequence variant.

Lung biopsy may be necessary in situations where the lung disease is very severe or rapidly progressive and there is insufficient time to wait for the results of genetic testing, or when genetic studies are ambiguous or negative. The histopathology findings of surfactant dysfunction described above are consistent with SP-B or ABCA3 deficiency, although one cannot distinguish between the two based on lung histopathology. Electron microscopy should be performed on all biopsy samples from infants suspected of SP-B or ABCA3 deficiency, as the characteristic ultrastructural findings of each disorder may establish the diagnosis. EM requires special handling and fixation of the tissue in order to not extract lipids and preserve lamellar body morphology. Specific recommendations for the handling of lung biopsy tissues have been published *(101)*. EM should also be performed on autopsy samples of children who die from neonatal lung disease, particularly when genetic testing was not performed, in order to potentially establish the diagnosis and appropriate counsel families regarding recurrence risk. Specific immunohistochemical staining for the surfactant proteins or ABCA3 may also aid in interpretation, however, such studies are mainly confined to research labs at the present time.

Conventional Management and Treatment

Current treatment options for SP-B- and ABCA3-deficient infants are limited. Distinguishing between the two disorders in severely affected neonates is important as children with ABCA3 deficiency may survive the initial period of lung disease, whereas SP-B deficiency is almost always fatal. Appropriate supportive care should be provided to these infants until a firm diagnosis is established. Unfortunately, little can be

done to alter the course of SP-B-deficient children. Surfactant replacement may provide transient improvement, but multiple repeat doses are required, and the beneficial effects are usually not sustained *(102)*. High-dose corticosteroids may also yield transient improvement, but do not halt the progression of the lung disease. Although an alveolar proteinosis component contributes to the lung pathology, total lung lavage is ineffective as it does not correct the underlying metabolic defects *(103)*. Currently lung transplantation remains the only therapeutic option for infants with SP-B deficiency *(104, 105)*.

Similarly, many infants with ABCA3 deficiency may fail to respond to maximal medical management. Surfactant replacement therapy has not been formally evaluated in ABCA3-deficient infants. ABCA3 expression is increased by corticosteroids in vitro and thus there may be a role for steroids in treating children with milder disease with presumed partial deficiency in which an augmentation of ABCA3 expression and function may be beneficial *(106)*. Hydroxychloroquine has been used to treat infants with ILD, particularly with the histopathology of DIP *(107)*. However, its clinical efficacy has not been formally evaluated nor is its mechanism of action in benefiting children with ILD clear.

Lung transplantation is currently the only effective treatment for SP-B deficiency and has also been employed for children with ABCA3 deficiency *(28)*. This option needs to be carefully considered and individualized for each family weighing risks and benefits. Short-term risks include surgical complications, infection, and acute rejection; and long-term problems include the need for chronic immunosuppression, infection, rejection, obliterative bronchiolitis, and need for re-transplantation. The procedure is only performed in small infants at a limited number of medical centers. This will often require the transfer of an unstable patient and relocation of the family for an indefinite period. Limitations of donor availability may mean a prolonged wait until a suitable organ is procured, and the infant may die awaiting the procedure. The family will need a strong social support network to deal with both the pre-operative period and post-operative medical regimen. Medical, social, or economic considerations can all provide barriers to transplantation, and it may not be a realistic option for many families. The 5-year survival rate was 48% for infants transplanted for SP-B deficiency, similar to that of infants of comparable age transplanted for other conditions *(105)*.

Given the bleak outlook for infants with SP-B deficiency and those with ABCA3 deficiency with progressive hypoxemic respiratory failure, once a diagnosis is firmly established, compassionate care should remain an option for these children and their families. As these disorders are autosomal recessive conditions, if the responsible mutations on both alleles can be identified, then prenatal diagnosis may be an option for future pregnancies *(108)*. Given the lethal nature of these disorders, preimplantation genetic diagnosis may also be considered for some families.

Future Therapeutic Targets and Directions

Better therapeutic options for children with SP-B and ABCA3 deficiency are needed. As both disorders involve disruption of surfactant metabolism within the cell, gene replacement therapy may be needed to effectively treat infants in whom mutations completely preclude production of functional protein. Preliminary studies with possible vectors for SP-B have been reported, but significant obstacles to gene replacement therapy include

delivering an effective dose at the correct time and achieving a sustained response without significant adverse host responses. Additionally, as these disorders usually have their onset at birth, such treatment would ideally begin prenatally or early in the newborn period. However, in the absence of a family history, the diagnosis is unlikely to be established until several weeks or months have passed, at which point affected children may already have irreversible lung injury. For older children with the milder form of ABCA3 deficiency, studies are needed to determine the efficacy and risks of currently available treatments. Therapies to facilitate proper protein folding and transit through the cell may benefit some SP-B- and ABCA3-deficient patients depending on their genotypes and the functional consequences of the responsible mutations. As ABCA3 is expressed in other organ systems, its role in other organs and whether there are functional consequences of ABCA3 deficiency for those organ systems remains to be determined.

While an estimate of the incidence of SP-B deficiency is available and the natural history is unfortunately predictable, much less is known with respect to ABCA3. The population frequency of ABCA3 mutations and the incidence and prevalence of disease have yet to be determined. For those children who survive the neonatal period, the course is variable, and factors that modify the course of the disease and markers for prediction of disease severity and outcome are currently unknown. While genetic testing may provide a diagnosis, there is a need for biomarkers in both peripheral blood samples and BALF samples that can used as aids to diagnosis and to follow disease severity and response to treatment. The sensitivity, costs, and turnaround times for genetic testing can be improved. Efforts need to be directed to develop an evaluation algorithm and non-invasive tests that allow for specific early diagnosis and obviates the need for lung biopsy.

Finally, the roles of both common and rare variants in *SFTPB* and *ABCA3* in modifying the effects of other more common lung diseases, including respiratory distress syndrome, bronchopulmonary dysplasia, asthma, and cystic fibrosis are currently unknown and, given the importance of these proteins in normal surfactant metabolism, is an important area for future investigations.

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12

Pulmonary Capillary Hemangiomatosis

Edward D. Chan, Kathryn Chmura, and Andrew Sullivan

Abstract Pulmonary capillary hemangiomatosis (PCH) is a rare lung disorder characterized by proliferation of thin-walled capillary channels that infiltrate the walls of alveolar septae, pulmonary blood vessels, airways, and pleura. In its most dramatic presentation, it manifests as dyspnea, hemoptysis, pulmonary hypertension, and right heart failure although PCH-like lesions may be found incidentally in lung tissues. In patients who present with pulmonary hypertension of unclear etiology, PCH should be considered in the differential diagnosis particularly in the presence of centrilobular pattern of ground-glass opacities, enhance septal lines, pleural effusion, and/or adenopathy on imaging. The etiology of PCH is unknown although histologic abnormalities indicate that the pathogenesis involves dysregulation of angio- or vasculogenesis that is distinct from idiopathic pulmonary hypertension. There is no effective treatment for PCH. Anecdotal reports indicate that alpha-interferon or doxycycline may have some efficacy. Given the very low incidence of PCH, it is plausible that effective anti-angiogenic agents developed for other diseases (i.e., cancer) may be used with some reasonable level of efficacy before the pathogenesis of PCH is more fully elucidated. Lung transplantation should be considered for severe cases.

Keywords: alveolar hemorrhage, angiogenesis, cor pulmonale, pulmonary hypertension

Introduction

Pulmonary capillary hemangiomatosis (PCH) is a rare lung disorder characterized by proliferation of thin-walled capillary channels that infiltrate the walls of alveolar septae, pulmonary blood vessels, airways, and pleura (Figure 12.1) *(1–3)*. PCH most commonly presents with dyspnea or hemoptysis followed by relentless pulmonary hypertension, right heart failure, and death *(1, 4)*. First described by Wagenvoort in 1978, less than 100 cases have been reported in the medical literature. Due to its infrequency, little is known about its pathogenesis and only anecdotal case reports exist to guide in its treatment.

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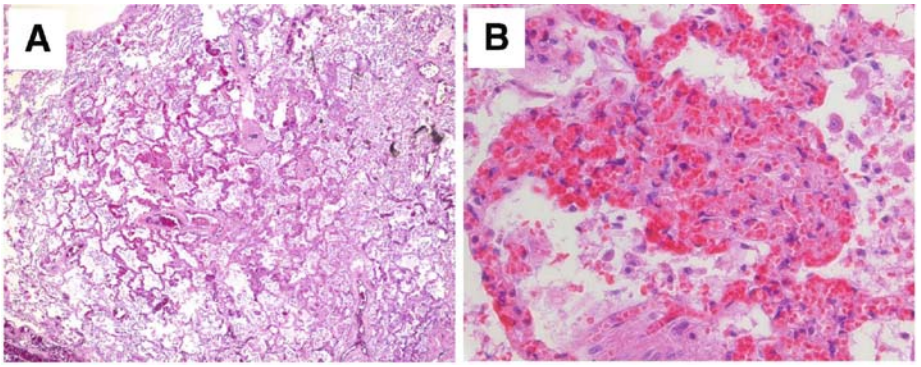

Figure 12.1 (**a**) Histopathology of the lungs revealed proliferating sheets of capillaries with invasion of the arteries, veins, airways, and pleura (H&E, 40×). (**b**) A higher power view of the capillary tufts of PCH (H&E, 400×)

Rapidly advancing knowledge in the field of angiogenesis and resultant therapeutic agents that modulate vascular growth will hopefully lead to more effective therapies against this rare and usually fatal disease.

Epidemiology/Genetics

Lung histopathology consistent with PCH has been described in patients ranging in age from infancy to the seventh decade of life *(1, 5, 6)*. No gender bias has been detected. While PCH was reported to occur in three siblings in a pattern consistence with autosomal recessive inheritance, most cases appear to be sporadic *(2)*. No formal screening for sub-clinical disease in relatives of probands has been attempted.

PCH-like lesions can be found incidentally in the lungs of individuals who are not suffering from the full clinical syndrome. In a study of 140 patients – mostly elderly males – who did not die from complications of pulmonary hypertension, isolated tufts of redundant/proliferating capillaries were found in the lungs of eight (5.7%) *(7)*. Given the low incidence of PCH, it is highly unlikely that any of these individuals would have eventually developed the clinical syndrome. These findings indicate that an isolated histologic abnormality is insufficient to make the diagnosis of PCH.

Molecular Pathogenesis

The etiology of PCH is unknown. The majority of cases occur without a recognizable precipitating factor although cases have been reported in association with a *Mycoplasma pneumoniae* infection, underlying autoimmune disease, or following lung transplantation *(7–9)*. The striking and distinct histologic abnormalities found in patients suffering from PCH lead one to naturally assume that the molecular pathogenesis of the disease arises from dysregulation of angio- or vasculogenesis. Few studies have been published that attempt to elucidate the molecular basis of this defect. In comparison to PCH, idiopathic or primary pulmonary hypertension (PPH) has undergone significant molecular analysis with findings that have led to effective treatment. Investigators have begun

to compare molecular findings within PCH foci or lesions to the plexiform lesions of PPH. In one study using a single PCH lung sample for immunohistochemical staining, there was an increase in the expression of markers associated with cellular proliferation (MiB-1) and angiogenesis (vascular endothelial growth factor) as shown in Figure 12.2 *(4)*. Unlike PPH–plexiform lesions, PCH foci did not demonstrate a loss of cell suppression markers peroxisome proliferation-activated receptor-gamma (PPAR-γ) and caveolin-1 (Figure 12.3) *(4)*. These findings suggest a distinct molecular pathogenic process for PCH when compared to PPH. Despite the likelihood that PCH and PPH arise from distinct molecular processes, investigators have reported that PCH lung tissues exhibit a relative deficiency of nitric oxide synthase (NOS) *(10)*. This is also similar to that seen in PPH–plexiform lesions. Studying lung samples of six patients with PCH, those with loss of endothelial NOS demonstrated morphologic development of pulmonary hypertension while those with preserved endothelial NOS did not *(10)*. Given the very low incidence of PCH, it is plausible that anti-angiogenic agents developed for

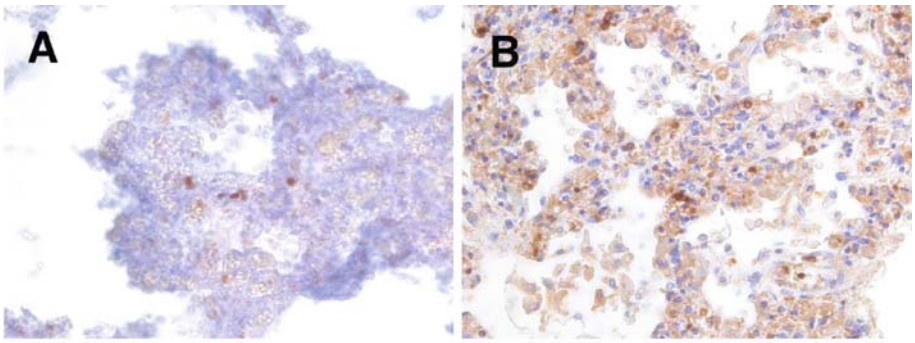

Figure 12.2 Immunohistochemical analysis of a PCH lung with cellular markers known to be *increased* in the plexiform lesions of PPH. (**a**) MiB-1 staining and (**b**) VEGF expression in the lungs of a patient with PCH. Original magnification for all is 400×

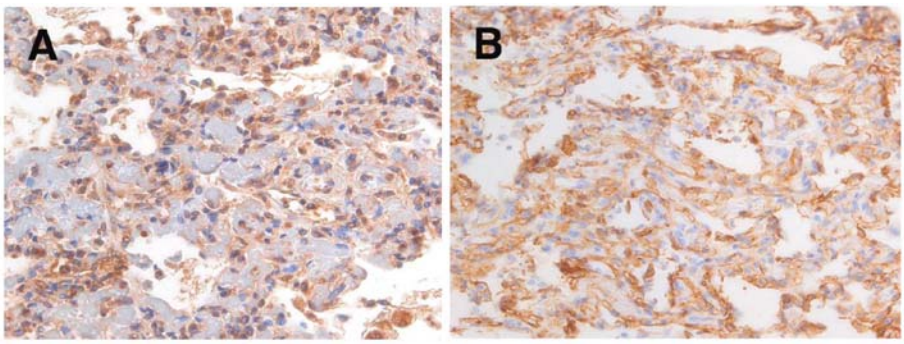

Figure 12.3 Immunohistochemical analysis of a PCH lung with cellular markers known to be *decreased* in the plexiform lesions of PPH. (**a**) PPAR-γ is constitutively present in the PCH lesions. (**b**) Caveolin-1 remains expressed in the endothelial cells of the capillary tufts of PCH. Original magnifications are 400×

other diseases (i.e., cancer) may be trialed in PCH while the pathogenesis of PCH is more fully elucidated.

Animal Models

Currently, no animal models have been used for the study of PCH.

Clinical Presentation

In 2002, Almagro and colleagues collected clinical information on all the 37 cases of PCH reported in the English medical literature up to that time *(1)*. They found that the most common complaint of patients with PCH was dyspnea (69%). Less common signs and symptoms included hemoptysis (33%), fever (24%), and chest pains or syncope in a few patients. Over one-half of cases developed cor pulmonale secondary to pulmonary hypertension. Common physical signs in patients with PCH include hypoxia, crackles on pulmonary auscultation, and the presence of pleural effusions which are often hemorrhagic. Rarely, patients will exhibit digital clubbing. In addition, physical exam is often dominated by signs of right heart failure. The most common cause of death reported was cor pulmonale with additional mortality attributed to respiratory failure, hemoptysis, and sudden cardiac arrest. Strikingly, the mean time to death after diagnosis was 3 years.

Although both PPH and PCH may exhibit evidence of pulmonary hypertension on chest radiograph, the findings in PPH are usually limited to the pulmonary arteries. In contrast, PCH is often characterized by ground-glass infiltrates and/or a diffuse bilateral reticulonodular pattern in addition to the enlarged central pulmonary arteries *(11)*. Though these findings may be subtle enough to be missed on a routine chest radiograph, they are almost always present on high-resolution computerized tomography of the chest. Pulmonary function tests typically reveal restrictive disease with a low carbon monoxide diffusion capacity *(1)*.

Diagnosis

The most common misdiagnosis in patients with PCH is PPH *(11)*. Others include pulmonary fibrosis, sarcoidosis, arteriovenous malformation, hemosiderosis, pulmonary embolism, lymphangiectasis, hemangioendotheliosis, and pulmonary veno-occlusive disease (PVOD) *(1)*. Distinguishing PCH from PPH can be difficult. A well-documented observation in the medical literature is that patients with post-capillary forms of pulmonary hypertension (PCH and PVOD) have a high risk of developing life-threatening pulmonary edema when challenged with vasodilators such as calcium-channel blockers or prostacyclins *(12)*. Any physical signs of left ventricular dysfunction or pulmonary congestion such as crackles or a pleural effusion should preclude the use of a vasodilator trial or treatment. In addition, chest images of patients with pulmonary hypertension should be evaluated with care for signs of PCH and PVOD. Resten and colleagues *(13)* reported thin-section CT findings on 73 adult cases of pulmonary hypertension and identified radiographic signs associated with poor outcomes

upon vasodilator trial and/or treatment: centrilobular pattern of ground-glass opacities, enhance septal lines, pleural effusion, and/or adenopathy. Thus, it is highly recommended that patients with these radiographic findings avoid vasodilator trial or treatment. Pulmonary imaging does not help to distinguish PCH from PVOD.

Ultimately, the diagnosis of PCH can only be made through histologic examination of lung material. Unfortunately, for the majority of cases reported, this has been performed at necropsy. Gross examination of the lung will often reveal multiple hemorrhagic plaques with firm nodular areas *(14)*. On histologic examination, PCH is characterized by an excess of capillary-like vessels which can infiltrate the walls of alveoli, pulmonary blood vessels, airways, lymph nodes, and pleura (Figure 12.1) *(3, 7)*. Enlarged/congested capillaries have at times been mistakenly interpreted as PCH foci. This can be avoided by looking for actual invasion of structures by the aberrant vessels as well as documenting multiple parallel rows of redundant capillaries within alveolar walls. Reticulin or CD34 staining can enhance the small vessels architecture *(4, 7)*.

When the diagnosis of PCH is suspected, the clinician is left with the difficult dilemma of whether the risk of lung biopsy in an often very ill patient is justified in a disease where efficacious treatment is limited. To date, the collective experience with PCH has yielded no consensus definitive recommendations. One important caveat is that patients with significant pulmonary hypertension are at an increased risk of serious consequences from surgical biopsy.

Treatment

Treatment for PCH is limited. Immunosuppresants such as prednisone and cyclophosphamide are ineffective. Alpha-interferon, known to have both anti-viral and anti-angiogenic properties, was associated with disease resolution in two children with PCH *(9, 15)*. However, these young patients had relatively mild disease with normal or near-normal gas exchange. More recently, a patient with PCH that was resistant to alpha-interferon had resolution after treatment with oral doxycycline *(16)*. The investigators posited that doxycycline interfered with matrix metalloproteinase activity and therefore effectively inhibited the dysregulated angiogenesis seen in PCH. Of note, these patients that responded to medical treatment were young (12–20 years of age), and none had significant hypoxemia or pulmonary hypertension. However, a 62-year-old man with PCH who had moderate pulmonary hypertension (45/22 mmHg) remained stable at 36 months with alpha-interferon treatment *(1)*. A number of patients with PCH have successfully undergone orthotropic lung transplantation with no evidence of recurrence *(1)*. Thus, patients with PCH demonstrating significant pulmonary hypertension that is not responsive to alpha-interferon, doxycycline, or other anti-angiogenic agents should be strongly considered for lung transplantation.

Some have advocated anticoagulation in cases of pulmonary capillary hypertension such as PCH and PVOD. The rationale for this recommendation is the notion that the pulmonary hypertension in PCH is due, in part, to pulmonary venule stasis and coagulation from the invading and compressing capillary-like vessels *(7)*. This practice obviates the need to distinguish between PCH and PVOD. Unfortunately, anticoagulation in PCH can be fatal due to hemoptysis or hemothorax and should be approached cautiously *(4, 17)*.

Lastly, it is important to re-emphasize that patients with PCH or suspected of having another form of post-capillary pulmonary hypertension should not undergo vasodilator trial or treatment.

Future Directions

Because of its extremely rare occurrence, clinical research regarding PCH will likely never progress beyond the realm of case reports or small case series. Nevertheless, newly diagnosed individuals may still benefit from an ever increasing molecular understanding of angiogenesis. Of the two agents so far reported to be associated with improvements/reversal of disease progression, both are purported by the authors to have some level of anti-angiogenic properties *(9, 16)*. More direct and powerful inhibitors of angiogenesis are currently available as pharmaceuticals and the therapeutic repertoire will undoubtedly grow. In the United States, the only Food and Drug Administration-approved drug originally developed to directly target an angiogenesis pathway is bevacizumab (Avastia®). This monoclonal antibody against human vascular endothelial growth factor (VEGF) has an indication for the treatment of certain malignancies. Its off-label use in the treatment of macular degeneration may signify its future utility with non-malignant vascular abnormalities *(18)*. For example, it has been proposed as an agent for recalcitrant and debilitating endometriosis *(19)*. It is the authors' belief that a trial of bevacizumab should be attempted in a severe case of adult PCH diagnosed pre-mortem or pre-transplant. Such compassionate use of the drug is justified given the dire outcomes associated with adult PCH.

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13

Anti-glomerular Basement Disease: Goodpasture's Syndrome

Gangadhar Taduri, Raghu Kalluri, and Ralph J. Panos

Abstract Goodpasture's syndrome is an exemplary rare lung and kidney disease that has led to significant discoveries in human biology. The initial observations that antibodies directed against glomerular basement membrane (GBM) caused glomerulonephritis stimulated evaluation of the components of the basement membrane, elucidation of the reticular collagen network, and identification of different types of collagen. The α3[IV] NC1 domain of type IV collagen is the antigenic epitope that initiates a complex autoimmune reaction culminating in the clinical manifestations of Goodpasture's syndrome. Immunization with α3[IV] collagen has provided an experimental model that has led to fundamental discoveries into the genetic and immune processes precipitating and modulating autoimmune diseases. Initial evaluation of the autoimmune process focused on humoral mechanisms, but more recent studies suggest that the cellular immune process is activated and plays a key role in the pathogenesis of Goodpasture's syndrome. Hemoptysis occurs in nearly all patients and renal histopathology demonstrates crescentic glomerulonephritis in the majority of cases. Immunofluorescence microscopy reveals the pathognomonic finding of linear deposition of IgG along with the glomerular capillaries and in the lung parenchyma. The diagnosis of anti-GBM disease is confirmed by the presence of circulating antibodies against basement membrane antigen in the correct clinical setting. Based on the pathogenetic mechanisms, therapeutic modalities include both induction and maintenance regimens: induction or initial therapy removes the pathogenic anti-GBM antibody by plasmapheresis and maintenance therapy reduces antibody production by immunosuppression. The prognosis of patients with anti-GBM disease depends on the level of renal dysfunction at presentation.

Keywords: Goodpasture's syndrome, anti-glomerular basement membrane disease, type IV collagen, pulmonary–renal syndrome, autoimmune disorder

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Introduction

The investigation of the pathogenesis and biochemical, cellular, and immune mechanisms causing Goodpasture's syndrome is an archetypal example of how the evaluation of a rare disease can further the understanding of important biological processes. Initial clinical observations associated pulmonary hemorrhage with glomerulonephritis and subsequent immunohistochemical studies demonstrated linear distribution of antibodies along glomerular and alveolar basement membranes in these patients. Further studies showed that autoantibodies eluted from the kidneys of individuals with Goodpasture's syndrome produced comparable linear deposition along normal renal glomerular basement membrane. Similar immunofluorescent staining patterns were present in experimental models of nephritis provoked by antibodies to basement membrane. Two subsequent serendipitous observations linked Goodpasture's syndrome with Alport's syndrome: (1) antibodies from patients with Goodpasture's syndrome did not react with the glomerular basement membrane in renal specimens from individuals with Alport's syndrome and (2) Goodpasture's syndrome was only observed in patients with Alport's syndrome after renal transplantation *(1, 2)*. These observations suggested that the native kidney in individuals with Alport's syndrome lacked an antigenic determinant within basement membrane found in the transplanted kidney.

Intensive research studies identified the α3[IV] NC1 domain of type IV collagen as the antigenic epitope that provokes a complex autoimmune reaction. (Other studies showed that Alport's syndrome is caused by mutations in the type IV collagen gene, and these patients may lack this portion of the type IV collagen molecule.) Initial evaluation of the autoimmune process focused on humoral mechanisms but more recent studies suggest that the cellular immune process is activated and plays a key role in the pathogenesis of Goodpasture's syndrome. Animal models of Goodpasture's syndrome are frequently used to investigate the cellular and cytokine pathways underlying autoimmune processes. Thus, Goodpasture's syndrome is an exemplary rare or "orphan" disorder that has expanded our insights into multiple and diverse biological processes.

This chapter will review the biochemical, genetic, and immunologic processes that cause Goodpasture's syndrome and then discuss its clinical presentation, evaluation, and management.

History

Ernest Goodpasture *(3)* described an 18-year-old man with hemoptysis and renal failure during the influenza pandemic of 1919. Based on this report, Stanton and Tange *(4)* coined the eponym Goodpasture's syndrome in their description of a series of men with glomerulonephritis and hemoptysis in 1958. Interestingly, the patient originally described by Goodpasture had focal necrosis of the spleen and intestinal hemorrhage suggesting systemic vasculitis. Thus, Goodpasture's patient most likely did not have the disease that has become known as Goodpasture's syndrome because vasculitis is not a usual feature of this disorder and suggests a different process.

Goodpasture's syndrome is usually used to refer to the triad of pulmonary hemorrhage and glomerulonephritis in the presence of circulating antiglomerular basement membrane antibodies, whereas Goodpasture's disease is the presence of glomerulonephritis and antiglomerular basement membrane antibodies without pulmonary

hemorrhage. Demonstration of circulating antiglomerular basement membrane antibodies with or without pulmonary or renal involvement is often designated antiglomerular basement membrane (anti-GBM) antibody disease.

Basement Membrane and Collagen Biochemistry

Basement Membrane

Basement membrane is a complex system of collagenous and noncollagenous proteins that provides structural and functional support to overlying cells (Figure 13.1) *(5)*. In addition, components of the basement membrane modulate cellular function and differentiation through specific receptors and signaling mechanisms *(5)*. Noncollagenous proteins include laminin, enactin, perlecan, and other minor components. The laminin protein family has 11 genetically distinct chains that assemble into 15 trimeric combinations and are the most abundant noncollagenous proteins. Laminins are assembled from three polypeptide chains designated the laminin α, β, and γ chains. Laminin-11 is the predominating laminin isoform composed of $\alpha 5 \beta 2 \gamma 1$. Laminin has a major role in cell–matrix interaction, which is mediated by connections with components of the basement membrane and also with cell surface receptors *(6)*. Nidogen or enactin is important for cardiac and pulmonary basement membrane function. Perlecan is a heparan sulfate proteoglycan that is ubiquitously present in basement membranes and is also found within connective tissues outside the basement membrane *(6)*. Nidogen and perlecans help stabilize the network of type IV collagen and laminin that composes the structural framework of the basement membrane *(6)*. Minor noncollagenous components of the basement membrane confer tissue specificity and may promote unique interactions with adjacent cells that direct cellular differentiation and function.

Collagen

Basement membrane is composed of collagen, which is arranged in different architectural patterns (Figure 13.2). The collagen in turn is made of glycoprotiens. Basement membrane offers structural and functional support to the cells. Type IV collagen is abundant in the basement membrane and type I collagen is the most common protein in the rest of the body.

There are six distinct type IV collagen genes that encode unique proteins known as α-chains (α1–α6). Each α-chain is 400 nm long and is composed of an N-terminal 7S domain (26 kDa, 28 nm), a triple-helical collagenous domain (120 kDa, 320 nm), and C-terminal noncollagenous globular domain (NC1) (25 kDa, 52 nm). The 7S domain and collagenous portions contain glycine, proline, hydroxyproline or lysine, and hydroxylysine amino acid sequences. The NC1 portion lacks hydroxyproline but is rich in cystine and lysine. The building blocks of the collagen type IV network are protomers, aggregates of three α-chains that assemble in a fixed combination: $\alpha 1 \alpha 1 \alpha 2$ and $\alpha 3 \alpha 4 \alpha 5$ and possibly $\alpha 1 \alpha 1 \alpha 5$, $\alpha 1 \alpha 2 \alpha 5$, or $\alpha 5 \alpha 5 \alpha 6$. The NC1 domains of the protomers dimerize in specific combinations to form linear arrays. Only three unique NC1 hexamers are formed: $\alpha 1.\alpha 1.\alpha 2$ [IV]–$\alpha 1.\alpha 1.\alpha 2$ [IV], $\alpha 3.\alpha 4.\alpha 5$ [IV]–$\alpha 3.\alpha 4.\alpha 5$ [IV], and $\alpha 1.\alpha 1.\alpha 2$ [IV]–$\alpha 5.\alpha 5.\alpha 6$ [IV]. Only $\alpha 1.\alpha 1.\alpha 2$ [IV]–$\alpha 1.\alpha 1.\alpha 2$ [IV] and $\alpha 3.\alpha 4.\alpha 5$ [IV]–$\alpha 3.\alpha 4.\alpha 5$ [IV] networks are found in both lung and kidney. Four 7S domains

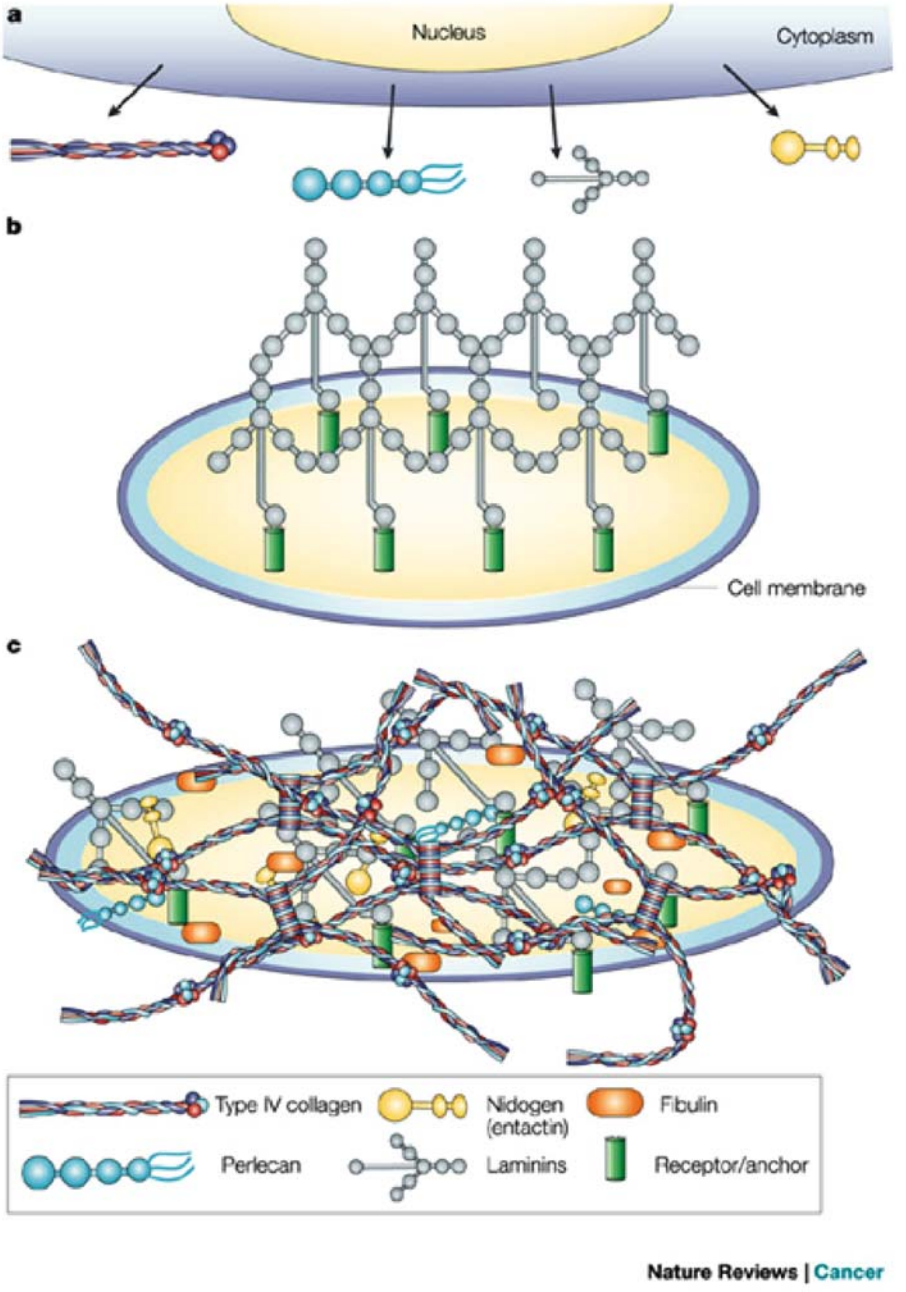

Figure 13.1 Structure of basement membrane: Basement membrane is a complex system of collagenous and noncollagenous proteins that provides structural and functional support to overlying cells. Noncollagenous proteins include laminin, enactin, perlecan, and other minor components

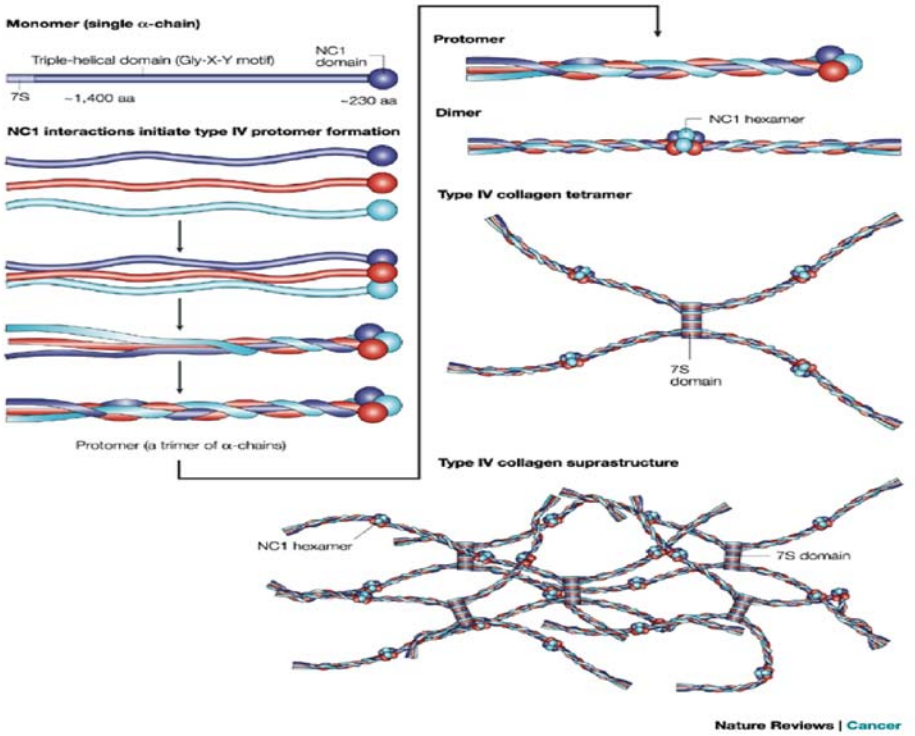

Figure 13.2 Structure of collagen IV: Structure of collagen is composed of protomers which are composed of two α1 chains and one α2 chain and characterized by a 7S triple-helical domain at the N-terminal containing N-linked carbohydrate moieties, followed by a long triple-helical collagenous domain and a noncollagenous NC1 trimer at the C-terminal. Six genetically distinct α-chains are arranged into three triple-helical protomers that differ in their chain composition. Interruptions in the Gly-Xaa-Yaa amino acid sequence at multiple sites along the collagenous domain confer flexibility, allowing for looping and supercoiling of protomers into networks, strengthen with interprotomer disulfide bonds. In the extracellular matrix, collagen IV protomers form networks through dimerization at their C-terminal NC1 domains and through tetramer formation at their N-terminal 7S domains

assemble into a tetramer to confer the quarternary grid-like configuration to the collagen [IV] network.

Pathogenesis

Antigenic Determinants

Goodpasture's disease is a classical example of an immune complex-mediated disease. Experiments passively transferring plasma or glomerular elutant from animals that developed GN after immunization with basement membrane components demonstrate that the antigenic determinant is located within the globular NC1 domain of the α3[IV] collagen (Figure 13.3) *(8)*. These anti-GBM antibodies bind protein produced by cells transfected with α3[IV] collagen cDNA confirming the antigenic determinant *(9, 10)*. The antibodies are typically IgG but sometimes may be IgA or IgM *(11, 7)*. Occasionally, antibodies may be directed against other α-chains but these antibodies are not pathogenic *(12)*.

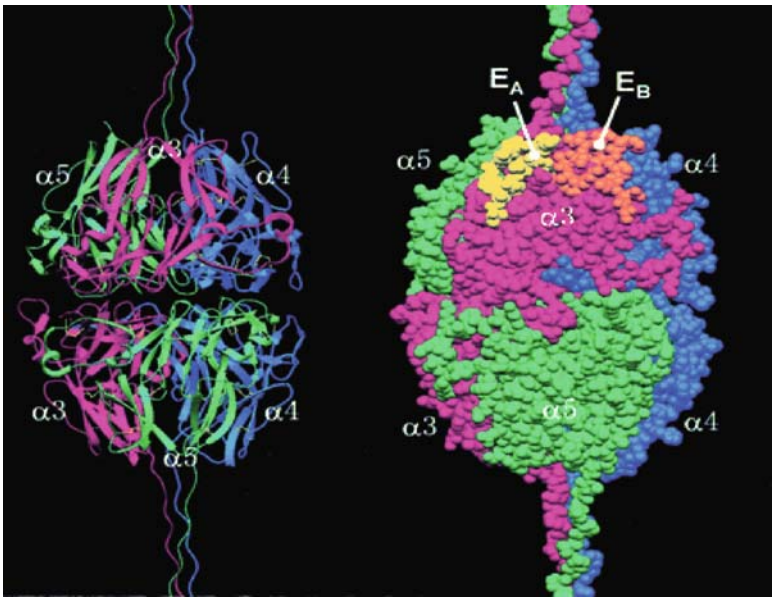

Figure 13.3 Atomic structure of the $\alpha 3.\alpha 4.\alpha 5$ NC1 hexamer. The three NC1 monomers of each protomer interact to form a trimeric cap that, in turn, interacts with the trimeric cap from another protomer to form the hexamer. Each NC1 monomer has a novel three-dimensional fold of the polypeptide chain, characterized mainly by β-sheets, shown by the ribbon diagrams (*left*). The sites for the E_A and E_B epitopes are shown on the space-filling model of the hexamer

Revelation of Cryptogenic Antigens

The autoimmune reaction is triggered by exposure of cryptic determinants within the NC1 carboxy-terminal domain through disruption of the NC1 hexamer (Figure 13.4). The triggering event is known in only a few instances *(13)*. Various insults such as infections, environmental toxins, ischemia, neoplasm, and mechanical factors such as lithotripsy have been implicated clinically as potential provocative factors in the revelation of the Goodpasture antigenic determinant *(14, 15)*. Experimentally, Chemical modifications such as exposure to reactive oxidative species can expose the cryptic antigens *(16)*. Anti-GBM antibodies bind rapidly and with high affinity to the Goodpasture's antigen and have slow dissociation rates *(8)*. In general, antibody production is short lived *(13)*.

Autoantibody Production/Humoral Immune Response

The generation of antigen-specific immunity involves both T cells and B cells. Previous studies have proven the importance of both autoantibodies and cell mediated immunity during the induction and effector phases of the autoimmune response *(17)*. Antigen dose, availability of costimulation and the presence of disease susceptible genetic background are important for the activation of the B and T cells *(18–21)*. Presence of the antibody directed against the N terminus of the NCI domain correlates better with

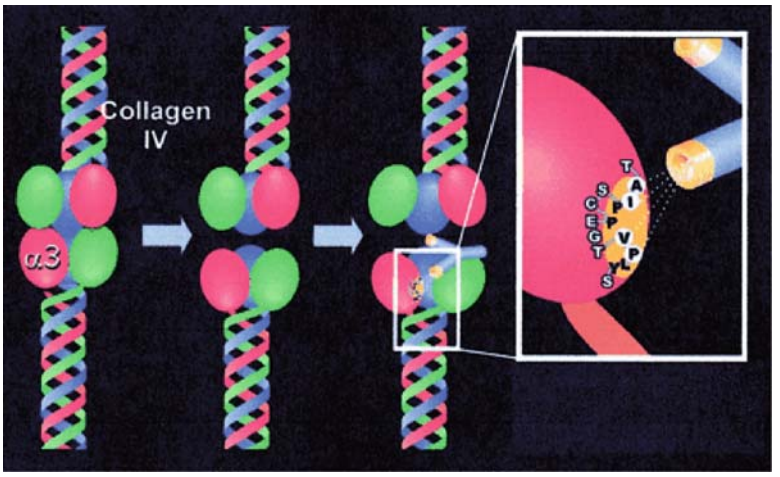

Figure 13.4 The identity and the cryptic nature of the E_A epitope of the GP antigen. The immunodominant E_A epitope was localized to a site, residues 17–31 (TAIPSCPEGTVPLYS), near the junction of the N terminus of the NC1 domain and the triple-helical domain of the α3 chain of collagen IV. The epitope is cryptic, being inaccessible to GP antibodies until the hexamer is dissociated

renal injury than does the presence of antibodies directed against other areas of the NCI domain *(12)*. Antibodies against the α3[IV] chain may exist in normal individuals but they are not pathogenic because they have low avidity and are composed of different IgG subclasses.

Cellular Immune Response: Role of Th1 Response

T cells can be classified as T helper 1 [Th1] and T helper 2 [Th2] based upon their cytokine production profile. The balance between the Th1 and Th2 responses directs the immune mechanism of different diseases *(22)*. Experimental evidence suggests a Th1 response in anti-GBM disease *(23, 24):* 1) the inflammatory infiltrate in the kidney consists of macrophages and lymphocytes, 2) IgG1 isotype is the most abundant antibody in this disease, *(23, 25)* and 3) a Th1 response induces a severe crescentic pattern of glomerular inflammation *(26)*.

Current hypotheses suggest that, initially, a strong Th1 response to the autoantigen is launched with only a weak accompanying Th2 response. Exposure of the cryptic epitope leads to the binding of autoantibodies to the GBM and high amounts of bound antibodies precipitate fissures in the GBM that initiate glomerular inflammation. Antigen specific Th1 effector/memory cells are attracted to the site of inflammation, causing a strong delayed-type hypersensitivity reaction that may be responsible for the majority of the kidney damage *(17)*.

Regulation of Antigen Tolerance

Antigenic tolerance is regulated by genetic factors and T-cell immunity. The mere presence of anti-GBM antibodies may not lead to disease unless a compatible MHC haplotype and nephrogenic T-cell repertoires are present *(24)*. The pattern of glomerular

injury induced by an injected antigen is regulated by the balance of T helper cell subset activation. A Th1 response induces a severe crescentic pattern of glomerulonephritis (GN) that is T helper cell and interferon (IFN)-α dependent *(17, 26)*. CD40/CD154 signaling plays a key role in initiating Th1 responses and may direct Th1 effector responses. The role of CD40 in the development of GN was assessed in a murine model of anti-GBM antibody-induced GN *(27)*. In this model, expression of CD40 by nonimmune renal cells regulates Th1 effector responses by inducing Th1 chemokine production *(27)*. IL-12 is pathogenetic but IFN-α is protective *(28)*. Interleukin [IL]-10 plays a pivotal role in regulating the Th1/Th2 balance of immune responses. Exogenously administered IL-10 suppresses nephrogenic Th1 responses, inhibits macrophage function, and attenuates crescentic glomerulonephritis *(29)*. CD25 cells regulate the immune response by suppressing the humoral and cellular immune response *(30)*.

Genetic Susceptibility

Major histocompatibility complex (MHC) class II molecules bind to α3[IV] NC1 antigenic epitopes, promote T-cell recognition, and thus regulate the humoral response. The prevalence of HLA–DR15 and DR4 is increased in individuals with Goodpasture's syndrome whereas the prevalence of DR1 and DR7 is decreased *(31)*. The absence of a specific amino acid motif within the DR1 domain may explain the lower incidence of anti-GBM disease in African Americans *(32)*. In a mouse model of human anti-GBM disease, all MHC haplotypes develop anti-α3[IV] NC1 antibodies after immunization with the α3 NC1 domain of type IV collagen but nephritis and lung hemorrhage only occur in MHC H-2s, b, and d haplotypes. These haplotypes are associated with an IL-12/Th1 like T-cell phenotype *(33)*. Other experiments using two strains of rats (Wistar Kyoto – WKY and Lewis – LEW) showed that both mice strains develop anti-GBM antibodies after immunization with collagenase-stabilized rat GBM (csGBM), but only WKY rats develop crescentic glomerulonephritis *(34)*. The antibody titer is higher and more specific in WKY rats compared with LEW rats. Passive transfer of antibodies from WKY with experimentally induced glomerulonephritis into untreated animals causes renal disease in WKY but not LEW rats *(34)*. These experiments illustrate the importance of the autoimmune and also inflammatory response to the deposited antibody in the development of anti-GBM disease. They also suggest a genetic basis of susceptibility to the development of Goodpasture's disease.

Epidemiology

Renal manifestations are a major criterion for the diagnosis of Goodpasture's syndrome. Depending on the type of renal injury, the prevalence and incidence of Goodpasture's disease varies. Acute glomerulonephritis (GN) is a rare presentation and Goodpasture's disease is estimated to cause less than one in a million cases of acute GN *(13)*. Anti-GBM disease causes approximately 20% of all rapidly progressive glomerulonephritis *(35)*. If concomitant pulmonary involvement is present, anti-GBM disease may be present in 50% of cases *(36)*. In one series of renal biopsies, anti-GBM disease occurred in 10% of crescentic GN cases *(13)*.

There is a slight male predilection in younger patients with anti-GBM disease, whereas females predominate in the older age group *(37)*. The younger group more

frequently presents with the full constellation of pulmonary and renal symptoms, while the older age group may present with only isolated features of glomerulonephritis *(37, 38)*.

In a few series, anti-GBM disease has occurred in clusters, suggesting a possible common triggering event such as pulmonary infection or injury *(39)*. Anti-GBM disease has also been reported after the urinary tract obstruction and lithotripsy that may have caused glomerular injury *(14, 15)*. These clinical associations suggest that pulmonary or renal injury by different inciting events may reveal the epitope that elicits the formation of anti-GBM antibodies initiating the immune response or allows existing antibodies to react with the uncovered cryptic antigen *(14)*. Similarly, anti-GBM disease may occur after renal transplantation in individuals with Alport's syndrome or with hereditary GN. In both of these disorders, the α3[IV] collagen chain is aberrantly expressed and lacks the Goodpasture antigen whereas the donor kidney has normal expression of the α3 chain *(40)*.

Clinical Manifestations

Renal

Renal involvement varies from normal renal function, mild urinary sediment abnormalities to rapidly progressive renal failure or acute renal failure. In rapidly progressive renal failure or acute renal failure, urinanalysis demonstrates proteinuria (usually in a subnephrotic range) and urine sediment consisting of dysmorphic red cells, red cell casts, white cells, and granular casts. In more mild disease, the urinary sediment may demonstrate hematuria and proteinuria with normal or mildly decreased glomerular filtration rates. Milder forms may progress rapidly to renal failure.

Pulmonary

Hemoptysis occurs in nearly all patients with Goodpasture's syndrome *(41–44)*. The severity of hemoptysis varies from minimal blood-streaked phlegm to massive hemorrhage. Less than 10–20% of patients do not experience hemoptysis but blood may be present in bronchoalveolar lavage fluid. Characteristically, the fluid becomes progressively more hemorrhagic as the lavage progresses suggesting that the hemorrhage is emanating from the alveoli and not more proximal airways. Hemoptysis precedes renal manifestations in over half of the patients *(45–46)*.

Constitutional symptoms such as fevers, chills, arthralgias, or skin rashes occur rarely in Goodpasture's syndrome *(47)*. Their presence suggests a systemic vasculitis or rapidly progressive GN. Fatigue, breathlessness, and weakness may occur especially in patients with significant anemia.

On physical examination, most patients appear pale *(43, 44)*. Auscultation of the lungs reveals crackles or rhonchi in up to half of all patients with Goodpasture's syndrome. Lower extremity edema is present in approximately one-third of patients. In one series of 54 patients, 10 (29%) had no abnormal findings *(44)*. Physical examination findings such as skin rashes, arthritis, or myopathy suggest processes other than Goodpasture's syndrome *(48)*.

Imaging and Physiologic Studies

Approximately one-quarter of patients with Goodpasture's syndrome have normal chest X-rays *(44)*. Diffuse alveolar filling in an acinar pattern is the most frequent finding. Chronic alveolar hemorrhage may cause a reticulonodular pattern due to interstitial fibrosis *(49)*. Atelectasis and consolidation may also occur. Pleural effusions are frequently observed and suggest fluid overload, possibly related to renal failure *(48)*. The chest CT scan typically reveals ground-glass opacification diffusely. Consolidation may also be present.

The diffusing capacity for carbon monoxide is increased in patients with Goodpasture's syndrome due to the binding of inhaled carbon monoxide by intra-alveolar blood *(48)*. Spirometry and lung volume measurement are usually not helpful in the evaluation of patients with pulmonary hemorrhage.

Pathology

Renal

A renal biopsy should be considered for histopathological confirmation of the diagnosis unless it is clinically contraindicated. Kidney biopsy may also help guide therapy and provide prognostic information. Light microscopy demonstrates crescentic glomerulonephritis in the majority of cases. Immunofluorescence microscopy reveals the pathognomonic finding of linear deposition of IgG along the glomerular capillaries. Some cases show tubular staining caused by circulating anti-tubular basement membrane antibodies that bind to α3[IV] NC1 present in the tubules. Electron microscopy in RPGN shows characteristic breaks in the glomerular basement membrane (GBM). These rents allow fibrin and cellular elements to enter Bowman's space and initiate crescent formation.

The pattern of linear immunofluorescent basement membrane staining also occurs in diabetic nephropathy and fibrillary glomerulonephritis *(50,51)*. The clinical manifestations, presence of circulating anti-GBM antibodies, light microscopy, and ultrastructural features help to distinguish these disorders from Goodpasture's syndrome. Diabetics will have history of diabetes, lack anti-GBM antibodies, and light microscopy reveals glomerulosclerosis without crescents *(50)*. Fibrillary glomerulonephritis demonstrates characteristic fibrils on electron microscopy and anti-GBM antibodies are absent *(51)*.

Pulmonary

Grossly, the lungs of patients with Goodpasture's syndrome are dense and consolidated with hemorrhage and petechiae along the pleural surface *(43)*. The major histopathological findings are red blood cells and hemosiderin-laden macrophages within the alveolar spaces. Neutrophilic capillaritis, thickening of the alveolar septae by edema and neutrophilic infiltration, is frequently present but is obscured by the alveolar hemorrhage *(52, 53)*. Diffuse alveolar damage, occasionally with hyaline membrane formation, may also occur *(52)*. Interstitial fibrosis is usually patchy and mild *(52, 53)*. Ultrastructural studies demonstrate widening of the basement membrane, loss of type I cells, and alveolar type II cell hypertrophy and hyperplasia *(46)*.

Immunohistochemical staining demonstrates linear immunofluorescence along alveolar walls and can be detected in lung tissue obtained by transbronchial or open lung biopsy *(52, 53, 54, 55)*. Staining may be falsely negative due to patchy immunofluorescence in transbronchial biopsies or falsely positive due to autofluorescence of lung tissue *(54, 56)*. In general, kidney tissue is superior to lung tissue for the demonstration of linear immunofluorescence.

Laboratory Studies

General

The laboratory evaluation depends on the site and severity of organ involvement. Renal and pulmonary laboratory evaluation should occur whenever anti-GBM disease is considered. Anemia is seen in acute pulmonary hemorrhage or in patients with recurrent pulmonary hemorrhage. Serum complement level is usually maintained in the normal range and the erythrocyte sedimentation rate is usually not elevated.

Renal

Renal evaluation consists of urinanalysis, laboratory studies, imaging, and renal biopsy. Urinanalysis shows dysmorphic red blood cells, casts, albumin, and subnephrotic-range proteinuria. Rarely nephrotic-range proteinuria is described in patients with a subacute presentation. Serum creatinine will be elevated in patients with crescentic glomerulonephritis and has a good correlation with the number of crescents. Radiological evaluation shows normal or enlarged kidneys.

Pulmonary

Pulmonary evaluation consists of sputum analysis and culture, physiologic studies, chest imaging, and lung biopsy. Sputum frequently reveals red blood cells but no evidence of infection or neoplasm. The diffusing capacity for carbon monoxide is frequently elevated but other measures of lung physiologic function such as spirometry and lung volumes are not helpful. Chest imaging studies including chest X-rays and chest computed tomography are not specific and often reveal alveolar opacifications caused by alveolar hemorrhage. Lung biopsy may demonstrate linear immunofluorescence along the basement membrane.

Serologies: Sensitivity/Specificity of Assays

The diagnosis of anti-GBM disease is confirmed by the presence of circulating antibodies against basement membrane antigen in the correct clinical setting. Indirect immunofluorescence or enzyme-linked immunoassay (ELISA) methods are used for detection of anti-GBM antibodies *(57, 58)*. Indirect immunofluorescence is laborious, requires technical expertise, and has high false-negative rates of 40% *(57, 58)*. ELISA methods are simple and repeatable and have a sensitivity of up to 60–100% *(57, 58)*. Low antibody titers may cause false-negative results *(59)*. The use of native or recombinant α3[IV] antigen in the ELISA can increase sensitivity to 95–100% and specificity

to 91–100% *(58)*. Unpurified Goodpasture antigen may cause false-positive results *(57, 60, 61)*. Western blot test can also be used to detect the presence and confirmation of anti-GBM antibodies *(60)*. The clinical features must be considered when interpreting serological studies.

Pulmonary–renal disease also occurs in ANCA-positive vasculitis such as Wegener's granulomatosis or microscopic polyangiitis. The presence of nonpulmonary or renal features suggests vasculitis as systemic manifestations do not occur in anti-GBM disease.

Approximately one-quarter of patients with anti-GBM disease also have circulating antibodies to antineutrophil cytoplasmic antibody (ANCA) *(11)*. The presence of both anti-GBM antibodies and ANCA indicates a better prognosis *(62, 63)*.

Acute

Based on the pathogenesis of anti-GBM disease, therapeutic modalities include induction and maintenance regimens: induction or initial therapy removes the pathogenic anti-GBM antibody and maintenance therapy suppresses antibody production.

The clinical syndrome of rapidly progressive renal or respiratory failure requires prompt diagnosis and initiation of supportive therapies such as dialysis or mechanical ventilation with contemporaneous antibody removal and immunosuppression. Early initiation of treatment may prevent the development of end-stage renal failure caused by crescentic glomerulonephritis *(14, 38)*.

Antibody removal is accomplished by large volume plasmapheresis daily for 2–3 weeks. Plasma exchange utilizes replacement of serum proteins with human albumin and fresh frozen plasma is added to normalize coagulation abnormalities *(64–66)*. In a prospective randomized study comparing immunosuppression with and without plasmapheresis, the mortality rate was 11% in the group treated only with immunosuppressives and 0% for those receiving combined therapy *(67)*. This difference approached but did not achieve statistical significance. If renal function does not improve and anti-GBM antibody titers do not decrease, plasmapheresis is continued *(38)*.

Immunosuppressive therapy prevents the production of anti-GBM antibodies and retards immune-mediated tissue injury. Steroids combined with cyclophosphamide are the most frequently used immunosuppressant regimen *(13)*. Steroids are initiated as intravenous pulse doses (15–30 mg/kg intravenously infused over 20 min, maximum dose 1 g) daily for 3 days, followed by oral prednisolone (1 mg/kg daily maximum 60–80 mg). Steroids are tapered according to the disease status. Cyclophosphamide is usually given orally, 2 mg/kg (maximum 100 mg/day). Intravenous cyclophosphamide is advised for those patients who are noncompliant, cannot tolerate oral therapy, and those with severe renal failure. Intravenous therapy is associated with less bladder toxicity *(68)*.

Plasmapheresis and immunosuppressive therapy should be initiated in all patients with pulmonary hemorrhage independent of renal involvement, patients with renal dysfunction not requiring immediate renal replacement therapy, patients with ANCA positivity, and patients requiring dialysis on presentation *(62–63, 69)*. Because it may be difficult to assess clinical status in patients presenting with end-stage disease, it may be worthwhile attempting immunosuppressive therapy for 2–3 weeks to determine whether the renal injury is reversible *(70)*.

Maintenance

The duration of induction therapy depends on the clinical response of the patient. Anti-GBM antibodies should be monitored every week until they are not detectable on two occasions *(38, 37)*. As the antibody response is usually self-limited and transient, 6–9 months of treatment may be required for total cessation of antibody production *(13, 71)*. After attaining remission, maintenance therapy may be initiated with steroids and less toxic azathioprine and continued for at least 6–9 months *(38, 37)*. If the disease is less aggressive and anti-GBM antibodies are persistently not detectable, the duration of therapy may be limited to 2–3 months. If anti-GBM antibody remains present despite therapy, the antigenic specificity of the antibody for the Goodpasture antigen should be confirmed and maintenance therapy continued. Recurrence of clinical symptoms or anti-GBM antibody requires resumption of induction therapy.

Experimental/New Therapies

Novel therapies such as immunoadsorption using a sepharose-coupled sheep anti-human IgG column remove circulating anti-GBM antibodies but will require further validation *(72)*. Rituximab, a chimeric monoclonal antibody to the pan-B lymphocyte antigen CD20, has been used successfully to treat Goodpasture's syndrome refractory to immunosuppressive therapy *(73)*. T-cell suppression by co-stimulatory pathway blockade prevents crescentic glomerulonephritis *(74)*.

Intranasal or oral administration of Goodpasture antigen causes tolerance and prevents glomerulonephritis in experimental animal models *(75, 76)*.

Prognosis/Outcome

The prognosis of patients with anti-GBM disease depends on the level of renal dysfunction at presentation. Anti-GBM disease can progress rapidly to end-stage renal disease requiring kidney transplantation. Need for dialysis is associated with a poor prognosis *(37)*. Before renal transplantation can be considered, anti-GBM antibody levels should be undetectable for at least 12 months and disease activity quiescent for at least 6 months after stopping immunosuppressive therapy *(77–80)*. Recurrence of IgG immunofluorescent staining along the basement membrane occurs in approximately 50% of renal transplant recipients but most of these patients are asymptomatic *(81)*. Clinically symptomatic recurrence with hematuria and proteinuria is very rare after proper pretransplant evaluation. The low recurrence of anti-GBM disease after renal transplantation can be explained by the self-limited nature of Goodpasture's syndrome and pre- and post-transplant immunosuppression *(82)*. Graft loss secondary to recurrence of anti-GBM disease is rare *(83)*.

Summary

Goodpasture's syndrome is an archetypal rare lung and kidney disease that has led to significant discoveries in human biology. The initial observations that antibodies directed against GBM caused glomerulonephritis stimulated evaluation of the components of the basement membrane and the elucidation of the reticular collagen network

that forms the framework upon which the basement membrane is constructed. New collagen types were discovered and the structural domains of collagen determined. Further studies identified the antigenic epitopes inciting the development of antibodies to type IV collagen. Immunization with α3[IV] collagen has provided an experimental model that has led to fundamental discoveries into the genetic and immune processes precipitating and modulating autoimmune diseases.

Understanding the pathogenetic mechanisms of anti-GBM disease brought about the development of highly successful therapies that are based on the removal of circulating antibodies and the reduction of antibody production and interdiction of immune-mediated tissue injury by immunosuppressive therapy. Prognosis depends on the level of renal function at the time of diagnosis. Early institution of therapy prior to the development of irrevocable renal and pulmonary injury portends a favorable outcome. Antibody production is usually short lived and recurrence is infrequent.

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14

Primary Ciliary Dyskinesia

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Abstract Primary ciliary dyskinesia (PCD) is a rare, genetically heterogeneous disorder of motile cilia. In PCD, genetic abnormalities of ciliary ultrastructure and function impair mucociliary clearance, which results in recurrent infection of the lung, as well as the middle ear and sinuses. The genetic defects in respiratory cilia are also frequently shared by specialized cilia (embryonic nodal cilia), which direct the asymmetry of thoracic and abdominal organs; thus, situs inversus and/or situs ambiguus occurs in ~50% of PCD patients. Sperm tail (flagellar) structures are also affected, and most PCD males are infertile. There has been exciting progress in defining the molecular pathogenesis of PCD, and clinical genetic screening tests have been established for two genes (*DNAI1* and *DNAH5*) that are common causes of PCD. Diagnostic testing now involves a combination of methods, including the measurement of nasal nitric oxide (nNO), which facilitates the identification of more PCD patients and expands our understanding of the PCD clinical phenotype. Early recognition and therapeutic intervention are likely to revolutionize clinical care and likely benefit long-term outcomes. Future efforts will continue to focus on further defining the molecular basis of PCD and exploring the interface/overlap of PCD with other genetic disorders involving "sensory" ciliopathies.

Keywords: primary ciliary dyskinesia (PCD), clinical manifestations, genetic basis, diagnosis, treatment, nasal nitric oxide, *DNAH5*, *DNAI1*, situs inversus, heterotaxy, mouse model of PCD

Introduction

Mucociliary clearance is the most important "innate" defense mechanism in the human conducting airways to protect the lung from the adverse effects of inhaled particles and microbes *(1)*. Effective mucociliary clearance requires the integrated actions of airway epithelia to regulate ion (and water) transport, mucus secretion, and the coordinated activity of respiratory cilia to clear the mucus. Under normal circumstances, ~200 motile cilia are present on each ciliated airway cell, which comprise ~60–80% of the conducting airway epithelium. These cilia beat in a coordinated manner to clear

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mucus, reflecting the complex structure in cilia of nine peripheral microtubular doublets and two central microtubules *(2)*.

Genetic ("primary") defects in the structure and function of motile cilia are the cause of primary ciliary dyskinesia (PCD) *(3)*. Defective mucociliary clearance in PCD patients results in recurrent (chronic) infection of the conducting airways, which ultimately results in bronchiectasis. Since motile cilia also line the sinuses and the Eustachian tube, genetically defective cilia also lead to recurrent infection in these sites in PCD patients *(4, 5)*.

The major therapeutic challenges in PCD reflect ongoing infection and inflammation in the lungs, sinuses, and middle ear *(6, 7)*. Empiric therapeutic approaches have been derived from treatment regimens developed and tested in patients with other (non-PCD) etiologies of abnormal mucus clearance and chronic bacterial infection, such as cystic fibrosis. However, it is not clear that all (or even most) of these therapies translate to clinical benefit in PCD, and therapeutic clinical trials in PCD are warranted, as soon as sufficient numbers of patients and participating centers can be identified. Moreover, the age of onset and early course of lung disease in PCD are not well defined, and a prospective study has just been initiated in infants and young children with PCD (http://rarediseasesnetwork.epi.usf.edu/; S. Davis and M. Rosenfeld). If this study demonstrates that the onset of lung disease typically occurs in many (most) PCD patients early in life (before the age of 5 years), it will revolutionize the clinical approach to young PCD patients.

In addition to cilia being the motive force for mucociliary clearance, conserved ciliary-type structures are important for male reproduction and embryologically derived orientation of asymmetric organs (heart, liver, spleen, gut, etc.) *(8, 9)*. Sperm tails are propelled by a cilia-like axonemal structure, and sperm motility is reduced (or absent) in most males with genetic defects in respiratory cilia. A highly specialized subtype of motile cilia (embryological nodal cilia) plays a key role in directing the normal asymmetry of thoracic and abdominal organs; thus, genetic defects in motile cilia are associated with situs inversus totalis in ~50% of PCD patients (Kartagener syndrome). A subset of PCD patients have heterotaxy (situs ambiguus), and many of those have congenital heart disease *(10, 11)*.

Although some ultrastructural and functional ciliary defects were initially described over 30 years ago, we have only recently begun to fully describe the clinical phenotype and understand the molecular pathogenesis of PCD *(3, 12)*. This delay in progress reflects several factors, including the rarity of this disease, the challenges in making a firm diagnosis of PCD, the lack of a focused voluntary health organization (the PCD Foundation was established in 2002), and the previous absence of a national infrastructure to assist in the study of the disease. Recent advances in molecular genetics have defined that mutations in two genes (*DNAI1* and *DNAH5*) are relatively common causes of PCD, and a clinical screening test for mutations in these genes has recently been established *(13–16)*. This chapter will discuss the recent explosion of clinical and genetic information about PCD, which reflects the coordinated efforts of a newly formed PCD Foundation, an NIH initiative in the area of rare lung diseases, and the collaborative spirit of multiple international investigators.

Epidemiology

PCD is a rare, genetically heterogeneous disorder, i.e., mutations in any one of multiple genes that play a role in cilia structure or function can cause the disorder. PCD is usually

an autosomal recessive genetic disorder. It is estimated that as many as 15,000 people in the United States may suffer from PCD, based on extrapolations of the prevalence of dextrocardia plus bronchiectasis in population surveys *(17, 18)*. However, the number of people with a defined diagnosis of PCD is much less, which likely reflects (at least in part) the difficulties of establishing a diagnosis without the aid of genetic testing. With the recent establishment of an NIH (ORD/NCRR) consortium to study genetic disorders of mucociliary clearance (http://rarediseasesnetwork.epi.usf.edu/), and the advent of improved screening techniques [measurement of nasal nitric oxide (nNO) and genetic testing] *(5, 19, 20)*, we are beginning to recognize a range of clinical phenotypes in PCD and an increasing number of patients with a well-defined diagnosis. Although it is stated that PCD affects all ethnicities, there is a paucity of African-Americans who have been diagnosed through the Consortium; whether this reflects a low prevalence of PCD in African-Americans, or a referral bias, is not known.

The vast majority of PCD is an autosomal, recessive trait, although there are a few reports of other inheritance patterns *(3)*. The pattern of disease in one family suggested an autosomal, dominant, or X-linked (dominant) inheritance *(21)*, and another family displayed X-linked recessive mental retardation and PCD, in conjunction with *OFD1* mutations *(22)*. Finally, others have reported an X-linked complex phenotype disorder involving retinitis pigmentosa, sensory hearing defects, and PCD, reflecting mutations in RPGR *(23–25)*.

A major focus of the NIH-sponsored (Consortium) research initiative in rare genetic diseases of the lungs is to extend the use of nasal NO as a screening test for PCD and to define additional disease-causing genes for PCD. Nasal NO screening tests will increase identification of at-risk subjects, and definition of new genetic etiologies will allow the development of broader genetic testing for PCD. Taken together, these approaches will aid the diagnosis of PCD and increase the number of available subjects for clinical trials of therapies used in other disorders (such as cystic fibrosis) and/or novel therapeutic approaches.

Genetic Basis and Molecular Pathogenesis of PCD

Overview: PCD was the first human disorder linked to cilia structure and function. Recently, there has been an explosion in the knowledge of multiple genetic disorders related to defective ciliary (axonemal) structure and/or function; collectively, these are called "ciliopathies." These include hydrocephalus, polycystic kidney diseases, polycystic liver disease, some forms of retinal degeneration, nephronophthisis, Bardet-Biedl syndrome, Alstrom syndrome, Meckel-Gruber syndrome, Joubert syndrome, laterality defects (reviewed in Ref. *26*), and Jeune syndrome *(27)*. In this chapter, we will review only genetics of PCD.

Genetics of PCD: PCD is a genetically heterogeneous disorder, owing to the complexity of the ciliary axonemal structure, which is highly conserved through evolution. Defining genetic causes of PCD from a candidate gene is challenging but has resulted in important insights. Due to the conservation of the axonemal structure phylogenetically, studies in the flagella of the lower organisms can provide clues about the mammalian axonemal structure. One such extensively studied organism is a bi-flagellate, unicellular algae *Chlamydomonas reinhardtii*, which has multiple motility mutants described and characterized (Figure 14.1). Human orthologues of the genes involved in the motility mutants of the *Chlamydomonas* are good candidates to study for human PCD. In fact,

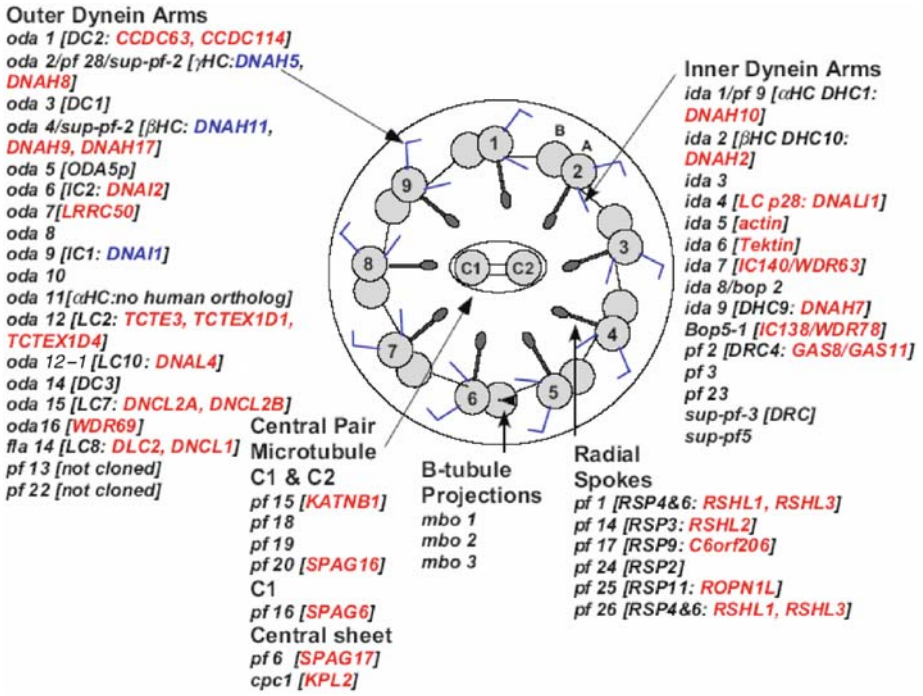

Figure 14.1 Schematic showing cross section of the flagellar axoneme. Some of the *Chlamydomonas* mutants and the corresponding human orthologous genes are shown. Human orthologous genes are marked in *red* and genes with mutations known in PCD are marked in *blue*. Corresponding *Chlamydomonas* genes are marked in *black* (adopted and modified from Refs. *46, 150–165, 100, 166–169*). DC, docking complex; DHC, dynein heavy chain; DRC, dynein regulatory complex; HC, heavy chain; IC, intermediate chain; LC, light chain; RSP, radial spoke protein

selection of candidates, assisted by *Chlamydomonas*, resulted in identifying the first PCD-causing gene *(28)*. Since then, it has been used to select multiple genes for their possible involvement in PCD. Below are the details of genes involved in human PCD.

DNAI1: This 20 exon gene encodes an axonemal dynein intermediate chain 1 (chromosome 9p13-p21) and was the first gene identified as disease-causing in PCD *(28)*. *DNAI1* was cloned by the candidate gene approach. Subsequent studies revealed that ~10% of >200 PCD patients harbored mutations in this gene, which increased to 14% if only patients with ODA defect were considered *(28–31)*. There are 18 mutant alleles for *DNAI1* known (Table 14.1), but a mutation cluster was observed in four exons (1, 13, 16, and 17), which became the basis for clinical molecular genetic testing. One splice mutation (IVS1+2_3insT) represented ~55% of all *DNAI1* mutant alleles, due to the founder effect *(31)*.

DNAH5: Using homozygosity mapping together with the candidate gene approach, Olbrich and colleagues identified *DNAH5* (chromosome 5p15) as a causative gene in a large inbred Arab family with PCD *(32, 33)*. *DNAH5* consists of 79 exons and encodes a heavy-chain dynein of an ODA that is orthologous to the γ-HC of *Chlamydomonas*. Large-scale mutation studies involving 134 unrelated families showed that ~28% of all PCD patients harbor mutations in this gene. The mutation detection rate went up (49%)

Table 14.1 Mutations of *DNAI1* gene in PCD patients *(28–31)*.

Exon/intron #	Mutation	Type of mutation	Number of unrelated patients with mutation
Intron 1[a]	c. IVS1+2_3insT (p. splice/truncation)[b]	Splice/truncation	18[c]
Exon 5	c. 282_283insAATA (p. G95NfsX24)	Frameshift	1
Exon 6	c. 463delA (p. T155LfsX18)	Frameshift	1
Intron 7	c. IVS7-2A>G	Splice	1
Exon 10	c. 874C>T (p. Q292X)	Nonsense	1
Intron 10	c. IVS10-4_7delGTTT	Splice	1
Exon 13[a]	c. 1212T>G (p. Y404X)	Nonsense	1[c]
	c. 1222G>A (p. V408M)	Missense	1
	c. 1307G>A (p. W436X)	Nonsense	1
Exon 16[a]	c. 1490G>A (p. R468_K523del)	Splice/deletion	1
	c. 1543G>A (p. G515S)	Missense	2
Exon 17[a]	c. 1612G>A (p. A538T)	Missense	2
	c. 1644G>A (p. W548X)	Nonsense	1[c]
	c. 1657_1668del (p. T553_F556del)	Frameshift	1
	c. 1703G>C (p. W568S)	Missense	1
	c. 1704G>A (p. W568X)	Nonsense	1
Exon 19	c. 1926_1927insCC (I643PfsX47)	Frameshift	1
Intron 19	c. IVS19+1G>A (p. A607_K667del)	Splice/deletion	1

[a] Included in the clinical molecular genetics test panel
[b] Founder mutation
[c] Additional one patient identified with this mutation (UNC Unpublished data)
c, nucleotide change and p, protein change

when only PCD families known to harbor ODA defects were considered *(13, 34)*. There were 43 different mutations identified showing allelic heterogeneity (Table 14.2), but a mutation cluster was seen in five exons (34, 50, 63, 76, and 77), accounting for 53% of all *DNAH5* mutations. This mutation cluster (five exons) is part of the clinical molecular genetic test for PCD. Immunofluorescence studies carried out in patients known to harbor biallelic mutations revealed that mutant *DNAH5* was expressed in the respiratory epithelial cells but failed to localize along the axonemal shaft *(3)*. In addition, sperm immunofluorescence analysis from a male patient (harboring biallelic truncating mutations in *DNAH5*) was the same as normal sperm; hence, the authors concluded

Table 14.2 Mutations of *DNAH5* gene in PCD patients *(13, 33)*.

Exon/intron #	Mutation: base change (amino acid change)	Type of mutation	Number of unrelated patients with mutation
Exon 3	c. 232C>T (p. R78X)	Nonsense	1
	c. 252T>G (p. Y84X)	Nonsense	1
Exon 7	c. 832delG (p. A278RfsX27)	Frameshift	1[c]
Exon 11	c. 1427_1428delTT (p. F476SfsX26)	Frameshift	1
Exon 12	c. 1627C>T (p. Q543X)	Nonsense	1
Exon 13	c. 1730G>C (p. N549_R577delfsX5)	Splice/truncation	1
Exon 14	c. 1828C>T (p. Q610X)	Nonsense	1
Intron 17	c. IVS17+2T>C	Splice	1
Exon 25	c. 3905delT (p. L1302RfsX19)	Frameshift	1
Intron 27	c. IVS27+1G>A	Splice	1
Exon 28	c. 4360C>T (p. R1454X)	Nonsense	1
	c. 4361G>A (p. R1454Q)	Missense	1
Exon 32	c. 5130_5131insA (p. R1711TfsX36)	Frameshift	1
	c. 5147G>T (p. R1716L)	Missense	1
Exon 33	c. 5281C>T (p. R1761X)	Nonsense	1
	c. 5482C>T (p. Q1828X)	Nonsense	1
Exon 34[a]	c. 5563_5564insA (p. I1855NfsX6)	Frameshift	4
	c. 5599_5600insC (p. L1867PfsX35)	Frameshift	1
Exon 36	c. 6037C>T (p. R2013X)	Nonsense	1
Exon 41	c. 6791G>A (p. S2264N)	Missense	1
Exon 43	c. 7039G>A (p. E2347K)	Missense	1
Exon 45	c. 7502G>C (p. R2501P)	Missense	1[c]
Exon 48	c. 7914_7915insA (R2639TfsX19)	Frameshift	1
	c. 7915C>T (p. R2639X)	Nonsense	1
Exon 49	c. 8029C>T (p. 2677X)	Nonsense	1[c]
	c. 8167C>T (p. Q2723X)	Nonsense	1
Exon 50[a]	c. 8314C>T (p. R2772X)	Nonsense	1
	c. 8404C>T (p. Q2802X)	Nonsense	1[c]
	c. 8440_8447delGAAC-CAAA (p.E2814fsX1)	Frameshift	2
Exon 51	c. 8528T>C (p. F2843S)[b]	Missense	2
Exon 53	c. 8910_8911delATinsG (2970SfsX7)	Frameshift	1
Exon 60	c. 10226G>C (p. W3409S)	Missense	1
Exon 62	c. 10555G>C (p. G3519R)	Missense	1
Exon 63[a]	c. 10815delT (p. P3606HfsX23)[b]	Frameshift	7[d]

Table 14.2 (continued)

Exon/intron #	Mutation: base change (amino acid change)	Type of mutation	Number of unrelated patients with mutation
Exon 67	c. 11528C>T (p. S3843L)	Missense	1
Exon 73	c. 12614G>T (p. G4205V)	Missense	1
Intron 74	c. IVS74-1G>C (p. S4304DfsX6)	Splice/frameshift	1
Intron 75[a]	c. IVS75-2A>T	Splice	1
Exon 76[a]	c. 13194_13197delCAGA (p. D4398EfsX16)	Frameshift	1[c]
Intron 76[a]	c. IVS76+5G>A[b]	Splice	2
Exon 77[a]	c. 13426C>T (p. D4476X)	Nonsense	1
	c. 13458_13459insT (p. N4487fsX1)	Frameshift	4[c]
	c. 13486C>T (p. R4496X)	Nonsense	1[c]

[a] Included in the clinical molecular genetics test panel
[b] Founder mutation
[c] Additional one patient identified with the mutation (UNC Unpublished data)
[d] Additional nine unrelated patients identified with the mutation
c, base change and p, protein change

that DNAH5 mislocalization was not present in sperm flagella *(35)*. In addition, it was noted that a PCD male patient with biallelic (compound heterozygous) nonsense mutations was able to father four children without any fertility assistance. He passed on one mutant *DNAH5* allele to the three children whose DNA was available for testing *(36)*.

DNAH11: This gene maps to human chromosome 7p21 and is a homologue of β-HC dynein of *Chlamydomonas* ODA. A patient with paternal uniparental isodisomy of chromosome 7 presented with cystic fibrosis and was homozygous for the common (deltaF508) mutation in *CFTR*. In addition, this patient also presented with situs inversus totalis, but the presence of cystic fibrosis made it difficult to also make a diagnosis of Kartagener syndrome. The human *DNAH11* orthologue in *Chlamydomonas* causes motility defects; hence, *DNAH11* was tested as a candidate for the presence of situs inversus in this patient. Results revealed that this patient harbored a homozygous nonsense mutation in *DNAH11* (R2852X). Since the mouse mutants for *lrd* (orthologue of *DNAH11*) caused only situs inversus and no characteristic PCD respiratory phenotype, this gene was classified as causing situs inversus *(37)*. In the same study, six unrelated patients with compatible linkage to the same chromosome region *(38)* were also analyzed and no pathogenic mutations were identified, although one heterozygous variant could not be excluded *(37)*. Recently, a large German family with five members affected with Kartagener syndrome was ascertained. Affected individuals had abnormal ciliary beat frequency, but normal dynein arm structure, as evaluated by electron microscopy and immunohistochemistry. All the affected subjects had biallelic compound heterozygous truncating mutations in *DNAH11 (39)*. These data support the notion that mutations in *DNAH11* are causative of PCD in a subset of patients with normal dynein arms, and studies with large number of patients are warranted.

TXNDC3: This gene encodes thioredoxin–nucleoside diphosphate and resides on chromosome 7p15. The homologue (*IC1*) in sea urchin encodes a component of sperm

ODA. In addition, *Chlamydomonas* LC3 and LC5 of ODA are homologous to *TXNDC3*. Due to the involvement of *TXNDC3* in axonemal structure, Duriez et al. *(40)* tested this candidate gene in 41 unrelated PCD patients. No pathogenic mutations were found in 40 families, but one family had a nonsense mutation (L426X) on one allele inherited from the mother and a common variant (c. 271-27C>T) on the trans allele inherited from the father. The frequency of the common variant is 1% in the non-PCD subjects, but this variant occurs near the branch point in the intron that is involved in the splicing. It was hypothesized that the presence of a nonsense mutation on one allele and a variant on the other was causative of PCD in this patient. Further studies revealed that *TXNDC3* had two transcripts, a full-length isoform and a novel short isoform TXNDC3d7, with the in-frame deletion of exon 7. It is the short transcript TXNDC3d7 that presumably binds to the microtubules. The levels of TXNDC3d7 were reduced in the PCD patient carrying the variant on one allele, thereby affecting the ratio of the two isoforms. Transallelic inheritance of a nonsense mutation and a pathogenic variant is consistent with the disease phenotype segregating in a recessive mode of inheritance.

ODF1 and *RPGR*: Occasionally, mutations in the genes not primarily affecting ciliary motor function, and co-segregating with other syndromes, have been seen in PCD. Mutations of *OFD1* were seen in a Polish family with multiple affected males presenting with X-linked mental retardation and PCD *(22)*. Mutations in *RPGR* have been noted in multiple families presenting with X-linked form of retinitis pigmentosa co-segregating with PCD *(41, 42)*. OFD1 and RPGR are both localized to the ciliary base and do not affect the axonemal motor proteins *(41, 22)*.

No PCD mutations: No mutations have been identified in a number of genes tested in unrelated PCD patients. These include ODA genes *DNAH17* ($n = 4$) *(43)*, *DNAH9* ($n = 2$) *(44)*, *DNAI2* ($n = 16$) *(45, 46)*, *DNAL1* (n–86) *(47)*, *DNAL4* ($n = 54$) *(48)*, *TCTE3* ($n = 36$) *(49)*, *DYNLL2* ($n = 58$); IDA genes *DNALI1* ($n = 61$) *(48, 50)*, *DNAH3* ($n = 7$) *(51)*, *DPCD* ($n = 51$) *(52)*, *DNAH7* ($n = 1$) *(53)*; central pair genes *SPAG6* ($n = 54$) *(43)*, *SPAG16* ($n = 7$) *(41, 42, 46, 54)*; and a ciliary gene *FOXJ1/HFH-4* ($n = 8$) *(55)*.

Linkage studies: Conventional family-based linkage studies are hard to perform in PCD, due to the presence of extensive locus heterogeneity, even within the families with identical ultrastructural defects. A large–scale, genome-wide linkage analysis of 31 multiplex families (70 affected individuals) from Europe and North America did not yield any major PCD locus, despite the fact that the sample size was powerful to detect the linkage if 40% of the families were linked to one locus *(38)*. This study did show some potential loci on multiple chromosomal regions. Other studies involving consanguineous families or isolated inbred populations from Arab families, Faroe Islands, and Druze have mapped PCD loci (chromosomes 19q13.41–13.42, 16p12.1–12.2, 15q13.1–15.1, respectively), with LOD scores of greater than 3, but no pathogenic gene has yet been determined *(56, 57)*.

Proteomics: In order to circumvent the challenges presented by genetic heterogeneity, other methods to define PCD loci need to be undertaken. One such methodology is ciliary proteomic analysis. In brief, ciliary proteins from a control and a PCD patient (with specific ultrastructural abnormalities) are isolated and compared. Proteins missing from cilia of PCD patients are identified by mass spectrometry of the corresponding protein from cilia of the controls. *DNAH7* emerged as a candidate gene because it was absent by ciliary proteomics analysis in a PCD patient, but full cDNA sequencing did not reveal causative mutations *(53)*. Additional proteomic analyses are likely to identify other PCD candidate genes.

Animal Models

Overview: There are several animal models for PCD, including dogs *(58–62)*, pigs *(63)*, cats *(64)*, rats *(65)*, and mice. Many are naturally occurring, but some are generated by gene knockout methods. In this section, the emphasis is given to mouse models, where genotype and phenotype correlations have been most extensively studied (see Table 14.3).

Table 14.3 Mice table.

Mouse gene mutation	General mouse phenotype	Mutations in human orthologue in PCD patients # (%)	References
Mdnah5$^{-/-}$ (insertional mutation in mouse *DNAH5*)	Recurrent respiratory infections, post-natal death, situs abnormalities, hydrocephalus, immotile cilia, ciliary ODA defects	38 (134 analyzed) (28%)	*(13, 33, 66)*
Dnahc5$^{del593-/-}$ (in-frame deletion of 593 amino acids in mouse *DNAH5* by ENU mutagenesis)	Respiratory cilia missing ODA, dyskinetic cilia, 25% situs solitus, 35% situs inversus totalis, 40% heterotaxy with congenital heart defects		*(72)*
lrd (*iv/iv*) spontaneous point mutation (in mouse *DNAH11*)	36% situs inversus totalis, 26% situs ambiguus, no ciliary ultrastructural defects, no infertility	One family with Kartagener syndrome and compound heterozygous for *DNAH11* mutation.	*(170)*
lrd (*lgl/lgl*) spontaneous large deletion (in mouse *DNAH11*)	Situs abnormalities, limb defects, craniofacial abnormalities, abnormal brain development	One subject with uniparental isodisomy, cystic fibrosis + situs inversus and biallelic nonsense mutation of *DNAH11*.	*(37, 171)*
lrd$^{-/-}$ (mouse *DNAH11*) *targeted deletion*	Situs abnormalities, immotile nodal cilia	None (6 analyzed)	*(37, 39, 73)*
Dpcd/poll$^{-/-}$	Chronic sinusitis, situs inversus, hydrocephalus, ciliary IDA defects, male infertility	None (51 analyzed)	*(52, 76)*

Table 14.3 (continued)

Mouse gene mutation	General mouse phenotype	Mutations in human orthologue in PCD patients # (%)	References
Pcdp1$^{-/-}$ nm1054 recessive mutation with 400-kb deletion	B6 mouse strain, severe hydrocephalus, and perinatal death. 129 mouse strain, mild to no hydrocephalus, male infertility, mucus accumulation in sinuses with reduced ciliary beat frequency. No situs inversus or ear disease	Not done	*(80)*
hy3 (spontaneous 1-bp deletion in *Hydin*)	Severe hydrocephalus, perinatal death. Normal ciliary axoneme, but one of the central microtubules lack specific projection. Reduced ciliary beat frequency, in ependymal and tracheal cilia	Not done	*(81–84)*
Hydin$^{-/-}$ (insertional mutation in mouse *Hydin*)			*(82–84,172)*
Tektin-t$^{-/-}$	Male infertility, frequent bending of sperm, sperm motility defects, sperm flagella, and tracheal cilia with IDA defects	Not done	*(96)*
Foxj1/Hfh4$^{-/-}$	Pre and post-natal growth failure, situs abnormalities, hydrocephalus, absence of 9+2 cilia, defective ciliogenesis	None (8 analyzed)	*(55)*
Mdhc7$^{-/-}$ (human *DNAH1*)	Male infertility, sperm immotility, ciliary beat frequency reduced in trachea without ultrastructural defects, only one globular head in IDA3 complex instead of two	Not done	*(97, 98)*
Spag16 chimeras (chimeras for *Spag16L* + *Spag16S*)	Most of the males infertile, very few fertile males but mutant allele is never transmitted to the progeny	Two subjects without PCD from a family heterozygous for mutation	*(54)*

Table 14.3 (continued)

Mouse gene mutation	General mouse phenotype	Mutations in human orthologue in PCD patients # (%)	References
Spag16L$^{-/-}$	Loss of central pair and doublet disorganization	None (five analyzed)	*(46, 100, 101)*
Spag6$^{-/-}$	50% mortality by 8 weeks after birth, hydrocephalus, male infertility, sperm motility defects, abnormal sperm morphology, 20% females infertile	None (54 analyzed)	*(43, 100, 102)*
Spag6$^{-/-}$/ *Spag16L*$^{-/-}$ double knockout	Growth retardation, hydrocephalus, 100% mortality by 5 weeks after birth, pneumonia, normal ultrastructure for cilia from brain and lung, no lateralization defects	Not done	*(103)*

ODA, outer dynein arms; IDA, inner dynein arms.

Mdnah5-*deficient mouse*: Homozygous *Mdnah5*$^{-/-}$ deficient mice were generated by transgenic insertional mutagenesis of an unrelated gene *(66)*, which led to out-of-frame premature truncation. These mice have classical features of PCD and are the only known mouse model in which the orthologous PCD gene in human is known to have disease-causing mutations for (classic) PCD. These mice have ciliary "immotility," situs abnormalities, and recurrent respiratory infection. The *Mdnah5* gene (79 exons) codes for a ciliary outer dynein arm (ODA) protein; ultrastructural analysis of mouse (and human) cilia revealed the absence of ODA. Mice heterozygous for *Mdnah5* did not show the PCD phenotype, consistent with an autosomal recessive mode of inheritance. Almost all homozygous mutant mice developed hydrocephalus and died perinatally. *Mdnah5* is expressed in the ependymal cells lining the brain ventricles and the aqueduct, and partial ODA deficiency was noted in the ependymal cilia of the knockout mice *(67)*. Hydrocephalus is occasionally reported in human PCD *(68–71)*, which suggests that ependymal dysfunction also contributes to human hydrocephalus *(67)*. Interestingly, Tan et al. *(72)* identified homozygous mice with an in-frame deletion of 593 amino acids (exons 7–17) during the ENU mutagenesis screen. These mice, called *Dnahc5del*^{593}, had dyskinetic cilia, and ultrastructure analysis of respiratory cilia revealed ODA defects. In addition, 35% of *Dnahc5del*^{593} mice presented with situs inversus totalis and 40% had heterotaxy with congenital heart defects that led to post-natal lethality.

lrd-*deficient mouse (*iv *mice and* lgl *mice)*: *Left–right dynein* (*lrd*) codes for a microtubule-based motor protein, which is expressed symmetrically at e7.5 in the ventral cells of the node of the embryo, but a striking asymmetric expression pattern is observed at e8.0. The homozygous mutant, generated by targeting the ATP-binding site (motor function domain), resulted in the random determination of situs *(73)*: 48% situs solitus; 38% situs inversus totalis; and 13% situs ambiguus. Additionally, two

spontaneous mouse mutants involving *lrd* have been described. One is the inversus viscerum mutant (*iv/iv* mouse) with a missense point mutation causing substitution of glutamic acid by lysine in *lrd*. Another is *legless* (*lgl*), which has a large deletion, including *lrd* gene *(74)*. Both *lgl/lgl* and *iv/iv* mice present with situs abnormalities similar to what is found in the *lrd* nullizygous mice *(74)*. None of the *iv/iv* mice had any respiratory problem or axonemal ultrastructural or ciliary functional defects; plus, fertility was not compromised. In addition to the situs abnormalities in *lgl/lgl* mice, they also had limb defects, craniofacial malformations, and abnormal development of the brain *(75)*. The severity of phenotype in *lgl/lgl* mice compared to the *iv/iv* mice likely reflects the more severe *lrd* mutation in *lgl/lgl* mice *(74)*. Mutation of *DNAH11* was found in a family with clear-cut diagnosis of Kartagener syndrome with normal dynein arms *(39)*; hence, it is still a candidate for PCD.

Dpcd- *and* Poll-*deficient mouse*: DNA polymerase lambda (*POLL*) is a member of X-family of polymerases that is important for maintenance of DNA integrity during replication, repair, recombination, and mitosis. Mice with homozygous deletion of *poll* were created using homologous recombination *(76)*; surprisingly, these mice had the classic PCD phenotype, including chronic suppurative sinusitis, hydrocephalus, situs inversus, and male infertility. Ultrastructural analysis of respiratory cilia showed defective IDA. Approximately half of the nullizygous mice died by 3 weeks of age, and 70% died by 9 weeks. Careful examination of the targeting construct revealed deletion of the first exon of another gene *Dpcd* (deleted in a mouse model of PCD), which was transcribed in the opposite direction *(52)*. Thus, these mice *(76)* were double knockouts for $poll^{-/-}/Dpcd^{-/-}$. Interestingly, in a separate study *(77)*, knockout mice generated using only catalytic domain of *poll* (where *Dpcd* was intact) were phenotypically normal, with no evidence of PCD. Taken together, it appears that *poll* is not a candidate for human PCD, but the phenotype observed by Kobayashi et al. *(76)* may indeed be due to the loss of *Dpcd*. In order to test the role of *DPCD* in human PCD, 51 unrelated PCD patients (15 with sole IDA defects) were analyzed; however, none harbored any mutations *(52)*. Thus, these results indicate that mutations in *DPCD* may not account for a large number of PCD cases.

Pcdp1-*deficient mouse*: A recessive, pleiotropic nm1504 mouse mutant consists of deletion of six genes on chromosome 1 spanning ~ 400 kb genomic region *(78, 79)*. The PCD-like phenotype was abolished by transgenic rescue with a novel protein Pcdp1. Pcdp1 is expressed in spermatogenic cell types; the protein is localized in flagella and in motile cilia of mice and humans. The severity of phenotype differed by genetic background; B6 (homozygous) mutant mice died perinatally due to severe hydrocephalus, but 129 mutant mice had little hydrocephalus. Both 129 and B6129F1 mice were infertile. Histological evaluation showed the absence of mature spermatozoa in seminiferous tubules, and only a few sperm had a visible tail. In addition, these mice had mucus accumulation in the sinuses without any inflammation, likely reflecting impaired mucociliary clearance. Ultrastructural studies on respiratory cilia showed normal dynein arms, but beat frequency was reduced by ~25%. These mice did not have situs inversus or otitis media. This gene has no orthologue in *Chlamydomonas (80)*. The protein has no identifiable domains or structural motifs, except that amino acids 53–231 have 22% identity with the central apparatus protein Hydin (see below). Since these mice had no situs abnormalities, it appears that Pcdp1 does not play a role in nodal cilia and left–right asymmetry; perhaps its function is associated with the central microtubule pair. *Pcdp1* is a candidate gene for PCD patients with normal ciliary ultrastructure.

Hydin-*deficient mouse*: Two mouse mutants of *Hydin* are known. First, the *hy3* mutant, described by Gruneberg *(81)*, had a spontaneous homozygous 1-bp deletion-causing premature truncation signal, resulting in loss of 89% of the full-length Hydin gene product *(82, 83)*. *OVE459* mouse mutant is characterized by homozygous genomic rearrangement within the *Hydin* caused by insertional mutation *(82, 83)*. Both Hydin-mutant mice present with lethal communicating hydrocephalus and die perinatally. Situs inversus was not present in *hy3/hy3* mice *(84)*. *Hydin* is evolutionarily conserved and the protein is localized to ependymal cells as well as cilia/flagella *(82, 85, 86)*. *Hydin*-mutant mice had normal axonemal dynein arms and radial spokes but lacked C2b projections in the central complex, which led to the ciliary bending defects and reduced beat frequency *(84)*. Gene knockdown experiments using RNA-mediated interference in *Chlamydomonas* led to short, paralyzed flagella lacking the C2b projection of the C2 microtubule *(87)*. *Hydin* is a component of the central pair and essential for the flagellar motility; hence, it remains a candidate for the human PCD. *Hydin* has two paralogous copies in humans: a full length on chromosome 16q (86 exons) and a duplicon on chromosome 1q lacking seven exons. Both paralogous copies are expressed and have sequence identity *(88)*, which makes it challenging to perform mutation analysis in PCD patients.

Tektin-t-*deficient mouse*: Tektins are constitutive proteins involved in the structural complexity and stability of axonemal microtubules in cilia, flagella, basal body, and centriole *(89–92)*. Tektins are conserved from *Chlamydomonas* to mammals and are thought to play an important role in the formation and movement of flagella and cilia *(93–95)*. Mutant mice were made by insertion of a gene-trapping vector *(96)*. Homozygous mutants (deficient in *tektin-t*) were viable, but with male infertility. The defective spermatozoa were able to fertilize eggs in vitro; hence, male infertility was caused by defective motility. Morphology of sperm was abnormal, including a motility defect reflecting defective IDA by ultrastructure analysis. Tracheal cilia had IDA and motility defects. Surprisingly, no respiratory phenotype or situs information was reported in these mice *(96)*. Given that these mice exhibited male infertility corresponding to an IDA defect and a functional defect in cilia, this gene remains a candidate gene for testing in human PCD for patients with IDA defects.

Mdhc7-*deficient mouse*: *Mdhc7* (human *DNAH1* or *HDHC7*) is a dynein heavy chain that encodes a component of IDA3 *(97)*. Homozygous *Mdhc7* knockout mice, generated by targeted disruption, were viable and did not show any respiratory distress or laterality defects *(98)*. Nullizygous male mice had motility defects, were infertile, and did not produce any offspring, but females were fertile. Tracheal ciliary beat frequency was reduced by 50% in *Mdhc7* nullizygous mice, but ultrastructure analysis showed no gross defect in axonemal structure. *Mdhc7* homozygous mutant mice had only single globular head in IDA3, compared to two heads in wild type *(97)*. This gene has not been tested in PCD patients but may be a candidate gene where ciliary ultrastructure is normal.

Foxj1/Hfh4 *(hepatocyte nuclear factor 4)-deficient mouse*: *FOXJ1*/*HFH4* (hepatocyte nuclear factor 4) belongs to winged-helix/forkhead family of transcription factors, and homozygous null mice were created by targeted disruption of *Foxj1*/*hfh4 (99)*. The mice had hydrocephalus and situs abnormalities (situs inversus totalis and heterotaxy), and nullizygous mice were devoid of cilia; hence, this gene is important for the development of cilia. This gene has been tested in eight PCD patients who had either linkage to the chromosome locus 17q23 or aciliogenesis, but no deleterious mutations were detected *(55)*. Since it was studied in only a few patients, it remains a candidate for PCD in patients with no cilia.

Pf20/Spag16 *(sperm-associated antigen 16)-deficient mouse*: The PF20/SPAG16 gene product contains conserved WD repeat regions and is located along the length of the C2 microtubule on the inter-microtubule bridge connecting the two central microtubules. In *Chlamydomonas*, mutant pf20 leads to the absence of the entire central apparatus and paralyzed flagella. To study *Pf20/Spag16*, Zhang et al. attempted to create a homozygous null mouse, using targeted disruption of *Spag16 (100)*, but could generate only chimeric mice that had male infertility due to impaired spermatogenesis and marked disorganization of sperm axonemal structure. Further insight came from the recognition that *Pf20/Spag16* encodes two transcripts (2.5 and 1.4 kb), termed as *Spag16L* (full length) and *Spag16S* (short), respectively. To explain their roles, Zhang et al. *(101)* created *Spag16L* (*Spag16_pr1*) homozygous null mice where SPAG16L but not *Spag16S* was eliminated. The resulting homozygous male mice were infertile, with a low sperm count and significant motility defect, but the ultrastructure of the flagellar axoneme was normal. Thus, SPAG16L deficiency impairs the function of the sperm tail without causing gross structural changes. These animals had no evidence of PCD; hence, *SPAG16L* does not appear to play an important role in respiratory cilia function. Additionally, five unrelated PCD patients with central pair defects did not harbor mutations in this gene *(46)*.

Pf16/Spag6 *(sperm-associated antigen 16)-deficient mouse*: *Spag6* is orthologous to the PF16 of *Chlamydomonas* that is located along the C1 microtubule and contains a conserved armadillo repeat, which is important for protein–protein interactions, including PF20 *(100)*. Mutant *Chlamydomonas* lacking pf16 results in paralyzed flagella, and the C1 microtubule is destabilized with the loss of C1-associated polypeptides. Sapiro et al. *(102)* created the *Spag6* homozygous null mice by gene targeting. Hydrocephalus was seen in ~50% of the mice, and all male mice were infertile, reflecting abnormal sperm motility and morphology. However, ultrastructure of tracheal and ependymal cilia of the nullizygous mice appeared normal. Human *SPAG6* has been studied in 54 PCD patients, but no mutations have been found *(43)*.

Spag6/Spag16L *double knockout mouse*: Since Spag6 and Spag16 are both localized in the central apparatus, Zhang et al. *(103)* generated double knockout mice (nullizygous $Spag6^{-/-}$ and $Spag16L^{-/-}$) to study the combined deficiency. The double mutant mice had more severe phenotypes, compared to the *Spag6* or *Spag16L* alone, i.e., there was 100% mortality by the age of 5 weeks, and severe hydrocephalus. Both these proteins were absent in the brain of the double mutants. In addition, these mice had pneumonia, accompanied by hemorrhage, edema, and atelectasis, whereas the lung phenotype was not observed in the mice nullizygous for each gene. Ultrastructure analysis of cilia from brain and lung of nullizygous double mutants revealed normal axonemal structure. Furthermore, no cilia-related phenotype or lateralization defects were observed in these mice. Thus far, no PCD patient has been found to harbor mutations in *SPAG16* or *SPAG6*, alone or in combination.

Clinical Manifestations of PCD

Overview: PCD is a multisystem disease with a broad range of clinical signs and symptoms, which vary among patients and by age *(5)* (see Table 14.4). The most prominent features include respiratory distress in full-term neonates, laterality defects (situs inversus totalis or heterotaxy, i.e., situs ambiguus), recurrent oto-sino-pulmonary infections,

Table 14.4 Clinical manifestations of PCD.

Clinical feature	Prevalence[a]
Chronic productive cough	+++++
Bronchiectasis (adults)	+++++
Evidence of chronic sinusitis by CT scan	+++++
Otitis media	+++++
Neonatal respiratory distress	+++++
Infertility (male)	+++++
Rhinosinusitis	+++++
Bronchiectasis (children – age dependent)	++++
Wheezing	++++
Situs inversus totalis	++++
Heterotaxy/situs ambiguus	++
Congenital heart disease (related to heterotaxy)	++
Lithoptysis	+
Hydrocephalus	+

[a] +++++, >60%; ++++, 40–60%; +++, 20–40%; ++, 5–20%; +, <5%

bronchiectasis, and male infertility *(5, 6, 9)*. The triad of chronic sinusitis, bronchiectasis, and situs inversus totalis, known as Kartagener syndrome (MIM# 244400), was recognized over 50 years ago, but identification of other manifestations has evolved more recently *(104)*. The respiratory disease results from dyskinesis of motile cilia. Typical symptoms in children are a chronic productive cough and persistent rhinorrhea *(7)*. Dysfunction of the cilia that line the epithelium of the middle ear and the Eustachian tube leads to chronic suppurative otitis media, which can result in scarring of the tympanic membrane and frequently hearing loss. Fifty percent of PCD patients have situs inversus totalis because dysfunction of the nodal cilia during embryogenesis leads to random right–left organ asymmetry *(105)*. Situs inversus totalis is sometimes diagnosed in utero, with ultrasound, or may be diagnosed at birth if respiratory symptoms prompt clinicians to order a chest radiograph. In rare cases, situs inversus is not diagnosed until adulthood. Infertility is diagnosed during adulthood in males who have immotile sperm reflecting the same ultrastructural and genetic defect(s) as seen in the respiratory cilia. Other rare clinical features, such as hydrocephalus, may also be present.

Neonatal respiratory distress: More than 80% of term neonates with PCD have symptoms such as tachypnea, increased work of breathing, and retractions *(106, 107)*. The neonatal lung contains fluid that must be cleared rapidly to allow adequate gas exchange; thus, respiratory distress in PCD implies that ciliary function is critical for effective clearance of fetal lung fluid. Other mechanisms that are important in removing this fluid include chest compression during vaginal delivery, and salt and water absorption. The differential diagnosis of tachypnea in a term infant includes transient tachypnea in the newborn, meconium aspiration, aspiration pneumonia, congenital heart disease, persistent fetal circulation, and sepsis. Neonates with PCD can also present with atelectasis and/or pneumonia *(106)*.

Several case studies have reported respiratory distress in term neonates that were later diagnosed with PCD. In 1981, Whitelaw described neonates with tachypnea, retraction, rales, and dextrocardia, and brought to our attention that PCD should be considered in these patients *(108)*. Holzmann found that 9 out of 10 patients with PCD

had neonatal respiratory distress and required prolonged hospitalization at birth from 10 days to 5 weeks. In all nine cases, other causes of respiratory distress, such as hyaline membrane disease, aspiration, neonatal pneumonia, metabolic disorders, and cardiovascular abnormalities, were excluded *(109)*.

Infants who are diagnosed with PCD at birth and receive early intervention can develop normally and complications can be minimized. Hossain describes a newborn with nasal congestion and hypoxia that did not improve with supplemental oxygen or antibiotics; however, after establishing the diagnosis of PCD by ciliary ultrastructural studies and initiating treatment with chest physiotherapy, the child had a rapid clinical improvement *(110)*.

Rhinosinusitis: In one population studied, all patients described symptoms of upper airway disease including daily and year-round nasal congestion, rhinitis, facial pain, and anosmia. In this cohort, 65% of children and 47% of adults with PCD had sinusitis, defined by a history of prior surgery or radiographic studies showing chronic sinusitis *(5)*. In another study, 76% of children had rhinitis *(107)*. On physical exam, nasal polyps, mucostasis, and edematous nasal mucosa were common. All patients who had a CT scan of the sinuses had evidence of mucosal thickening *(5)*.

Lower respiratory tract: The most common symptom in PCD is a chronic productive cough frequently associated with purulent sputum production *(7)*. Patients usually present during early childhood with a daily wet cough and recurrent episodes of bronchitis and/or pneumonia, reflecting poor clearance of airway secretions by mucus ciliary clearance. Even though cough clearance is retained as a defense mechanism, affected individuals have recurrent airway infections *(7, 107, 111, 112)*. Patients typically have crackles on physical exam, and clubbing may be present in some adults. Wheezing is also commonly present, especially in children *(7, 107)*.

Chronic/recurrent lower respiratory tract infections lead to bronchiectasis. In one series, all adults ($n = 29$) and 56% of children ($n = 16$) with PCD had bronchiectasis, predominantly in the middle and lower lobes, based on high-resolution CT scans *(113)*. The right middle lobe was most commonly affected in both the adult and the pediatric patients. The upper lobes were the least affected. The distribution of the bronchiectasis was central or diffuse; there were no patients with isolated peripheral bronchiectasis *(113)*. It was previously thought that bronchiectasis occurred during late childhood and adulthood; however, in a recent retrospective study of children with PCD (median age 4 years old), 96% had evidence of bronchial wall thickening on HRCT, and 73% had evidence of bronchiectasis *(114)*.

The common bacterial pathogens obtained from sputum cultures vary between adult and children. In 80% of children, *Haemophilus influenzae* was present in sputum, compared to 22% in adults. Similarly, *Staphylococcus aureus* was more prevalent in children (46% versus 14% of samples from adults). In adults, *Pseudomonas aeruginosa* (both smooth and mucoid strains) was more prevalent *(5, 7)*. Additionally, nontuberculous mycobacterium (NTM) is recovered in PCD sputum samples *(5, 115)*.

Ultimately, recurrent airway infections result in impaired lung function. In a cross-sectional study, PCD children (<18 years of age; median age, 8 years) had a predicted FEV_1 of 85% compared to 60% in PCD adults (median age, 36 years) *(5)*.

Otitis media: Cilia play an important role in the Eustachian tube to protect against middle ear infection *(116)*. The Eustachian cilia have similar structure to the cilia on the bronchial mucus membrane. It was first discovered in 1975 that patients with

Kartagener syndrome had poor pneumatization of the mastoid cells and conductive hearing loss *(117)*. Coren et al. reviewed the cases of 55 children and 51% had otitis media and 25% had associated hearing loss *(107)*. Later, Noone et al. described that 95% of PCD patients had a history of recurrent otitis media requiring multiple treatments with antibiotics *(5)*. Common signs and symptoms of middle ear disease in PCD patients are ear pain associated with infections, sensation of fullness, aural discharge, and hearing loss *(118)*. The consequences of impaired MCC in the Eustachian tube are more pronounced in children, with a clear relationship between age of the patient and middle ear disease. In the infant, the Eustachian tube is short and horizontal relative to the face but progressively angles downward with growth and elongation of the face. As the angle of the Eustachian tube changes with age, the pumping mechanism of the Eustachian tube may adequately remove mucus from the middle ear *(118, 119)*. Retained fluid in the middle ear is associated with a transient hearing loss. The transient hearing loss associated with PCD improves as the child grows, but later than in non-PCD children, with recurrent ear infections and transient hearing loss (12 years of age versus 9 years of age in normals) *(119)*. Some PCD patients with severe, chronic, and untreated ear infections may have permanent hearing impairment and require hearing aids.

Laterality defects: situs inversus and heterotaxy: Fifty percent of PCD patients have situs inversus totalis, in which there is a complete mirror image of the lateralization (asymmetry) of the internal organs. Heterotaxy (situs ambiguus) is a combination of situs solitus and situs inversus totalis, and comprises a broad spectrum of abnormalities *(10, 11)*. In a recent study, 6.3% of 337 PCD patients were identified to have heterotaxy. There was a higher prevalence of outer dynein arm (ODA) defects in PCD patients with situs inversus totalis and heterotaxy than in patients with situs solitus ($p < 0.001$) and a lower prevalence of inner dynein arm (IDA) and central apparatus defects. The distribution of different types of ciliary defects and genetic mutations in PCD patients classified by situs status supports the concept that the embryonic nodal cilia play a key role in organ lateralization and that the ODA may be more important than the IDA for nodal cilia function *(10)*.

Twelve of the 21 PCD patients with heterotaxy had cardiac or vascular malformations, including vascular anomalies, double outlet right ventricle, atrioventricular canal defect, atrial septal defect, aortic coarctation, subpulmonic stenosis, ventricular septal defect, and left ventricular outlet obstruction. There was a 200-fold higher prevalence of congenital heart disease related to heterotaxy in PCD as compared to the general population *(10)*.

Infertility: The vast majority of male PCD patients have sperm immobility or dysmotility with subsequent infertility. The ultrastructure of sperm flagella is similar to the ultrastructure seen in the cilia of the respiratory epithelium, i.e., nine microtubule doublets surrounding a central pair of microtubules. Therefore, the ultrastructural defects that cause dyskinetic cilia of the respiratory tract are also present in the sperm flagella, which lead to immotile sperm and consequently infertility *(9)*. Women may also be affected by fertility issues, although less commonly, due to ciliary dysfunction in the fallopian tubes *(120)*. We speculate that women with PCD are more likely to have ectopic pregnancies.

Uncommon features: Although rare, some patients with PCD have intrabronchial calcium deposition, with associated lithoptysis *(121)*. Hydrocephalus is commonly seen in mouse models of PCD and is thought to reflect dysfunction of the ependymal cilia; however, hydrocephalus is rare in PCD patients *(68, 70)*.

Diagnostic Approach

The diagnosis of PCD can be challenging; however, new diagnostic tools, including genetic testing, are under development. The gold standard for determining the ciliary ultrastructure defect has been via transmission electron microscopy (TEM) *(6)*. Many types of ultrastructural defects have been reported in patients with PCD but the most common is the absence of the outer dynein arm (ODA) and/or the inner dynein arm (IDA) (see Figure 14.2). The mean number of ODAs per cilium is reduced from 7.5–9 in normal subjects to <1.6 in patients with PCD, and the mean number of IDAs per cilium is reduced from 3–5 in control subjects to <0.6 in patients with PCD *(122)*. Isolated ODA defects are seen in approximately 30–45% of patients and isolated IDA defects are seen in 10–30% of patients. The IDA has many different isoforms that occur at intervals along the axoneme, and computer-assisted analysis of TEM cross-sectional photographs can improve IDA visualization *(123)*. As many as 57% of patients have been described as having both ODA and IDA defects *(5, 124–126)*. Other reported defects involve the radial spokes and the central pair. Loss of the central pair with and without transposition, and migration of a peripheral microtubule pair to the center of the cilia because the central pair is absent, has been described *(5, 127)*. Diagnosing PCD via TEM is challenging because few centers have expertise in processing and analyzing ciliary biopsies. In addition, some patients (as many as 15%) with the "classic" PCD phenotype (including situs inversus, neonatal respiratory distress, chronic sinusitis, and bronchiectasis) have no ultrastructural defects *(5, 123, 124, 126)*. In these patients, the diagnosis of PCD was based on a compatible clinical phenotype, including situs inversus totalis and heterotaxic defects, together with other adjunctive diagnostic tests such as high-speed measurements of ciliary beat frequency and waveform, measurements of nasal nitric oxide levels, or the use of immunohistochemical immunofluorescence studies *(5, 6, 112)*.

Primary genetic defects causing ciliary ultrastructural defects must be distinguished from acquired or secondary defects, which occur with infection or inflammation. There are multiple changes in ciliary ultrastructure that have been described as secondary changes, but loss of ODA and/or IDA has not been described *(128)*. Cultured respiratory epithelial cells can aid in distinguishing between primary and secondary ultrastructure abnormalities, but only a few centers perform these techniques and a significant amount of cell material is necessary *(126)*.

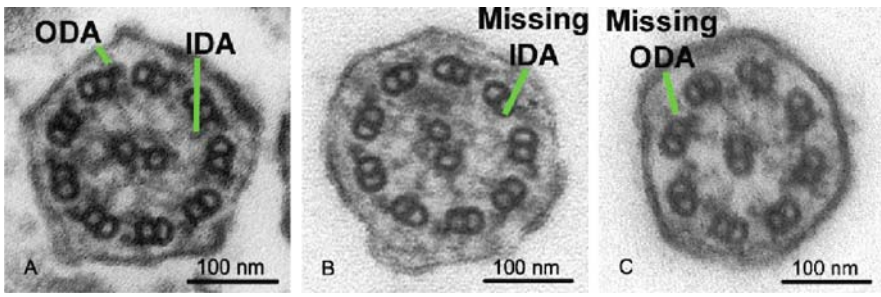

Figure 14.2 Electron micrographs (EM) of respiratory cilia. (**a**) Normal EM. Both ODA and IDA are present. (**b**) IDA absent. (**c**) ODA absent with transposition defect

Ciliary function can be assessed using high-speed video microscopy by people accustomed to scoring ciliary activity *(125)*. Ciliated epithelium obtained from brushing or scraping the inferior nasal turbinate is directly examined for ciliary beat frequency (CBF) and ciliary waveform. The normal range of CBF is 1 l–16 Hz at 37°C and varies with temperature and methodologies. It is important to avoid studying a patient during an acute respiratory infection, because acute infections can lead to secondary changes in the function of cilia *(6)*. Furthermore, examination with light microscopy is variable and can miss ciliary dysfunction.

A recently developed tool to aid in the diagnosis of PCD includes a noninvasive and easy-to-perform screening test that measures nasal nitric oxide (nNO) production. Several studies have shown that nNO is reduced in patients with PCD, although the exact mechanism is unknown *(5, 19, 20)*. The test is performed by inserting a NO sampling line into one nostril while the patient closes the soft palate. There is minimal overlap between the nNO levels in patients with PCD, compared to normal and most disease controls *(5, 20)* (see Figure 14.3). However, cystic fibrosis needs to be ruled out, since nNO levels in CF patients can overlap with levels of PCD patients. The ATS/ERS currently recommends sampling nNO via aspiration at a constant flow rate. This method is currently the most prevalent and best validated method and samples nasal NO in isolation from the lower respiratory tract. Closure of the soft palate is necessary to prevent leakage of nasal NO via the posterior velopharyngeal aperture *(129)*.

The latest tool introduced for diagnosis is a clinical genetic test that can identify mutant alleles in 25–30% of patients with PCD *(14)*. The genetic test sequences 9 exons that contain the most commonly occurring mutations in *DNAI1* and *DNAH5 (13–16)*.

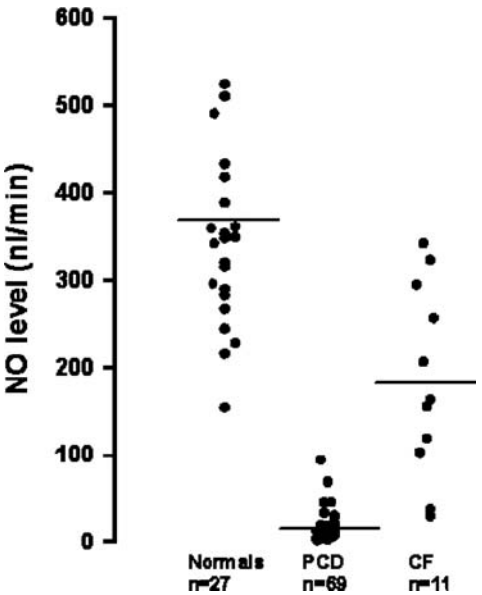

Figure 14.3 Nasal nitric oxide (nNO) levels in healthy normals ($n = 27$), PCD ($n = 69$ with defined ultrastructural defect), and CF ($n = 11$).

Splice mutations, nonsense, and frameshift mutations have been identified in these two genes (*DNAI1* and *DNAH5*), which encode for ODA proteins. Identifying two mutant alleles establishes a diagnosis, but the absence of a mutation or the presence of only one mutation does not rule out the diagnosis of PCD *(14)*.

Management/Treatment of PCD

The key to altering the clinical course of PCD is to make the diagnosis early and intervene systematically. Recent data has suggested that neonatal respiratory distress or tachypnea in a newborn can be the first signs of PCD *(5, 106)*. Early diagnosis during childhood may lead to earlier initiation of therapies that can delay, and possibly prevent, the occurrence of bronchiectasis. In contrast, a delay in diagnosis can lead to poorer outcomes *(107)*. Regular treatments of physiotherapy, combined with monitoring samples of sputum and directing antibiotic treatments to specific pathogens aggressively, can prevent lung damage and slow the decline of lung function *(130)*.

Managing lung disease and other complications of PCD is challenging because there are no standard evidence-based guidelines for treating PCD and no treatments are available to correct the ciliary dysfunction *(7)*. Clinicians can follow the general guidelines for treatment of non-CF bronchiectasis but must keep in mind the other systemic complications that are present in patients with PCD. The goals of treatment are to improve symptoms and prevent the progression of airway damage (Table 14.5).

A central focus of treatment is enhancing airway clearance. Several approaches may be used including physiotherapy, postural drainage, exercise, handheld positive expiratory pressure devices, and/or mechanical oscillatory vest percussion. Hypertonic saline has been used to improve cough clearance in patients with bronchiectasis; however, there is no specific research data on the efficacy of hypertonic saline in patients with

Table 14.5 Management principles for PCD lung disease.

1. Optimize airway clearance
   - Chest physiotherapy and postural drainage
   - Exercise
   - Handheld positive expiratory pressure devices
   - Mechanical oscillatory vest percussion
   - Nebulized hypertonic saline
   - Avoidance of cough suppressants
2. Prevent/control respiratory infections
   - Immunization (influenza, pneumococcal)
   - Training in good hand hygiene and infection control techniques
   - Monitoring routine sputum cultures (including AFB)
   - Aggressive antibiotic therapy directed by respiratory cultures
3. Avoid airway irritants
   - Avoid smoking/secondhand smoke
   - Avoid other respiratory tract irritants
4. Consider surgical options for severe lung disease
   - Lobectomy
   - Double lung transplantation

PCD. Patients should avoid smoking and other irritants that may increase mucus production. Cough clearance is the sole intact mechanism for mucus clearance; therefore, cough suppressants should be avoided.

Mucolytics, such a dornase alpha, recombinant human DNase I, have been well tolerated in patients with bronchiectasis caused by cystic fibrosis and have shown an improvement in lung function *(131, 132)*. Unfortunately, similar results have not been shown in patients with other causes of bronchiectasis. In a randomized control trial, O'Donnell et al. studied 300 patients with idiopathic bronchiectasis and found that dornase alpha had a negative effective on FEV_1 *(133)*. There was a 1.7% decline in FEV_1 in the placebo arm and a 3.6% decline in FEV_1 in the treatment arm ($p \leq 0.05$). Subjects in the treatment arm also had more pulmonary exacerbations *(133)*. No randomized control trials with dornase alpha have been done on patients with PCD, but we hypothesize that it will have similar results as in patients with idiopathic bronchiectasis.

Early management focuses on preventative and aggressive treatment of respiratory infections. Preventative strategies focus on immunizations against common respiratory pathogens (i.e., periodic pneumococcal and yearly influenza vaccination), teaching good hand hygiene, and other infection control maneuvers. Respiratory infections should be managed immediately and aggressively, using the sputum culture to choose appropriate oral, inhaled, or intravenous antibiotics *(5–7)*. Common pathogens isolated from sputum cultures of PCD patients are *H. influenzae*, *S. aureus,* and *P. aeruginosa (5)*.

Routine measures to monitor and assess lung disease include sputum cultures to track respiratory tract flora, pulmonary function tests, and chest radiographs. Screening for nontuberculous mycobacterium (NTM) in PCD may be as important as in cystic fibrosis. In one cohort, 15.5% of patients had positive acid fast bacilli cultures. Almost half (45%) of the patients who grew *Mycobacterium abscessus*, *MAC*, or *M. kansasii* either met the American Thoracic Society bacteriologic criteria for the diagnosis of NTM lung disease or had been previously treated at other institutions *(115)*.

Macrolides are immunomodulators that suppress inflammation without causing overt immunosuppression. For over 20 years, erythromycin has been used to treat panbronchiolitis, based on a Japanese study *(134)*. Four clinical trials performed on patients with cystic fibrosis have demonstrated clinical improvement after initiation of azithromycin *(135–138)*. These studies showed an improvement in lung function, a reduction in exacerbations with a decreased need for antibiotics or hospitalization, and a decrease in systemic inflammatory markers *(135–138)*. Although no studies have included patients with PCD, we hypothesize that patients with chronic *Pseudomonas* colonization may benefit from chronic macrolide therapy; however, prospective clinical trials need to be performed.

Surgical therapies, including lobectomy, are a possible treatment option for localized bronchiectasis, although the benefit is questionable and should be undertaken only after the involvement of experts. Lung transplant may also be an option for patients with end-stage lung disease *(139)*.

Treatment for sinusitis should mimic treatment for bronchitis and pneumonias, including routine cultures to identify pathogens, and early aggressive treatment of acute infections. Sinus irrigations with saline lavages or antibiotics have been used in addition to more invasive surgical treatments such as nasal polypectomy and surgical sinus drainage.

Complications of chronic suppurative otitis media include hearing loss. All young patients should routinely have their hearing tested for conductive hearing loss and treated appropriately with speech therapy and hearing aids. Another major complication of otitis media is tympanic membrane perforation. Current surgical treatment for chronic middle ear infections is placement of myringotomy tubes. However, there is also an association of tympanic membrane perforation with myringotomy tubes, as well as otorrhea and tympanosclerosis *(140)*.

Future Therapeutic Targets and Directions

The full spectrum of PCD lung disease is only beginning to be defined. The ability to extend our understanding of the pulmonary phenotype will occur when genetic testing is able to identify the majority of PCD patients, which is likely to occur in the near future. A prospective study has just been initiated in infants and young children with PCD (http://rarediseasesnetwork.epi.usf.edu/; S. Davis and M. Rosenfeld) to establish the age of onset of PCD lung disease. If the age of onset of lung disease is typically before 5 years *(141)*, then early identification and initiation of therapy might have great impact in this age group.

There is much to be learned about treatment for PCD lung disease from systematic study of therapy developed for lung disease in cystic fibrosis (CF), another genetic disease associated with defective mucociliary clearance and bronchiectasis *(142)*. Specifically, it seems likely that inhaled antibiotics, oral macrolides, and perhaps even inhaled hypertonic saline might be beneficial for PCD, as has been shown for CF *(143, 144)*. However, this is not proven and will require systematic evaluation, as soon as enough PCD patients are available in North America and Europe. It is also possible that DNase might be useful in PCD; however, studies in non-CF bronchiectasis have not shown benefit *(133)*.

For other (non-pulmonary) manifestations of PCD, there is also likely important benefit to systematic evaluation and intervention. For example, the morbidity from recurrent infection and inflammation of middle ear disease frequently leads to permanent hearing loss in PCD, but we currently do not understand the most appropriate, basic intervention, such as whether (or not) to place myringotomy tubes *(7, 140)*. Nor do we know if hearing deficits in PCD in childhood lead to developmental delay of speech and cognitive function. Sinus disease also has significant morbidity in PCD, but there has been little study of this problem.

For future directions, we anticipate that some PCD patients will have "STOP" mutations that are amenable to read through with small molecules, such as aminoglycosides or PTC124, as has been shown in CF and other disorders *(145–147)*. It also seems likely that advances in genetic testing will identify a PCD phenotype in patients who have mutations associated with residual ciliary function. If so, then these patients might respond to drugs that stimulate ciliary beat frequency, such as beta agonists. Finally, the discovery of the genetic basis of PCD is in its infancy. We anticipate that further advances in genetics will demonstrate overlap of PCD-like lung disease with "sensory" ciliopathies, such as polycystic kidney disease, as has recently been suggested *(148, 149)*. If true, the possibilities of therapeutic intervention become even more expansive and complex. For PCD, a new day is dawning, and molecular and genetic advances seem likely to have a beneficial effect on the diagnosis and treatment of this disorder.

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15

Pulmonary Alveolar Microlithiasis

Koichi Hagiwara, Takeshi Johkoh, and Teruo Tachibana

Abstract Pulmonary alveolar microlithiasis (PAM: OMIM265100) is an autosomal recessive disorder characterized by the intra-alveolar formation of microliths that are mainly composed of calcium phosphate. Microliths are found in about 80% of the alveoli. They grow very slowly and finally occupy most of the alveolar space. Mild-to-moderate chronic inflammation and fibrosis are observed mainly in the interstitium asymptomatic diagnosed of their diseases in their childhood where the disease is often discovered incidentally on a chest xray taken for a different purpose. The disease usually takes a chronic, slowly progressive course. Patients are generally free of symptoms until middle age, when respiratory insufficiency gradually develops. Many patients die of respiratory failure. In 2006, two independent researchers reported that homozygous loss-of-function mutations in the SLC34A2 gene is present in PAM patients. SLC34A2 encodes a type IIb sodium-dependent phosphate transporter that is expressed in type II alveolar cells. Loss of phosphate transporter function in alveolar type II cells is considered to be the cause of PAM.

Keywords: diffuse pulmonary shadow, phosphorus transporter, intra-alveolar microliths, autosomal recessive inheritance

History

Harbitz in 1918 *(1)* first described an extensive calcification in the lung consistent with PAM. In 1933, Puhr named the disease pulmonary alveolar microlithiasis *(2)*. Since then, more than 500 cases have been reported worldwide *(3–6)*. In 2006, two groups have reported that PAM is caused by a mutation in the type IIb sodium-dependent phosphate transporter gene *(7–9)*.

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Epidemiology

Frequency

PAM is a rare disease and its frequency is unknown. No sex predisposition has been observed. More than 600 patients have been reported worldwide: 115 patients in Japan (as of 2009); 79 patients in Turkey *(6)*; and 61 patients in Italy *(6)*. Familial occurrence is common. Out of 115 patients found in Japan, 56 cases are known to arise from 24 families; 2 sibs are affected in 17 families; 3 sibs are affected in 6 families; and 4 sibs are affected in 1 family. In 391 patients reported in the literature worldwide *(5)*, 139 are familial cases. There are 39 families with two affected sibs, 7 families with 3 affected sibs, 2 families with 4 affected sibs, 1 family with 5 affected sibs, and 1 with 2 patients in the cousins. A family in which six patients were clustered was reported in Turkey *(10)*. About 30% of the patients in Japan are from inbred families and born to parents who are not affected. A high frequency of horizontal transmission, accumulation of the patients in inbred families, and the absence of sex predisposition are all consistent with an autosomal recessive inheritance.

Clinical Manifestation

Signs and Symptoms

Patients do not have any subjective symptoms until middle age. The most common presentation of the disease is the incidental finding of an abnormality on chest X-ray. A decrease in the diffusion capacity (DLco) and restrictive ventilatory defects gradually becomes apparent. Physical examination lacks abnormal findings until the respiratory function is seriously impaired, when signs and symptoms of chronic respiratory failure emerge. Most patients die of respiratory failure.

Blood Study

Routine blood cell counts and biochemistry are typically normal: PAM patients do not show abnormalities in liver, kidney, and parathyroid functions. Hypercalcemia or hyperphosphatemia, which is often found in the patients with metastatic pulmonary calcification *(11)*, is not observed. Elevation in the serum surfactant protein A and D levels, which are found in a variety of diseases that accompany inflammation in the lung interstitium, correlates with the deterioration of the respiratory function and the progression of the disease in PAM *(12)*.

Bronchoalveolar Lavage Fluid (BALF)

Microliths that have characteristic lamellar structure are found in BALF *(10, 13–15)*. Also noted is an increase in the numbers of inflammatory cells.

Respiratory Function

In the early stages of the disease, patients have normal pulmonary function tests. Even when the chest X-ray shows a profusion of infiltrates such that the mediastinal contours

are obscured by the densely distributed small nodular opacities. A slight decrease in DLco and vital capacity may be the only abnormalities in the lung function tests. Nevertheless, the respiratory function gradually decreases over time. In the advanced stages, bullae and pneumothorax develop and calcified consolidation may be observed on the chest CT, associated with marked decreases in DLco, and %VC. Eventually, many PAM patients develop progressive hypoxemia and die of respiratory failure.

Radiological findings

Chest X-Ray

Characteristic radiographic manifestations of PAM consist of bilateral fine sand-like micronodulation and calcific densities in all lung fields. The opacities are often most dense in the middle and lower fields and often obliterate the mediastinal and diaphragmatic contours *(16)* (Figure 15.1). Individual deposits are sharply defined nodules measuring up to 1 mm in diameter *(16–18)*.

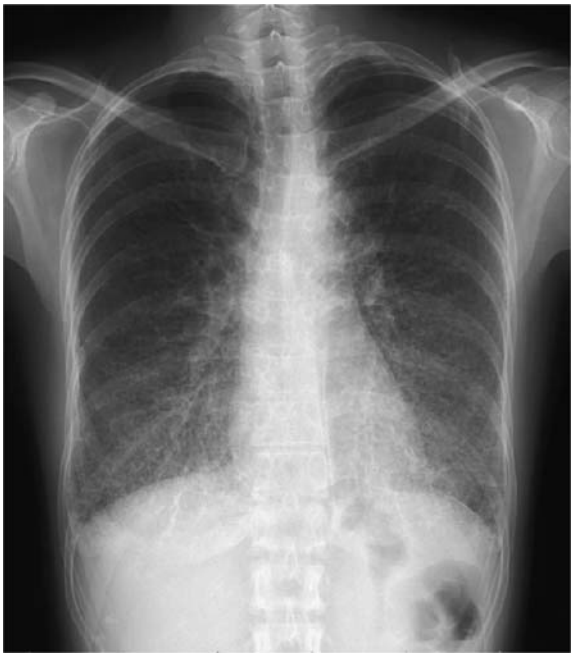

Figure 15.1 Chest X-ray of a 40-year-old female. Fine sand-like micronodules that have radiographic densities consistent with calcification are present in all lung fields. The mediastinal and diaphragmatic contours are obliterated

CT

On CT, small nodules with calcification densities, which are often confluent, are observed throughout all lung fields *(13, 18–22)* (Figure 15.2). Areas of ground-glass

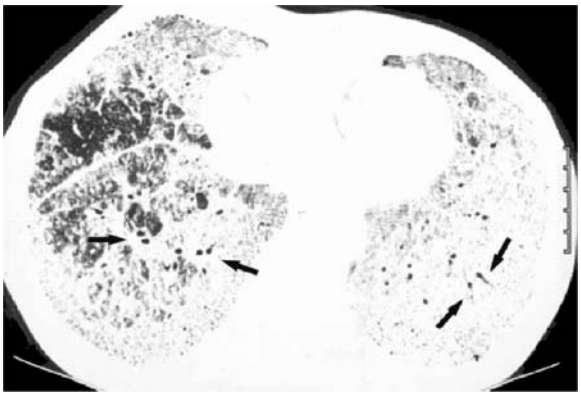

Figure 15.2 Chest CT of a 55-year-old male (window level –700 Hounsfield units (HU), window width 1,000 HU). Small calcified nodules, ground-glass opacities and consolidation with air bronchograms (*arrows*) are observed

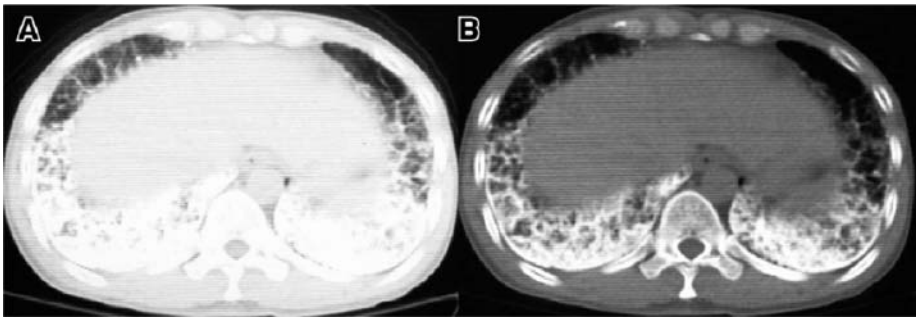

Figure 15.3 Chest CT at the diaphragm level. (**a**) Window level –300 HU, window width 1,000 HU. Ground-glass opacities and consolidation are observed. (**b**) Window level 50 HU, window width 1,000. Calcifications that have a reticular structure are observed in the consolidation

attenuation and consolidation are both observed, and in some cases the latter may contain air bronchograms (Figure 15.3). The opacities can become so profuse that the interstitium of the lung is highlighted in relief, producing a reticulonodular pattern with Kerley B lines or the appearance of interstitial thickening *(23, 24)*. True thickening of interlobular septa and bronchovascular bundles, accompanied by calcified nodules along these structures, is seen in many patients (Figure 15.4) *(25)*. Cysts less than 10 mm in diameter are found along the pleura, and bullae 1–8 cm in diameter are found in the apical regions. Calcified lines alongside the pleura are commonly observed (Figure 15.5).

Scintigram

The ^{99m}Tc bone scintigram shows marked pulmonary uptake in PAM *(26, 27, 30)*.

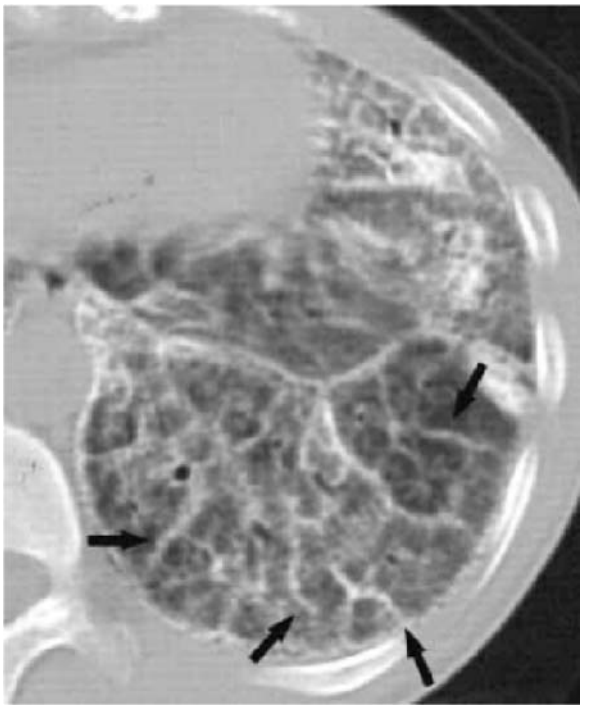

Figure 15.4 Chest CT of a 41-year-old female (window level –400 HU, window width 4,000 HU). *Left lower lobe*. Thickening and calcification of the interlobular septa are observed

Pathology

Lung tissue specimens reveal characteristic intra-alveolar microliths. The main component of the microliths is calcium phosphate *(28, 29)*. Their concentric lamellar structure is clearly observed by both light microscopy (Figure 15.6) and electron microscopy. Scanning electron microscopy reveals microliths of various sizes with globular, oval, or irregular shapes with an uneven surface *(28, 30–32)*. In the advanced stages of the disease, microliths are clustered along the bronchovascular bundles (Figure 15.7), in interlobular septa and in subpleural regions (Figure 15.8). Mild or moderate interstitial fibrosis and ossifications (Figure 15.9) are observed.

In the metastatic pulmonary calcification, calcification appears along the alveolar wall, while, in PAM, microliths are formed in the alveolar space. Metastatic pulmonary calcification often accompanies hypercalcemia and calcifications in kidney, heart, stomach, and other organs. These are not observed in PAM.

Diagnosis

Diagnosis

The diagnosis is suspected when the chest X-ray and the chest CT reveal the characteristic features of PAM. To establish the diagnosis, typical intra-alveolar microliths should

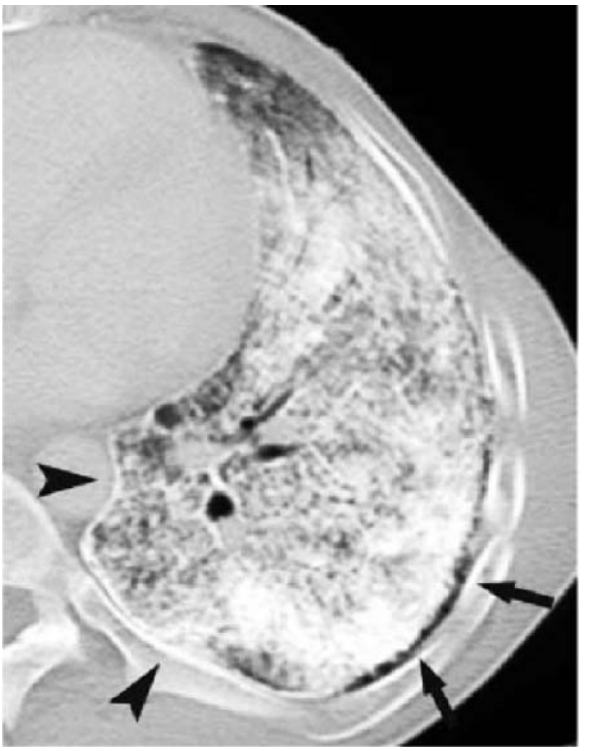

Figure 15.5 Chest CT of a 49-year-old male (window level 155 HU, window width 1,858 HU). *Left lower lobe*. Small cysts along the pleura (*arrows*) and linear calcification along the border of mediastinum (*arrowheads*) are observed

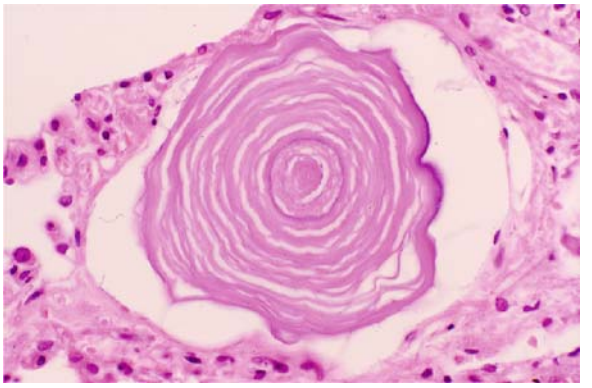

Figure 15.6 An intra-alveolar microlith. The typical lamellar structure is observed

be confirmed in lung tissue obtained by transbronchial biopsy, video-assisted thoracic surgery (VATS), or open lung biopsy. For precise pathological examination, a decalcification procedure should be employed before making thin sections. The presence of microliths in BALF strongly supports the diagnosis of PAM.

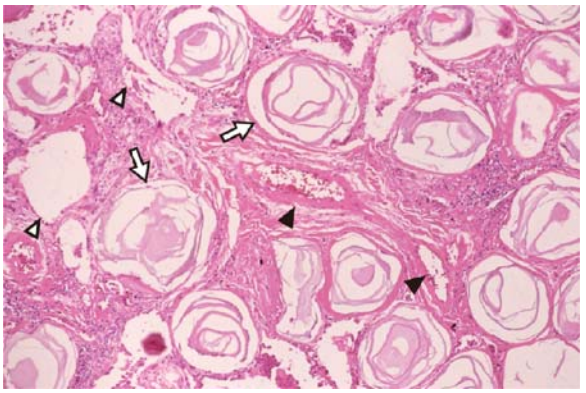

Figure 15.7 Microliths adjacent to the bronchovascular bundle. Numerous microliths (*arrows*) are observed together with small airways (*white triangles*) and pulmonary arteries (*black triangles*)

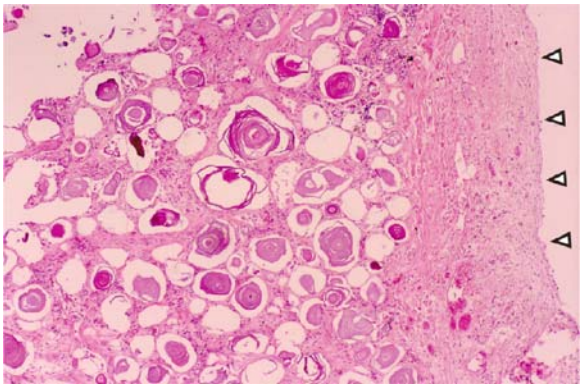

Figure 15.8 Microliths in the subpleural regions. Pleura is indicated by *white triangles*

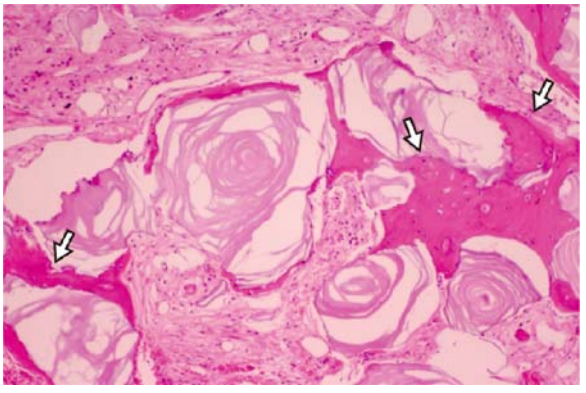

Figure 15.9 Ossification. The ossified area is indicated by *white triangles*

In order to establish a correct diagnosis, the possibility of other diseases which present with diffuse pulmonary shadows should be carefully excluded. Family members of each index case should be examined since multiple patients are often found in a single family.

Complications

In most cases, the lung is the only organ affected. A case with epididymal and periurethral calcifications was reported *(33)*. The causes of death for the 10 Japanese patients who were autopsied were respiratory failure due to PAM in 8; bile duct cancer in 1; and cerebral hemorrhage in 1.

Mode of Detection and Age of Diagnosis

Since PAM patients with early stage disease lack clinical symptoms, the diagnosis is often first suspected by abnormal chest X-rays. Among 115 Japanese patients, 98 were asymptomatic at the time of diagnosis. In 576 accumulated cases worldwide, 298 were asymptomatic *(6)*. The age of diagnosis varies considerably. In 115 Japanese patients, 59 were diagnosed under the age of 15, while 20 were diagnosed when they are over 40. In 576 accumulated cases, the peak ages of diagnosis were 10–29 years old, which are followed by 30–39 and then 40–49 *(6)*.

Procedures Used for Diagnosis

A definite diagnosis of PAM can be established only by pathological examination. In 115 Japanese patients, 42 subjects had their diagnosis confirmed by lung biopsy and 10 by autopsy. Among 576 accumulated cases worldwide, 270 were confirmed by lung biopsy and 59 were confirmed by autopsy *(6)*.

Prognosis

Among 53 Japanese cases that were followed up for 10–19 years, 3 died during the period and 50 are alive. Among 35 Japanese cases that were followed up for 20–49 years, 15 had died and 20 are alive. The main cause of death was respiratory failure. The cases stated above include eight autopsy cases: the diagnosis was established when they were 6, 7, 8, 10, 18, 33, 45, and 60 years old and the patients died when they were 43, 45, 56, 57, 32, 56, 55, and 76 years old. This indicates that PAM patients live long even when their diseases are noticed in childhood. PAM patients who were followed up for a long period of time are also found in the literature *(16, 17, 28, 29, 34, 35)*.

Therapies

At present, there is no therapy for PAM that effectively reduces or eliminates the microliths. Therapeutic bronchoalveolar lavage is not effective. There is a case report in which disodium etidronate was administered to a 9-year old patient for 1 year with regression of calcific densities on chest X-ray and CT *(36)*; however, the effect of the

drug has not yet been confirmed in other cases. Many of the patients are managed by home oxygen therapy and given drugs that alleviate pulmonary hypertension. Lung transplantation has been performed in several patients *(37–40)*: the list of the long-term survivors includes a patient who received lung transplantation 12 years ago when the patient was 32 years old *(37)* (personal communication) and a patient who received lung transplantation 7.5 years ago when the patient was 53 *(40)*.

The Gene Causing PAM

Mutation in the SLC34A2 Causes PAM

PAM is often found in inbred families and clusters in siblings. PAM occurs both in males and in females with similar frequencies. These inheritance patterns suggest that PAM is an autosomal recessive disorder caused by a disease gene with a high penetrance. In 2006, two independent groups reported that PAM is caused by an inactivating mutation of SLC34A2 *(7–9)*, a gene that encodes a type IIb sodium-dependent phosphate transporter *(41, 42)* (Figure 15.10). In one study Turkish researchers investigated a family with multiple consanguineous marriages and six affected patients *(10)*. They utilized the linkage analysis and the haplotype analysis to find the gene. In the other study Japanese researchers investigated three sporadic cases. They utilized an approach based on homozygosity mapping *(43)* which was modified so that it fits into the genome-wide single nucleotide polymorphism (SNP) analyses. A total of eight different SLC34A2 mutations have been reported so far: Six mutations produce truncated proteins, in two of which the loss of sodium transporter function was confirmed using *Xenopus* oocytes *(8)*. One mutation produces a protein with an amino acid substitution. One deletion deletes the promoter of SLC34A2 gene, and the SLC34A2 mRNA is not transcribed. These mutations were found to be homozygous in all patients, which suggests the role of the inbred marriage of their parents. In fact, in all three cases investigated by the genome-wide SNP analysis, SLC34A2 was found to be located in the candidate autozygous segments where two copies of the chromosomal segment are likely to be identical by descent (Figure 15.11) *(8, 44)*.

SLC34A2 and Phosphate Transport

Type II sodium-dependent phosphate transporters have three members: SLC34A1, SLC34A2, and SLC34A3. Inactivating mutations of SLC34A1 cause nephrolithiasis and osteoporosis associated with hypophosphatemia *(45)*, and those of SLC34A3 cause hypophosphatemic rickets with hypercalciuria *(46)*. These indicate the importance of sodium-dependent phosphate transporters in both calcium and phosphate metabolism. SLC34A2 is unique among the members in that it is the only member expressed in the lung *(41, 42)*. In other organs where SLC34A2 is expressed, SLC34A1 and/or SLC34A3 is also expressed. These explain the reason why the lung is the only organ affected in the PAM patients. In the lung, SLC34A2 is expressed only in the alveolar type II cells (Figure 15.12) *(8)*. SLC34A2 transports phosphate ion from the alveolar space into the cells *(47)*. Pulmonary surfactant is abundant on the surface of alveoli, and its essential constituents are phospholipids. Moreover, surfactant is metabolized by the alveolar type II cells and alveolar macrophages. Taken together, these observations suggest a compelling hypothesis for the pathogenesis of PAM *(8)*.

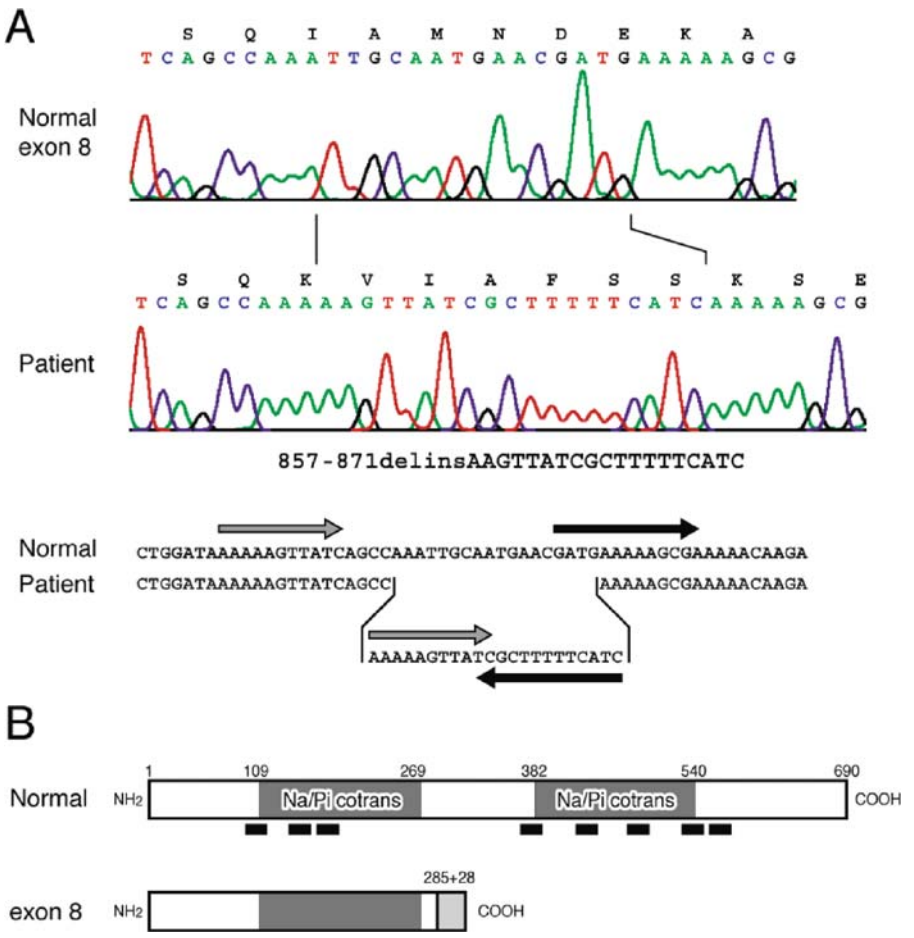

Figure 15.10 Mutation seen in a patient with PAM. (**a**) An aberrant sequence is inserted in exon 8 and shifts the reading frame. This causes a premature termination of SLC34A2 protein shown in (**b**). Na/Pi cotrans: a sodium phosphate cotransporter motif (pfam 02690). *Black bars* under the normal protein structure indicate the transmembrane domains

Alveolar macrophages digest outdated surfactant and release phosphate ion into alveolar space. Alveolar type II cells in PAM patients fail to take up the ion because they lack SLC34A2 function. Therefore, the concentration of phosphate ion increases and eventually leads to the formation of the intra-alveolar microliths. This hypothesis needs to be confirmed.

Therapeutic Consideration

The finding that SLC34A2 function is impaired in PAM suggests that inability to eliminating phosphate from the alveolar space is the cause of PAM. Therapies that reduce phosphate ion concentration in the alveolar space should be devised for the treatment of PAM.

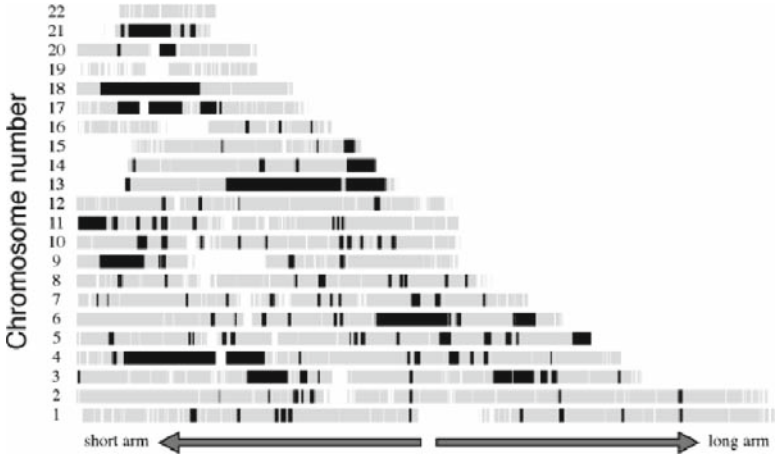

Figure 15.11 Candidate autozygous segments seen in a patient. Black bands are possible autozygous segments detected by the analysis of a genome-wide SNP genotyping. In autozygous segments, chromosome fragments of two homologous chromosomes are identical by descent and derived from a single chromosome of a single ancestor

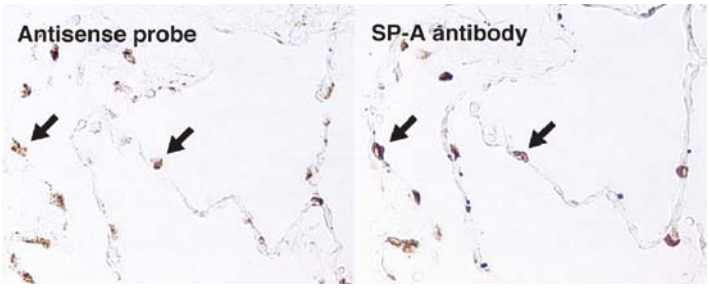

Figure 15.12 Expression of SLC34A2 in the alveolar type II cells. Serial sections of normal lung tissue were stained using different probes. *Left panel*: in situ hybridization using SLC34A2 antisense probe. SP-A: immunohistochemistry using an anti SP-A probe that is a marker molecule for the alveolar type II cells

Population Genetics

The small number of PAM patients and the observation that many of them are found in inbred families suggest that the frequency of inactivating mutations of the SLC34A2 gene is very small in the general population. In Japan, where approximately 100 patients have been found during the last 5 decades, we estimate the frequency of the gene is less than 0.001. The small number of patients worldwide also suggests that the frequency of the gene is low worldwide. It is known that the frequency of rare recessive disorders correlates well with the frequency of consanguineous marriages. Until recently, consanguineous marriages were frequent in Japan *(48)* and in the Middle East *(49)*. This may be the reason why these two regions have had many PAM patients.

Conclusion

PAM is an autosomal recessive disorder caused by a loss-of-function mutation in the SLC34A2 gene. PAM displays characteristic X-ray and CT findings and is not difficult to diagnose when lung biopsy specimens are obtained. An effective therapy that compensates for the lost SLC34A2 function, at least in the alveolar space, needs to be devised.

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16

Cystic Fibrosis

André M. Cantin

Abstract Cystic fibrosis (CF) remains the most common lethal disease associated with a single gene defect in populations of European descent. The gene that prevents CF is the cystic fibrosis transmembrane conductance regulator (CFTR), an ATP-dependent anion channel expressed mostly at the apical surface of epithelia lined with mucus secretions. CF results from a deficiency in CFTR amount and/or function. Fortunately, the clinical situation is rapidly improving for CF patients and their families. The mean age of survival has markedly increased in recent years. These improvements are attributable to the high quality of care that has evolved in the multidisciplinary treatment of secondary defects such as lung infections and malabsorption. Furthermore, the hope of finding a cure or control for individuals with CF is buoyed up by novel pharmacological approaches that directly address the primary defect in CFTR function. This review will examine various aspects of CF including its epidemiology, genetic basis and molecular pathogenesis, animal models, clinical presentation, diagnostic approaches, conventional treatments, and future therapeutic avenues to correct dysfunctional CFTR.

Keywords: bronchiectasis, airway infection, inflammation, mucus, hereditary lung disease, CFTR, epithelium, pancreatic insufficiency

Introduction

Cystic fibrosis (CF) is the most common fatal disease related to an inherited single gene defect *(1)*. Clinical manifestations are largely restricted to cylindrical tissues that have an epithelium lined with mucin-rich secretions *(2)*. The tissues most sensitive to CFTR dysfunction are the exocrine pancreas and the *vas deferens (3)*. Several other ductal tissues lined with mucous secretions are affected by CFTR deficiency including the liver, the large intestine, and sinuses. However, the organ linked to most of the morbidity and almost all of the mortality in CF is the lung. Severe CFTR deficiency can lead to progressive and irreversible destruction of the airways. The ensuing bronchiectasis places CF patients at risk of respiratory insufficiency and death.

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The median age of survival of CF individuals has increased on average 6 months every year over the past 20 years and currently approaches 37 years old in North America *(4)*. This improvement in survival is entirely attributable to interventions that have not addressed the basic defect of CFTR deficiency. As improvements in CF health care continue to impact on quality of life and survival, we have entered a new era in which therapies developed to directly correct the basic defect of CFTR dysfunction are being tested in patients *(5)*. Correction of CFTR function should markedly increase life expectancy of CF individuals.

Epidemiology

As of April 2008, more than 1,500 mutations were listed in the CFTR mutation database (http://www.genet.sickkids.on.ca/cftr/StatisticsPage.html). Among these mutations, the deletion of phenylalanine at the 508th amino acid (ΔF508) is by far the most common. The ΔF508 mutation is present on 70% of CF chromosomes in most Caucasian populations. Up to 89% of CF patients of North America carry this mutation on at least one of their chromosomes. This latter observation is of particular importance with respect to promising novel pharmacological approaches that may help restore partial CFTR function.

The incidence of CF varies greatly and is highest in Caucasian populations where it is estimated at between 1:2,500 and 1:3,200 depending on whether the incidence is calculated from clinical diagnosis or from newborn screening *(6)*. The incidence of CF can be much higher in certain populations such as French Canadians from the Saguenay/Lac St-Jean region, as well as inhabitants of a region of Brittany, a subset of Afrikaners and inhabitants of the Faroe Islands *(6, 7)*. The incidence of CF also varies from 1:1,700 to 1:6,500 in different European populations and is rare in Finland. Not only is the incidence of CF variable but also the distribution of the types of CFTR mutations differs greatly among populations. The ΔF508 mutation was the only one identified in a population from the Faroe Islands whereas this mutation accounts for only 1/3 of the CF-bearing alleles in the Ashkenazi community of Israel *(8)*. The frequency of CFTR mutations can also vary considerably within populations that are of similar ethnic origin and inhabit the same geographic area. The ΔF508 mutation accounts for 71% of CF chromosomes in patients in the Quebec City area whereas it is present on only 55% of CF chromosomes in patients from the nearby Saguenay/Lac St-Jean region in Canada *(6)*. Because new approaches to therapy are being designed based on the specific consequences of each mutation on CFTR function, it will become more important for clinicians to not only recognize the diagnosis of CF but also identify the mutations present on each CF chromosome of each patient within their care.

Genetic Basis and Molecular Pathogenesis

Cystic fibrosis is an autosomal recessive disease caused by mutations affecting the *Cftr* gene located on the long arm of chromosome 7 (7q31.2) *(9, 10)*. Following the identification and cloning of the *Cftr* gene, CFTR was found to be a 1,480 amino acid protein spanning apical membranes of various epithelial cells (Figure 16.1). The CFTR glycoprotein is a member of the adenine nucleotide-binding cassette (ABC) protein family and is unique for two reasons: first, it has a cytoplasmic regulatory (R) domain

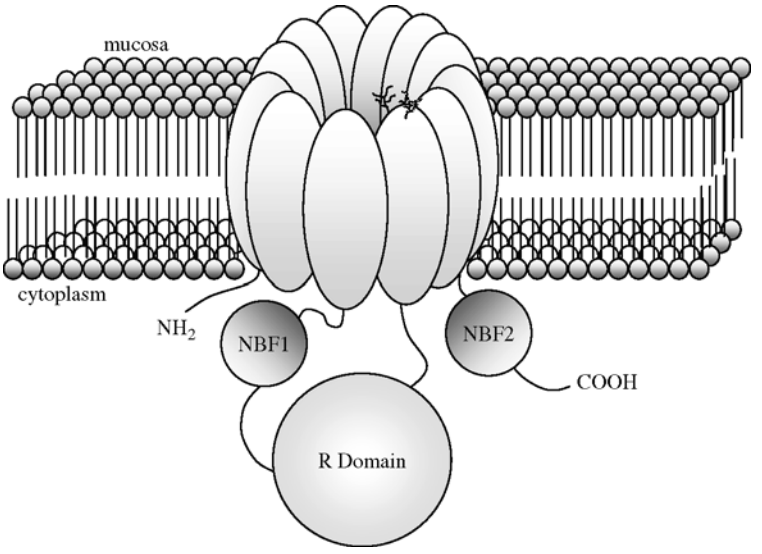

Figure 16.1 CFTR is a glycoprotein comprised of two transmembrane domains with six subunits that each form a pore permeable to select anions, particularly chloride and bicarbonate. The two nucleotide (ATP) binding folds (NBF1 and NBF2) have key interactions with the transmembrane domains allowing the channel to be either open (active gating) or closed (quiet). The most common *Cftr* gene mutation, ΔF508 results in a conformational change of NBF1 which leads to endoplasmic reticulum associated degradation before the diseased protein can reach the epithelial apical membrane. The R domain keeps CFTR in a quiet state until it is phosphorylated at multiple sites by protein kinase A. The degree of R domain phosphorylation which is further modulated by phosphatases will define the channel's open probability

containing potential sites for protein kinase A-mediated phosphorylation and, second, it functions as a cyclic adenosine monophosphate (cAMP)-dependent anion channel *(5)*. CFTR plays a key role in defining the water content at the surface of several epithelial tissues that have secretory and/or absorptive functions *(11, 12)*. As its name suggests, CFTR regulates epithelial anion conductance, primarily through the movement of chloride across apical membranes *(13)*. The movement of chloride anions through the CFTR channel plays a key role in both secretion and absorption processes associated with various tissues. In addition to its direct role in chloride movement, CFTR also plays a key role in the regulation of ion transport through other channels. The absence of CFTR function in epithelial cells of mucosal tissues results in a marked increase of sodium absorption through the epithelial sodium channel (ENaC) *(14, 15)*. The excessive sodium absorption through ENaC contributes not only to an increase in the baseline transepithelial potential difference characteristic of patients with CF but also to the accelerated absorption of water and dehydration of mucus within the diseased organs *(12)*. Excessive absorption of sodium in the airways of transgenic mice that overexpress ENaC can itself lead to cystic fibrosis-like lung disease *(16)*. The marked alterations in sodium, chloride, and water homeostasis of mucosal tissues in CF are compounded by the abnormal movement of bicarbonate anions *(17)*. Bicarbonate plays a key role in regulating the pH of epithelial surface fluid and mucous secretions *(18)*. Bicarbonate movement across apical membranes of epithelial tissues occurs directly through the CFTR channel, and the loss of this key function is thought to contribute significantly

to CF pancreatic disease and likely plays an important role in the pathophysiology of disease in other tissues such as the airways *(19, 20)*.

The molecular pathogenesis of CF is directly related to three factors: (1) the amount of functional CFTR defined by the CF patient's pair of *Cftr* alleles, (2) the impact of the environment (e.g., cigarette smoke) *(21)*, and (3) the genetic background other than the *Cftr* gene – often referred to as the modifier genes. Because the genetic basis of CF involves such a large number of mutations within the very large *Cftr* gene, the impact of these mutations on CFTR proteins varies greatly. It is therefore useful to define CF gene mutations as a function of their impact on *Cftr* gene expression as well as on CFTR protein abundance, structure, and function. The various effects of different gene mutations on the CFTR protein are directly related to the phenotype expressed in CF patients, particularly with respect to pancreatic function. Furthermore, consequences of different mutations on the CFTR protein have direct implications with respect to the development of molecular therapies for CF. The mutations can be conveniently grouped into six classes (Figure 16.2) defined by the effect of the *Cftr* gene defect on the CFTR protein *(22, 23)*.

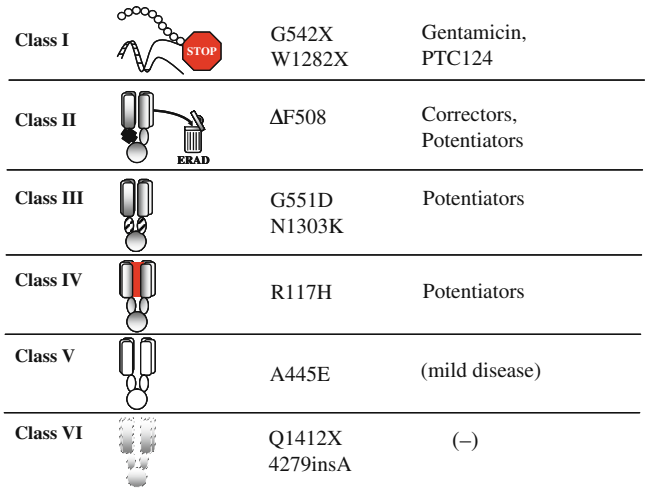

Figure 16.2 Different classes of CFTR mutations are based on the impact of the gene mutations on protein synthesis and function (please see text for details). The classification scheme was initially proposed by *(21)* and in a modified version by Wilschanski and Durie *(23)*. Examples of some of the more common mutations in each of the classes are shown. Therapeutic approaches tailored to correct CFTR defects defined by the various classes are shown. Although many of the approaches in development may be applicable to classes V and VI, there is no drug development specific to these classes

Class 1 mutations affect the proper synthesis of full-length functional CFTR protein due to the abnormal presence of a premature termination codon in the mRNA transcript. The incompletely synthesized CFTR protein is rapidly degraded by the control mechanism of the cell's endoplasmic reticulum compartment. Most of the mutations within class 1 are expected to produce a severe deficiency of CFTR protein since no full-length protein is synthesized. However, there are some class 1 mutations that are associated with a less severe phenotype due to a miss-splicing mutation that results in the production of a small amount of CFTR transcript.

Class 2 mutations result in a poorly folded protein or protein domain(s). Misfolded CFTR is recognized through the molecular chaperone machinery of the endoplasmic reticulum (ER) membrane and undergoes an arrest of its maturation. This abnormal CFTR protein which is not normally glycosylated will then be ubiquitylated and undergo endoplasmic reticulum-associated protein degradation (ERAD). The ΔF508 CFTR protein present in the majority of CF patients is the product of a class 2 mutation. Because this single mutation is found in almost 90% of patients in North America and most parts of Europe, great efforts are being focused on research to correct this protein trafficking problem. All *Cftr* gene mutations within class 2 are associated with a severe deficiency of CFTR abundance and function at the apical membrane of epithelial cells.

Class 3 mutations result in CFTR proteins that cannot be activated due to defective ATP binding and hydrolysis or altered coupling of ATP binding to the activation of CFTR. Proteins synthesized by a *Cftr* gene bearing a class 3 mutation are unable to respond to cAMP stimulation. The molecular consequence of these mutations is a severe functional defect in CFTR.

Class 4 mutations are associated with a protein that has faulty chloride conductance and most often represent alterations in the transmembrane domains. Since the consequences of class 4 mutations on the protein do not affect regulation of the channel by cAMP and since chloride conductance is preserved, albeit at a lower level, CF patients bearing a class 4 mutation on at least one of their chromosomes will often have a milder phenotype.

Class 5 mutations are also associated with milder phenotypes since these mutations do not affect the structure or the function of the CFTR protein. Mutations within class 5 will have an effect on the amounts of protein that are synthesized; however, it is estimated that approximately 5% of the normal levels of CFTR protein is sufficient to prevent the expression of many of the most significant manifestations in the CF lungs *(24)*. It has been suggested that class 1 and 5 be grouped together since both result in altered levels of mRNA *(1)*.

Class 6 mutations represent an additional class proposed by Haardt et al. that affect the protein stability due to the truncation of amino acid residues in the C-terminus *(25)*. Although the loss of the C-terminal residues does not affect the selectivity or the regulation of the chloride channel, it markedly reduces the stability of the protein at the apical membrane. The truncated protein can be reduced five- to sixfold and has been associated with the expression of a severe CF phenotype.

Approximately 15% of CF patients bear mutations within classes 4 and 5, thus leading to milder disease manifestations since some functional CFTR remains present at the apical surface of epithelial cells *(26)*. However, the correlation between genotype and phenotype in lung tissues is not as strong as it is in the pancreas. The weaker correlation between genotype and phenotype in the lung suggests that environmental (i.e., oxidative stress, inflammation, infection) and other genetic factors are involved in determining the respiratory prognosis. Among these environmental factors, certain pathogenic bacteria greatly affect the prognosis. The acquisition of *Pseudomonas aeruginosa* and certain genomovars of the *cepacia* complex play a key role in respiratory outcomes *(27)*. Genetic factors other than CFTR such as genes encoding host defenses such as mannose-binding lectin 2 (MBL2), glutathione metabolism, alternate anion channels, and transforming growth factor β (TGFβ) are all likely to define the course of CF lung disease. For example, low MBL2 gene expression coupled with high TGFβ producing gene expression is associated with more rapid deterioration of lung function in CF *(28)*.

Within the first few weeks of life, newborns with CF have increased numbers of neutrophils and bacteria in their airways *(29, 30)*. The presence of neutrophils and their products in the bronchoalveolar lavage fluid of CF children at such an early age has raised the question of whether inflammation is initiated by the basic defect in CFTR function or by the early contamination of CF airways with pathogenic bacteria. Subsequent studies of CF infants using bronchoalveolar lavage fluid have demonstrated that neutrophils, interleukin-8 (IL-8) and free neutrophil elastase could be found only in patients with infected airways *(31)*. The absence of inflammatory indices in patients with pristine airways suggests that bacteria are needed to initiate the airway inflammation associated with CF. However the inflammatory response associated with the presence of pathogenic bacteria in the CF airways is clearly exaggerated *(32)*. The ratio of neutrophils or IL-8 to the bacterial density measured in bronchoalveolar lavage fluid is significantly higher in CF patients when compared to that of children with non-CF chronic respiratory diseases. Furthermore, proteome-based analyses of bronchoalveolar lavage fluid from young children with and without CF have demonstrated significantly higher concentrations of neutrophil-derived proteins for a similar bacterial burden in patients with CF *(33)*. Several investigators have reported increases in activation of the nuclear transcription factor kappa B and cytokine release from cell lines expressing defective CFTR *(34–36)*. However, when similar studies were performed on primary cells derived from non-CF and CF lungs, no evidence of an intrinsic hyperinflammatory phenotype could be observed in relation to CFTR deficiency *(37)*. Because of the numerous reports of exaggerated cytokine release as well as abnormal signaling pathways in CF cells and because of the clinical evidence of the hyperinflammatory response to infection in CF patients, it is likely that under certain environmental conditions, CFTR deficiency is related to a hyperinflammatory response *(38)*.

Although a CFTR defect likely favors an exaggerated inflammatory response in various organs, airway infection with pathogenic bacteria clearly remains the major determinant in defining the prognosis of patients. Several distinct pathogenic hypotheses have been proposed to explain the increased susceptibility to lung infection that is associated with CFTR deficiency. Initial investigations of the airway surface liquid electrolytes suggested that CFTR deficiency is associated with an increase in the salt concentration *(39)*. One of the major first lines of defense against bacterial infections in mucosal epithelia is related to antimicrobial peptides and proteins such as human beta defensins (HBD), secretory leukocyte protease inhibitor (SLBI), lysozyme and lactoferrin *(40)*. These antimicrobial molecules are highly effective under normal physiological conditions but their antimicrobial properties are greatly decreased in the presence of high salt concentrations *(41)*. Although it is clear that high salt concentrations inhibit the antimicrobial properties of cationic peptides, measurements of ASL salt concentrations in vivo are fraught with great technical challenges. Studies using minimally invasive techniques based on fluorescent markers would tend to indicate that the ASL salt concentrations are similar in both CF and non-CF tissues *(42)*.

Another hypothesis that has been proposed to explain the increased susceptibility of CFTR deficient tissues to infection is related to observations indicating that the CFTR protein itself can act as a ligand for binding *P. aeruginosa* bacteria *(43, 44)*. The *P. aeruginosa* bacteria that are bound to CFTR at the apical surface of epithelial cells in the lung would then be engulfed by epithelial cells and eliminated through mechanisms that are not fully defined. The relative importance of this mechanism for clearing pathogenic bacteria and in particular *P. aeruginosa* is currently unknown *(45)*.

CFTR deficiency leads not only to lung infection but also to severe damage of the pancreas and obstruction of the bile duct within the liver as well as intestinal occlusion expressed as meconium ileus in 15% of infants with CF, and as the distal intestinal obstructive syndrome in several older patients particularly those with severe CFTR gene mutations. A unifying hypothesis that could explain at least in part the pathophysiology within all of these organs is related to the decreased hydration of mucus at apical membranes. The absence of functional CFTR in epithelial tissues at the mucous membrane interface clearly results in a decreased capacity of these tissues to secrete water in response to physiologic or supra-physiological stimulation *(11)*. It has been clearly demonstrated that the acute response of CFTR bearing mucosal tissues to certain aggressions such as cholera toxin or an oxidant burden results in a rapid and marked activation of CFTR channel function *(46, 47)*. The result of the activation of normal CFTR protein by these stimuli at the mucosal surface is to induce an abundance of watery secretions which is likely a defense aimed at flushing away either pathogenic bacteria or toxic substances. This capacity to increase water secretion when needed is lost in CF mucosal tissues. Another very important function of CFTR is to regulate the baseline absorption of water from the epithelial surface liquid layer. The interplay between CFTR function and absorption of sodium through ENaC is markedly abnormal in CF patients. The net result of this abnormality is a marked increase in absorption of water from the mucosa and a resulting concentration of mucosal proteins and substances at the apical surface of epithelia. In the presence of wild-type CFTR, the mucous layer is sufficiently fluid to allow the coordinated beating of cilia and a directional movement of particles at the surface of the epithelial layer. In contrast, CF tissues have a much lower surface liquid volume which results in the crushing of cilia under a dehydrated mucous layer *(12)*. The cilia present in the CF tissues no longer beat in a coordinated fashion, thus resulting in the stagnation of particles at the epithelial surface. These in vitro observations fit very nicely with histological studies of CF airway tissues in which mucus plaques appear to be glued to the CF airway epithelium and in many areas completely obstruct the small airways *(48)*.

The CF epithelial water hyposecretion and hyperabsorption hypotheses not only explain the pathophysiology of the CF lung but also can be transposed to other tissues directly affected by CFTR deficiency. In addition to marked abnormalities in water homeostasis of CF epithelial tissues, another major contributing factor to CF pathogenesis is the defect in secretion of bicarbonate. Bicarbonate is an essential anion for the regulation of surface fluid pH in all tissues expressing CFTR. However, the tissue that seems to be the most susceptible to improper bicarbonate secretion is the pancreas *(49)*. Although the mechanisms by which bicarbonate secretion deficiency can result in the various pathologies observed in CF tissues are not fully understood, one of the most likely targets of abnormal water and bicarbonate homeostasis is the mucins present at the mucosal surface of all tissues expressing CFTR.

Mucins represent the most abundant protein family within the mucus lining epithelial tissues that express CFTR *(50)*. The mucin superfamily comprises several genes encoding glycoproteins that are characterized by the presence of several mucin-like domains, which include proline, threonine, and serine, or PTS domains. These residues form the sites to which abundant oligosaccharide side chains are attached through to glycosylic bonds. The sugars present on the mucin proteins represent more than 70% of the glycoprotein mass and are heavily sialylated and sulfated. This latter property provides mucins with a net negative charge at physiological pH. The mucin superfamily

is comprised of at least 17 MUC genes, most of which are cell-tethered mucins *(51)*. The secreted polymeric mucins MUC2, MUC5AC, and MUC5B are located at a gene complex on chromosome 11p15.5. Their protein products comprise the vast majority of mucins found within the mucus present at the apical surface of CFTR bearing epithelial tissues. These mucins are packaged tightly within granules of epithelial secretory cells and kept in this compact configuration by high concentrations of calcium and a low pH. The extracellular chloride and bicarbonate concentrations likely allow the mucin proteins that have undergone exocytosis to unfold from their highly compact state. Once in the extracellular space, the mucins are highly hydrophilic and their physical properties will be directly defined by the water, salt, and pH present in the liquid at the apical surface of mucous membranes. Since CFTR regulates salt movement across epithelia, hydration of mucus, and secretion of bicarbonate, it is highly likely that there is a direct link between CFTR deficiency and abnormal physical properties of mucins.

Inflammation, oxidative stress, and proteases have been linked to goblet cell hyperplasia and submucosal gland hypertrophy accompanied by hypersecretion of mucins *(52–54)*. Oxidants and proteases both have been shown to not only induce goblet cell hyperplasia but also increase polymeric mucin gene transcription and exocytosis of mucin proteins that are stored in specialized granules *(55–58)*. Recent attempts to measure mucin proteins in CF secretions have resulted in the surprising observation of a decrease in mucin concentration within CF airway secretions *(59)*. However, the detection of the mucins in CF sputum using antibodies directed against specific epitopes is likely to underestimate CF airway mucin concentrations since mucins are highly susceptible to proteolytic degradation and CF sputum is a very rich source of proteases. Previous studies using methods that were not dependent on immuno-detection revealed that mucins comprised up to 18% of the non dialyzable solids in CF sputum suggesting that mucins are abundant in CF airway secretion *(60)*.

The combination of abundant mucin secretion and the loss of water leads to severe defects in mucus clearance as well as marked abnormalities in host defenses that are dependent on neutrophil killing of the bacteria *(61, 62)*. These pathophysiological changes set the stage for initial bacterial colonization and chronic infection of the CF airways (Figure 16.3). Once the chronic bacterial infection has taken hold, an exaggerated inflammatory response due to the CFTR defect will induce the recruitment and activation of blood-derived neutrophils. The neutrophil is a short-lived leukocyte with a very large cargo of hydrolytic enzymes and cationic proteins. Among these enzymes, neutrophil elastase is one of the most abundant. Neutrophil elastase is a serine protease capable of cleaving several key interstitial proteins within airway walls *(63)*. Neutrophil elastase is an omnivorous protease that can also cleave complement, complement receptor, and other key host defense proteins on phagocytic cells as well as important host defense proteins such as surfactant protein D *(64–69)*. The quantity of neutrophil elastase in the extracellular milieu of CF airway secretions largely exceeds the inhibitory capacity of its natural inhibitors within the airways such as α_1-antitrypsin, SLPI, and elafin *(70)*. This overwhelming protease burden results in the ongoing hydrolysis of structural proteins and host defense molecules while inducing further goblet cell and submucosal gland hyperplasia, mucin gene transcription and mucin protein exocytosis. This series of events further increases the susceptibility of CF lung tissues to bacterial infection thus maintaining and amplifying the vicious circle of airway infection, inflammation, and destruction.

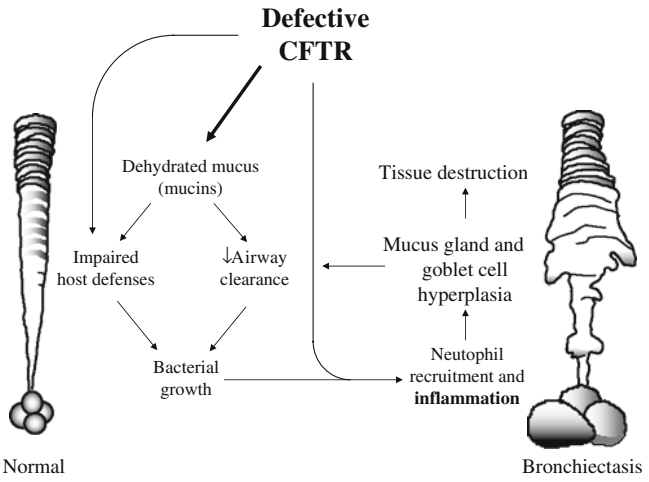

Figure 16.3 Summary of the pathophysiology of lung tissue destruction in patients with defective CFTR. CFTR dysfunction clearly leads to mucus dehydration and impaired airway clearance. Defective CFTR may also play a key role in the regulation of mucosal host defenses and inflammation. The ultimate outcome of CFTR dysfunction is the irreversible destruction of airway tissues known as bronchiectasis (illustrated at *right* of panel)

Another key area of investigation in the pathophysiology of CF is fatty acid metabolism. At least two aspects of fatty acid metabolism have been reported as having direct links to CFTR deficiency. First, the ratio of docosahexaenoic acid to arachidonic acid has been shown to be lower in the lipid membranes of tissues affected by CFTR deficiency *(71)*. Not only is this ratio decreased in CF patients but is also significantly decreased, albeit to a lesser extent, in obligate heterozygotes carrying a copy of a disease-causing mutation on one of their two CFTR genes. Heterozygote subjects are free from any symptoms or clinical manifestations of CF and therefore this observation suggests very strongly that the abnormal DHA:AA ratio is not a secondary defect but is directly linked to CFTR function. Furthermore, transgenic mice bearing a CFTR knockout genotype express similar DHA:AA abnormalities and recent reports indicate that it is possible to decrease liver pathology in these mice by supplementing the animals with DHA *(72)*. It is, however, important to note that abnormalities in other organs did not show any significant changes with DHA supplementation thus indicating that this CFTR-dependent abnormality in lipid metabolism is complex and merits further investigation.

A second lipid abnormality that has recently been suggested to be linked to CFTR deficiency is related to ceramide *(73, 74)*. Ceramides are a family of lipid molecules comprised of sphingosine and a fatty acid. These sphingolipids are present in cell membranes and have been shown to play important roles in regulating host defense responses to *P. aeruginosa (74)*. It has been recently reported that patients with CF and CFTR knockout mice have decreased plasma levels of ceramide as well as decreased ceramide levels in CF-affected organs. Furthermore, Fenretinide, a drug that has been shown to induce ceramide production in cells, was found to normalize ceramide levels in CFTR knockout mice *(73, 75)*. The CFTR knockout mice treated with Fenretinide also showed a significant improvement in their capacity to clear *P. aeruginosa* bacteria from their

CFTR deficient lungs. While these data are of great interest, further work is needed to define this lipid abnormality in CF patients and to understand its relative importance in the pathogenesis of CF organ damage as observed in the lungs and in other tissues.

Animal Models

Three years after the *CFTR* gene had been identified and cloned, investigators were successful in producing a transgenic mouse model of CF *(76)*. This first model known as the CFTRtm1UNC revealed that mice are highly dependent on CFTR function within the intestine since 95% of the KO mice died shortly after weaning. This high mortality rate required that the mice be given a special liquid protein diet in order to increase survival. Several features of this CF murine model recapitulate the disease phenotype recognized in the intestine of CF patients. The mice have an abnormal electrophysiological response, show a failure to thrive and upon histological examination, present evidence of intestinal obstruction with goblet cell hyperplasia, mucin accumulation, and eventually intestinal perforation accompanied by peritonitis. These features are quite similar to meconium ileus observed in approximately 18% of patients with the ΔF508 CFTR mutation. This UNC mouse model is therefore an excellent reflection of the intestinal pathology that can be observed in CF patients. In contrast, investigators were surprised to observe that the lung manifestations of disease in this mouse model were much milder and often absent. Subsequently, investigators have had partial success in reproducing the lung phenotype of hypersusceptibility to bacterial infections caused by agents such as *P. aeruginosa,* but reproducing the same lung phenotype as in CF patients remains a challenge. Several features may explain the differences in the lung phenotype between mice and humans with a CFTR deficiency. First, mice do not have the same density of submucosal glands in the tracheo-bronchial tree and many of these glands are localized at the upper most portion of the murine trachea but not in the lower airways. Second, as in humans, mice have alternate chloride channels within their airway tissues and it is possible that these alternate chloride channels play a more important role in the murine lung, thus compensating for the lack of CFTR function. Finally, the genetic background upon which mice are bred is another factor that contributes to the phenotypic expression within the lungs *(77)*.

Because of the very poor survival of CFTR knockout mice due to intestinal obstruction and perforation, a strategy was devised to specifically correct the CFTR deficiency within the gut of CFTR knockout mice. Human CFTR, under the control of the rat intestinal fatty acid binding protein gene promoter, was expressed in transgenic knockout mice and resulted in sufficient CFTR function to prevent lethality without evidence of expression in the lung *(78)*. This model conveniently improved survival while allowing one to study the phenotypic expression of CFTR deficiency in airway tissues. Although strategies such as the liquid diet and the specific intestinal correction of CFTR allow investigators to improve the survival and have better models of CF, the phenotypic expression of the disease remains imperfect and the typical lung changes observed with chronic CF are almost impossible to reproduce in these animals. There is therefore a great need for better animal models. One very promising strategy currently being developed at the University of Iowa is the CF pig *(79)*. Heterozygote male piglets bearing either the disrupted or the ΔF508 CFTR mutation have been generated and appear to be healthy. This major breakthrough raises the possibility that a CF animal

model with phenotypic changes in the lung and pancreas that are much more similar to those observed in humans may be forthcoming.

Clinical Presentation

The term cystic fibrosis of the pancreas was coined by Dorothy Hansine Andersen who in 1938 was the first to recognize the cystic lesions of the pancreas during an autopsy of a child who had presented symptoms similar to those of gluten intolerance or celiac disease *(80)*. A few years later, Paul di Sant'Agnese observed that many of the children suffering from heat prostration during the 1948 heat wave in New York had cystic fibrosis and were losing abnormally large amounts of salt in their sweat *(81)*. He went on to report the high sweat salt concentration as one of the cardinal features of CF, and to this day, the sweat chloride test remains the key procedure used to confirm a CF diagnosis *(82, 83)*.

The initial clinical presentation of CF varies greatly and spans the spectrum from meconium ileus with intestinal perforation at birth to an apparently indolent course throughout adulthood *(84)*. The most common modes of presentation are respiratory symptoms, failure to thrive, and meconium ileus. In the absence of newborn screening, a CF diagnosis can be delayed by several months and sometimes by years. An increasing number of patients are being diagnosed through newborn screening programs prior to clinical manifestations of the disease *(85)*.

Respiratory symptoms are initially dominated by chronic cough and repeated bouts of bronchopulmonary infections. As the child ages the cough is associated with viscous mucoid airway secretions. Occasionally more severe intermittent lung infections with fever and hemoptysis are observed, and these symptoms become more common with age *(86)*.

The underlying anatomical alterations in pulmonary CF are the destruction of airway walls accompanied by goblet cell and mucous gland hyperplasia *(87)*. These changes favor the dynamic compression of airways during exhalation, causing the characteristic expiratory obstructive syndrome that can be measured with pulmonary function tests. The forced expiratory volume in 1 s (FEV_1) and the forced vital capacity (FVC) decline over time and represent key indicators used to monitor lung disease progression and to decide when it is appropriate to refer the patient for lung transplantation *(88)*. As the obstructive airway disease progresses physical signs such as increased diameter of the chest, decreased breath sounds, and crackles can be observed. Signs of respiratory insufficiency with cyanosis and right heart failure are often present in patients awaiting lung transplantation.

Because of the increased airway resistance and the presence of cysts, individuals with CF are at increased risk of a pneumothorax. Pneumothoraces are most often observed in patients with more advanced disease *(89)*. Chest tube insertion and drainage represent the initial therapeutic approach. Recurrent pneumothoraces can be treated with video-assisted thoracoscopic surgery. If lung disease is too advanced to allow a surgical approach, chemical pleurodesis through a chest tube is a reasonable option. The choice of the pleural sclerosing agent can be difficult due to the high failure rate and pain associated with several of the options. Intrapleural quinacrine seems to be one of the more effective and well-tolerated sclerosing agents for recurrent CF pneumothoraces *(90)*.

The clinical course of CF is related to the causal agents of lung infection. Bacteria identified in the early course of disease include *Hemophilus influenzae* and *Staphylococcus aureus*. Subsequently *P. aeruginosa* acquisition occurs *(27)*. In the past, *P. aeruginosa* acquisition in the vast majority of CF children occurred before the age of 5 years and was considered to be an inevitable outcome of CF. *P. aeruginosa* was often said to "colonize" rather than infect the airways. Today we know that the presence of *P. aeruginosa* in CF airway secretions is always clinically significant and associated with an excessive inflammatory response fueled by the lack of CFTR function. Once chronic infection with pathogenic bacteria, particularly *P. aeruginosa* occurs, the clinical course is characterized by a chronic cough, the production of abundant viscous sputum often difficult to expectorate, and an accelerated decline in FEV_1.

CF adults will on average have one or two yearly episodes of respiratory exacerbations characterized by an increase in cough frequency, shortness of breath, colored and viscous sputum production, decreased appetite, weight loss, and tachycardia *(91)*. In some patients, halitosis is a symptom of lower respiratory tract infections that can have a significant impact on the quality of life. Fever is often absent but can be one of the signs of an exacerbation. Respiratory exacerbations will usually be accompanied by a decrease in FEV_1 and FVC, low oxygen saturation, and the chest radiography may or may not show increased opacities when compared to previous films.

Several pathogenic microorganisms contribute to specific clinical syndromes in CF patients. One of the most dramatic is the *cepacia* syndrome first recognized in the early 1980 s when *Burkholderia cepacia* (then know as *Pseudomonas cepacia*) acquisition was reported to be associated with an alarmingly high fatality rate *(92)*. Before any definitive evidence of transmission between persons with CF was available, clinicians at the CF center in Cleveland began segregating *B. cepacia* positive and negative patients and soon reported a sharp decline in the rate of acquisition of this microorganism *(93, 94)*. Transmission between patients was subsequently confirmed with ribotyping using restriction fragment length polymorphism banding patterns *(94)*. The implementation of strict infection control measures in CF clinics, in hospitals, and at social gatherings such as summer camps has since resulted in a marked decline of *B. cepacia* acquisition. Molecular microbiology has allowed the development of a classification scheme of the *B. cepacia* complex into nine species or genomovars *(95)*. The cepacia syndrome, characterized by a severe respiratory deterioration and death within months of bacterial acquisition, is mostly associated with genomovar III although a similar syndrome has been reported with other *B. cepacia* species *(96)*. Because of the poor post-surgical prognosis, some centers have been reluctant to offer lung transplantation to CF patients bearing genomovar III *B. cepacia* in their airways.

Viscous airway mucus constitutes an ideal niche for the aspergillus fungal genus, of which fumigatus is the most common pathogenic species. Up to a quarter of CF patients have aspergillus in their airway secretions *(97)*, and a significant proportion of them are allergic to this fungus *(98)*. The proximity of the aspergillus antigen and the host's antibodies leads to a type III Arthus reaction in which the antigen, antibody, and complement complex attract neutrophils to the airway wall. The ensuing release of neutrophil proteinases destroys the airway surrounding the impacted mucus plugs and causes pathognomonic proximal saccular bronchiectasis *(99)*. This disease process is known as allergic bronchopulmonary aspergillosis or ABPA, and its symptoms include respiratory exacerbations with cough and dyspnea, expectoration of rubbery-like brown plugs and bronchial casts. The radiological manifestations are characterized by

evanescent pulmonary infiltrates in the areas peripheral to mucus plugs, as well as tramlines and glove-finger shadows. Blood eosinophils and IgE levels are increased, and the skin prick test to the aspergillus antigen reveals a positive early phase reaction. Treatment often requires systemic corticosteroids to which antifungal can be added.

Atypical mycobacteria, methicillin-resistant *Staphylococcus aureus* (MRSA), *Stenotrophomonas maltophilia*, and *Alcaligenes xylosoxidans* contribute to an increasing number of resistant pathogenic organisms that are associated with specific clinical manifestations of CF. Patient-to-patient transmission of these organisms is a mounting concern and requires close attention to infection control measures *(100)*.

A characteristic feature of CF is clubbing of the digits. Clubbing is related to the increased right to left shunting of deoxygenated blood through a markedly increased intrapleural bronchial artery circulation associated with extensive bronchiectasis. The increase in the bronchial vasculature combined with severe airway infection and inflammation places the CF patient at risk of life-threatening hemoptysis from arterial bleeding. Patients will occasionally report having felt from where the bleeding originated; however, identification of the site of bleeding will normally be attempted using bronchoscopy and chest computerized tomography. An effective minimally invasive solution to significant hemoptysis is bronchial artery embolization (BAE), a treatment that is often definitive. Strikingly though, CF patients who undergo BAE subsequently have a much higher risk of dying or needing a lung transplantation than those with similar lung function impairment who have not required BAE *(101)*. It is not know whether this increased risk is due to the BAE itself or to more severe lung damage that is not reflected in the similar FEV_1 levels. For the few patients in whom recurrent bleeding occurs on the same side after embolization, the procedure can be repeated with a successful outcome.

Nasal polyps and chronic sinusitis are common manifestations of CF *(102)*. These inflammatory and infectious conditions observed in the CF lung are mirrored in the sinuses which bear a similar respiratory ciliated mucosa lined with mucus. Symptoms of sinus disease include nasal congestion, post-nasal drip, chronic cough, purulent nasal discharge, and anosmia. Radiography will reveal poorly developed sinuses with opaque material filling the sinus cavities in many patients *(103)*. Nasal polyps are common and originate from the sinus outlets. Nasal polyps, which can grow sufficiently to protrude from the naris, are consequences of sustained inflammation and can be removed surgically. Long-term treatment with regular saline solution irrigation and inhaled nasal corticosteroids is usually sufficient to prevent recurrence.

Nutrition and Gastrointestinal Manifestations

Nutrition. One of the major challenges for CF patients and their caregivers is ensuring that the patient's nutritional needs are met. Resting energy expenditure (REE) is markedly increased in the vast majority of CF individuals due to the increased work of breathing, inflammatory cell phagocytosis of bacteria, and a severe chronic lung inflammatory reaction. Increased REE is associated with accelerated lung deterioration *(104)*. The lung inflammatory cells release large amounts of catabolic cytokines such as tumor necrosis factor alpha, previously known as cachectin because of its cachexia-inducing effects *(105)*. Nutrition concerns are not limited to the maintenance of adequate caloric intake to ensure a healthy body mass index (BMI). Poor lipid-soluble (A, D, E, K)

vitamin absorption compounds several CF-related abnormalities including osteoporosis and liver-dependent coagulation factor deficiencies. Nutritional deficiencies also are thought to contribute to antioxidant deficiencies with respect to the increased oxidative stress associated with CF lung disease *(106)*. Certain antioxidant vitamin and mineral deficiencies may contribute to accelerate the deterioration of lung function.

Gastroesophageal reflux. Gastroesophageal reflux is a relatively common problem in patients with CF and may be associated with hiatal hernia, esophagitis, and esophageal stricture *(107)*. Patients with gastroesophageal reflux tend to have more severe CF lung disease. The prevalence of gastroesophageal reflux is very high in patients with end-stage lung disease who are listed for lung transplantation *(108)*. It is possible that micro-aspiration of acid reflux into the airways contributes to airway pathology – particularly in patients after lung transplantation *(109)*. Fundoplication surgery, but not proton pump inhibitor medication, in lung transplant recipients with documented gastroesophageal reflux has been reported to decrease both acute lung rejection and chronic bronchiolitis obliterans *(110)*. The latter is the major cause of morbidity and mortality in the years following lung allograft surgery.

Pancreatic insufficiency. Severe destruction of the exocrine pancreas occurs in up to 85% of CF patients at an early age and is often present at birth *(22)*. The 10–15% of patients with a pancreatic sufficient phenotype do not need dietary pancreatic enzyme supplementation to avoid steatorrhea and ensure normal weight gain and growth. The PS phenotype is strongly associated with genotypes within the type IV and V CFTR classes of mutations. In contrast to patients without residual pancreatic function, patients with pancreatic sufficiency are at increased risk of intermittent pancreatitis *(111)*.

Cystic fibrosis-related diabetes (CFRD). As CF patients are living longer the prevalence of CFRD is also increasing and is now estimated at more than 40% in patients over 30 years old *(112, 113)*. Glucose intolerance is present in up to 70% of CF adults *(114)*. The primary cause of CFRD is the insulinopenia associated with destruction of Langerhans islet cells. The total area of insulin-staining islet cells is reduced in the pancreas of CFRD compared to non-CFRD patients, a likely consequence of the severe cystic and fibrotic changes initiated in the exocrine pancreas. Inflammation, infection, and corticosteroid treatment of CF patients are factors that may contribute to insulin resistance that can compound the insulin deficiency associated with CFRD. CFRD is distinct from types I and II diabetes. Management of patients with CFRD must take into account several unique features such as their poor nutritional status, increased energy expenditure, increased caloric needs, decreased glucagon secretion, liver disease, low body mass index, decreased lipid levels, frequent infections, inflammation, delayed gastric emptying, abnormal intestinal transit, and decreased intestinal absorption *(115)*. A diagnosis of CFRD is associated with a more severe lung prognosis particularly in female subjects *(116)*. The recognition and treatment of CFRD is therefore important to prevent not only the micro-vascular consequences of diabetes but also the deleterious effects of diabetes on pulmonary function and nutritional status. Treatment of CFRD will include a high caloric intake to ensure that the patient's energetic needs are met and the use of insulin therapy. Oral hypoglycemic agents are not usually the therapy of choice for CFRD, and if they are used then liver function abnormalities related to CF must be carefully monitored and considered *(112)*.

Liver disease and gallbladder disease. Most patients with CF have hepatobiliary disease but only a minority will develop symptoms and significant clinical consequences

(22). As in almost all CF-affected tissues, the mucosal secretions within the bile ductules are viscous and cause focal obstruction of normal bile flow. A striking feature of CF-related liver disease (CFLD) is its focal, patchy nature characterized by the proximity of obstructed and normal biliary tracts. The stagnant flow results in precipitation of proteins with toxic bile salts in the ductules, focal periductal inflammation, hyperplasia, and fibrosis. Steatosis and patchy areas of biliary cirrhosis are common in CFLD. However, extensive multilobular cirrhosis and end-stage liver failure with portal hypertension, splenomegaly, and esophageal varices occur in a minority of patients and are more common in males with the ΔF508 or other severe classes of CFTR mutations *(117)*. Genetic modifiers such as the Z alpha-1 proteinase inhibitor and gain-of-function variants of the TGFβ pathway have been linked with more severe CFLD *(118)*. One of the major challenges in assessing CFLD is the absence of a direct correlation between biochemical markers or radiological indices and the extent of cirrhosis. Because of the focal, uneven distribution of disease, percutaneous needle liver biopsy is not necessarily reflective of a CFLD prognosis.

Another common site of CF disease is the gallbladder. Protein-rich viscous bile secretions favor the development of a non-functional microgallbladder, cholelithiasis, biliary tract sludge, and occasionally a distended gallbladder.

Distal intestinal obstruction syndrome (DIOS). Intermittent abdominal pain is one of the more common symptoms of CF and can be attributed to inadequate pancreatic enzyme supplementation, constipation, inflammatory bowel disease, or DIOS. Because of CFTR deficiency, the intestinal contents of the distal ileum are often thick dehydrated and poorly digested. The accumulation of paste-like intestinal contents in the distal ileum and proximal colon can lead to DIOS, an often acute and very painful intestinal obstruction syndrome *(119)*. DIOS is more common in patients with pancreatic insufficiency, a previous history of meconium ileus, severe classes of CFTR mutations, and in patients who have had major surgery. The abdominal pain is either diffused or located in the lower right quadrant where a mass can be palpated. DIOS also is expressed as a less severe, intermittent, and recurrent painful syndrome.

Other intestinal manifestations of CF. The thickened secretions within the intestine increase the risk of intussusception 10- to 20-fold in CF compared to the general population *(22)*. Intussusception is generally a serious medical emergency requiring rapid surgical intervention to prevent intestinal ischemia and necrosis in older adults without CF. In contrast, intussusception in CF patients is commonly intermittent and will most often resolve spontaneously. Intussusception in CF can also present as a fortuitous observation with a characteristic "doughnut sign" upon radiological or ultrasound examination. Rectal prolapse and intestinal bacterial overgrowth are other well-known manifestations of CF.

In the mid-1990s a new clinical entity, fibrosing colonopathy, was reported in younger patients taking exceptionally high doses of pancreatic enzyme supplements *(120)*. These patients had symptoms similar to inflammatory bowel disease with increased abdominal pain, intermittent bouts of intestinal obstruction, and the passing of blood and mucus in their stools. The anatomical defect was found to be an extensive inflammatory reaction associated with fibrotic submucosal ring-like stricture of the proximal colon that could extend throughout the colon. The association between fibrosing colonopathy and very high doses of pancreatic enzyme supplementation led to the conclusion that excessive enzyme dosage was a major contributor to this syndrome.

Cancer. Another much less common but severe manifestation of CF is cancer *(121, 122)*. CF patients have a 6.5-fold increase in the risk of gastrointestinal cancers such as colon and pancreatic adenocarcinomas as well as of cholangiocarcinoma, a hepatobiliary cancer. The increased risk of cancer is specific to the GI tract and not observed in other tissues bearing CFTR. The reasons for this are not clear but as CF patients are living longer, the incidence of GI cancers in CF is likely to rise and will require increased vigilance in multidisciplinary CF clinics.

Infertility. More than 97% of male CF patients are infertile. Male infertility is characterized by azospermia and is associated with a congenital absence of the vas deferens. The vas deferens is one of the tissues most sensitive to a lack of CFTR function. A large segment of males with azospermia and no other symptoms have mild forms of CFTR functional deficiency leading to congenital absence of the vas deferens or CBAVD *(123)*. In females a reduced fertility is observed due to thickened cervical secretions but fertility is often preserved. Whereas non-smokers who are heterozygote carriers of CFTR mutations have normal or slightly increased fertility, heterozygote smokers have a significant reduction in fertility, suggesting that environmental factors compound the effects of CFTR deficiency on fertility *(124)*.

Osteoporosis. It has been known for many years that patients with CF are at an increased risk of developing osteoporosis at a very early age *(125)*. Malabsorption of fat soluble vitamins, particularly D, but also K, was thought to be the major cause of osteoporosis. However, the very high frequency of persistent osteopenia despite adequate vitamin and calcium supplementation remained a mystery. A possible explanation may relate directly to CFTR deficiency itself as is suggested by studies of CF mice *(126)*.

Diagnostic Approach

CF will be suspected following one or more CF-related symptoms, a positive family history among siblings, or an abnormal newborn screening result *(82)*. A CF diagnosis must be confirmed by two abnormally elevated sweat chloride concentrations measured on separate days or by the identification of two recognized disease-causing mutations. In exceptional cases, the confirmation of a CF diagnosis cannot be made using these criteria and evidence of CFTR dysfunction through nasal potential difference measurements can be helpful. However, interpretation of nasal PD results must be done with care, since measurements can be abnormal in non-CF conditions such as cigarette smoke exposure *(127)*.

Delay in making a CF diagnosis is associated with less favorable outcomes such as failure to thrive and smaller head circumference. Because children cannot recover from these adverse impacts of a late diagnosis despite optimal therapy in later years, there is a recognized need to implement newborn screening services for the general population *(128)*. Newborn screening makes use of the immuno-reactive trypsinogen or IRT assay. Pancreatic damage in CF newborns is associated with the release IRT into plasma, and assays are sufficiently sensitive and specific to reliably measure this marker on blood samples obtained at birth using the Guthrie card. Basically, two CF newborn screening algorithms exist, the IRT/IRT and the IRT/DNA algorithms *(129)*. The detection limit of the IRT/IRT algorithm is set at a higher value to decrease the number of false-positive results and therefore has a slightly lower sensitivity than IRT/DNA, but has the

advantage of not detecting carriers. The IRT/DNA makes use of a detection assay that screens a panel of 30–40 mutations. Some centers will also add full-length CFTR gene sequencing if an abnormal IRT is associated with only one CF-causing mutation in the DNA test. Because the IRT detection limit is set to a lower value in this algorithm, more false positives are initially detected but these are rapidly identified with the DNA test. Also, a definite number of carriers will be detected, and expert genetic counseling services are needed. Most family members that learn fortuitously of their carrier status understand the implications of the new information and view the acquisition of this information favorably. Regardless of the algorithm, diagnosis must be confirmed by sweat testing. The sweat test must be performed shortly after a positive screen in order to decrease parental anxiety while waiting for confirmation or exclusion of CF *(129)*.

Conventional Treatment

Current therapy for CF is entirely focused on alleviating the consequences of CFTR deficiency in various tissues and organs. Because of the complex nature of CF, ideally all patients should benefit from the care provided by multidisciplinary CF clinic teams focused on quality improvement *(130)*. Wide consensus exists about the benefits of a multidisciplinary team approach to CF patients and it is largely felt that specialized clinics have been a major contributor to the marked increase in patient survival. Intensive multidisciplinary care and follow-up should be provided from the time of diagnosis. Diagnosis must made be as early as possible. Great effort is needed to ensure that the nutritional requirements of the infant are met such that growth, weight gain, and head circumference progress normally. Successful nutritional support includes attention to pancreatic enzyme and adequate lipid soluble vitamin and oligoelement supplementation.

Lung therapy. Lung health is such a major determinant of the CF prognosis *(131)*. Prevention and therapy of airway disease is key, and the Cystic Fibrosis Foundation has issued pulmonary therapy guidelines *(4)*. Chest physiotherapy airway clearance techniques form the cornerstone of conventional prevention and therapy of CF lung disease. Several techniques and devices for airway clearance are available, but limited comparative data make it difficult to adequately assess the relative value of each type of airway clearance technique *(132)*. However, all CF patients should be trained in one form of airway clearance technique and followed by professionals with expertise in CF chest physiotherapy.

Because bacterial infection and inflammation are most often present within the first months of life, aggressive treatment of respiratory infectious complications is essential. Symptomatic bronchopulmonary infections must be treated with systemic antibiotics. The first growth of *P. aeruginosa* is treated aggressively using systemic and/or nebulized antibiotics. Eradication of *P. aeruginosa*, at least transiently, is possible in most cases and it may be years before the bacteria is identified again in the patient's respiratory secretions *(133)*. Acute respiratory infections or exacerbations with *P. aeruginosa* should be treated with two antibiotics to which the bacteria are sensitive. The duration of therapy for pulmonary exacerbations is generally 14 days. Because of the increased volume of distribution and accelerated elimination of many antibiotics, dosage regimens of antibiotics in CF are different from those recommended in non-CF populations *(131)*. The persistence of *P. aeruginosa* in CF airways requires special attention and is

generally treated with high-dose inhaled tobramycin or occasionally with inhaled colistimethate. Chronic suppressive inhaled antibiotic therapy can favor the selection of resistant organisms in CF sputum; however, the benefits of such therapy clearly outweigh its risks. Inhaled TOBI®, a concentrated formulation of tobramycin developed for inhalation therapy cycled 28 days on/off, is associated with a decrease in hospitalization rates, as well as improved lung function *(134)*.

Chronic infection and inflammation of the CF lung carries with it a burden of dead and dying neutrophils and bacteria. These cells release massive amounts of DNA into the extracellular milieu. DNA is largely comprised of lengthy anionic carbohydrate chains known to increase mucus viscosity. Inhalation of a human recombinant DNase or dornase alpha solution improves the expiratory flow rates and is associated with higher well-being scores in quality of life assessments, particularly in patients with moderate to severe lung disease *(135)*. Few side effects other than voice changes are reported. Inhaled twice daily hypertonic (7%, 4 ml) saline solution is another generally well-tolerated inhalation therapy that has been shown to provide moderate improvements in forced expiratory airflow in CF patients but its efficacy is less than that of dornase alpha *(136)*. Hypertonic saline inhalation does not replace dornase alpha but can provide added benefit. However, one of the difficulties with current approaches to CF lung therapy is the increasing treatment burden for patients and families who must spend considerable time every day implementing the prescribed regimens *(137)*. Compliance with therapy is a major challenge that increases as the disease progresses.

Inhaled bronchodilator therapy provides clear benefit for many individuals with CF, but there is insufficient evidence to determine whether anticholinergic agents are beneficial *(138, 139)*. Inhaled corticosteroids should not be used routinely as there is not sufficient evidence that they reduce the time to exacerbation or improve lung function *(140)*. However, some CF individuals with reactive airways disease and reversible asthma-like airways disease will benefit from inhaled corticosteroids.

Systemic anti-inflammatory therapy with ibuprofen is of benefit particularly in younger patients *(141)*. The ibuprofen dose must be adjusted for each patient to assure appropriate serum levels since it is possible that low ibuprofen levels cause a paradoxical pro-inflammatory reaction. Finally, chronic oral therapy with macrolide antibiotics has been studied in CF following numerous reports of its benefits in diffuse panbronchiolitis in Japan, a non-CF airway disease characterized by chronic infection with *P. aeruginosa (142)*. Initially thought to act as anti-inflammatory agents that can suppress neutrophil chemotaxis, macrolides have since been shown to alter *P. aeruginosa* gene expression and biology and may increase the susceptibility of this organism to host defenses and antibiotic therapy. Chronic azithromycin therapy in CF was found to be associated with a small increase in lung function and a decrease in pulmonary exacerbations *(143)*.

Gastrointestinal therapy. Treatment of choice for DIOS is not surgical and it is important to distinguish this entity from surgical emergencies such as appendicitis. Large volumes of polyethylene glycol electrolyte solution and other hydration strategies to relieve the obstruction are most often successful *(144)*. Prevention of DIOS is possible with regular intake of small volumes of polyethylene glycol electrolyte solution, or a thiol-containing solution (acetylcysteine), or an osmotic peristalsis promoting agent such as lactulose.

Although portal hypertension and esophageal varices are relatively common, the long-term course of CFLD is largely benign in CF adults. There is no definitive

evidence that treatment of CFLD with ursodeoxycholic acid (UDCA) can prevent progression to end-stage liver disease. However, since UDCA therapy does improve liver enzyme abnormalities and decreases hepatobiliary symptoms, its use is common in CF patients with signs of CFLD *(144)*.

Treatment and prevention of osteoporosis is possible and necessary in CF patients. Regular monitoring of bone mineral density and 1,25(OH) vitamin D plasma levels guide the preventive interventions that include vitamin D and calcium supplementation as well as bisphosphonate therapy in selected patients. High doses of vitamin D supplementation may be needed to reach and maintain normal plasma levels in CF *(145)*.

Further Therapeutic Targets and Direction

Although great strides have been made in CF care, new therapies of secondary defects are likely to provide only marginal health improvements at the cost of increasing an already heavy treatment burden. Patients with CF need fewer, not more drugs. To achieve this goal, CF care will require that the underlying causative molecular deficiency be addressed. Since the discovery of the *CFTR* gene, investigators have cherished the hope that gene therapy would be a viable solution.

Gene therapy. The most recent clinical trial of gene therapy in cystic fibrosis was a placebo-controlled phase IIB trial in 102 CF patients with FEV_1 of 60% and higher *(146)*. The CFTR gene packaged in an adeno-associated virus vector tgAAVCF was delivered by aerosol therapy twice at 30-day intervals. The repeat administration was found to be safe; however, no changes were observed in spirometry, the primary outcome, or in sputum markers or days of antibiotic use. To date, no other gene therapy trial has shown better results.

Pharmacological Approaches

Purine receptor agonists. Denufosol is a P2Y2 purine receptor agonist with prolonged stability that can increase calcium-dependent chloride secretion in the airways. A phase II trial has shown that denufosol treatment not only is safe but also resulted in some improvement of lung function over the control group *(147)*. Larger studies are ongoing and will need to be analyzed before one can evaluate the true potential for this drug in CF.

Alternate chloride channel activation. Moli1901 (duramycin) is a polycyclic peptide that increases cellular calcium and can stimulate chloride secretion through alternate chloride channels. A phase II study of Moli1901 confirmed its safety in CF patients and interestingly, a statistically significant increase in the FEV_1 was observed despite this being a short (5 days) study in a small (24 patients) number of patients *(148)*. Further studies are planned.

ENaC inhibitors. Excessive sodium absorption is one of the direct consequences of dysfunctional CFTR. Sodium is absorbed through the epithelial sodium channel, ENaC, at the apical surface of airway epithelial cells. Amiloride is an ENaC inhibitor and has shown safety upon inhalation. Amiloride is not likely to be the optimal approach to block sodium and water absorption since it has a short half-life. Furthermore, a clinical trial has provided evidence that amiloride inhalation in CF was associated with a trend

toward lower lung function *(149)*. Alternate inhibitors of ENaC are currently being studied.

Premature termination codon. Several pharmacological approaches specific to the severe CFTR mutation class defects are at different stages of development, many with promising preliminary results. Class I mutations result in the premature termination of transcription due to an abnormal coding sequence on the *Cftr* gene. PTC124 is a novel orally available compound that allows the ribosomal reading through premature but not through normal termination codons *(150)*. CFTR nonsense mutation G542X results in premature termination of transcription without CFTR function. *Cftr*–/– mice expressing hG542X have been treated with PTC124 through the sub-cutaneous and oral routes *(151)*. The drug was well tolerated and resulted in the restoration of cAMP-dependent chloride currents in the intestine of hG542X *Cftr*–/– mice to 24–29% of the average cAMP transepithelial chloride current observed in wild-type animals. Although similar results could be obtained with the aminoglycoside gentamicin, PTC124 (or its equivalents) should be of greater interest since it is not expected to induce the toxicity associated with aminoglycoside therapy and can be delivered by the oral route. Furthermore, it is estimated that correction of as little as 10% of normal CFTR protein function will benefit CF patients *(24)*.

CFTR correctors and potentiators. Small molecule discovery programs aimed at restoring CFTR function are progressing at a very rapid pace *(152)*. New compounds of interest are conveniently classified as either correctors that improve protein folding/trafficking or potentiators that aid the cAMP-dependent function of abnormal CFTR protein present at the apical surface of epithelial cells *(153)*. High-throughput screening efforts are continuously expanding the list of promising compounds in each of these categories.

Among the first correctors tested was 4-phenylbutyrate but this compound has modest in vivo efficacy *(154, 155)*. More promising correctors under investigation include, among others, phosphodiesterase-5 (PDE-5) inhibitors *(156–158)*, the quinazoline VRT-325 *(159, 160)*, bisaminomethylbithiazoles *(161)*, aminoarylthiazoles, benzo(c)quinolizinium (MPB) *(162, 163)*, and α-glucosidase inhibitor miglustat *(164–166)*. Sildenafil and vardenafil are PDE-5 inhibitors that have also been shown to correct ΔF508 trafficking ex-vivo in nasal epithelial cells; however, the concentrations of sildenafil needed to correct CFTR are estimated to be several-fold higher than plasma concentrations obtained during erectile dysfunction therapy *(167)*. Structural analogs of sildenafil such as KM11060 hold promise as they are more potent correctors of ΔF508 CFTR than other PDE-5 inhibitors *(158)*.

Potentiators are of interest for CFTR mutations of both classes II and III. They include xanthines *(168)*, flavones *(169)*, tetrahydrobenzothiophene *(170)*, phenylglycine, and sulfonamide *(171)*. An increase in cAMP activation of ΔF508 CFTR using a potentiator in association with a corrector could provide enhanced CFTR function. Potentiators would also be important for class III mutations in which the protein is present at the apical membrane but is unable to regulate channel opening.

The Cystic Fibrosis Foundation announced in March 2008 that VX-770, an oral drug being developed by Vertex Pharmaceuticals Incorporated, showed promising results in a Phase 2a clinical trial for patients who carry the class III G551D CFTR mutation. The foundation reported that these CF patients showed significant improvement in lung function, nasal potential difference measurements, and sweat chloride levels after 14 days of therapy (http://www.cff.org/ information accessed March 27, 2008). This is

the first evidence in CF patients that an oral drug can correct at least in part, not only the basic defect of CFTR function but also its pathophysiological consequences. CF research has truly entered a new era in which correction of CFTR dysfunction is becoming a reality.

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17

Pulmonary Langerhans' Cell Histiocytosis – Advances in the Understanding of a True Dendritic Cell Lung Disease

Robert Vassallo

Abstract Pulmonary Langerhans' cell histiocytosis (PLCH) is a rare lung disease that generally, but not invariably, occurs in cigarette smokers. The pathologic hallmark of PLCH is expansion of Langerhans and other inflammatory cells in a bronchiolocentric fashion. The precise mechanisms by which smoking induces PLCH in susceptible individuals are not known, but likely involve a combination of molecular events resulting in enhanced recruitment and retention of Langerhans cells in small airways. PLCH is primarily a disease of small airways, with variable extension into the lung interstitial and vascular compartments. While cellular inflammation is evident early in the disease course, the more advanced stages are characterized by cystic lung destruction, cicatricial scarring of airways, pulmonary vascular remodeling, and emphysematous change. High resolution chest CT scanning is very useful in the diagnostic evaluation and may show nodular and cystic changes that are virtually pathognomonic. In several instances, lung biopsy (bronchoscopic or surgical) is necessary to establish a definitive diagnosis. All smokers with PLCH must be counseled on smoking cessation, while for selected patients, pharmacotherapy with corticosteroids or other agents may be indicated to prevent disease progression and preserve lung function. All symptomatic patients should be screened for the presence of pulmonary hypertension, which may respond to vasodilator therapy. The prognosis for a significant proportion of patients is relatively good, particularly if smoking cessation is achieved, and if longitudinal lung function testing shows stability. Pneumothoraces, secondary pulmonary hypertension, and the development of premature emphysema are important complications that shorten life expectancy. Lung transplantation may be indicated for patients with relentless progressive disease.

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The histiocytic syndromes are a collection of diseases associated with proliferative abnormalities involving cells belonging to the monocyte/macrophage and dendritic cell lineage. Individual diseases within the group of histiocytic syndromes vary widely with respect to natural history and clinical behavior. For instance, some histiocytic syndromes have a very benign natural course and require minimal treatment (such as a solitary bone lesion due to focal Langerhans cell histiocytosis), whereas other syndromes may be considerably more aggressive. The Histiocyte Society and the American Histiocytosis Association proposed a useful classification scheme for these disorders which takes into account the primary culprit cell and natural biology as criteria for classification (Table 17.1) *(1)*.

Table 17.1 Histiocytic disorders.

A. Disorders of varied biological behavior
Dendritic cell-related
Langerhans cell histiocytosis
Secondary dendritic cell processes
Juvenile xanthogranuloma and related disorders
Solitary histiocytomas of various dendritic cell phenotypes
Macrophage-related
Hemophagocytic syndromes [familial, primary or secondary]
Rosai-Dorfman disease
Solitary histiocytoma with macrophage phenotype
B. Malignant disorders
Leukemias [acute and chronic]
Extramedullary monocytic tumor or sarcoma
Dendritic cell or macropahge-related histiocytic sarcoma
Specific phenotypes

Modified from reference *(1)*.

The Langerhans cell histiocytosis (LCH) are a subgroup of the histiocytic syndromes in which specialized dendritic cells expressing surface CD1a antigens and intracellular Birbeck granules – a subgroup of dendritic cells referred to as Langerhans' cells – proliferate and infiltrate organ systems resulting in varying degrees of organ dysfunction *(2, 3)*. The term LCH was intentionally chosen by the histiocyte society to replace the term "histiocytosis X," in order to acknowledge the central role of the Langerhans cell as the key pathogenic cells in these diseases *(1)*. It is now apparent that the diseases formerly called "eosinophilic granuloma" are also part of the LCH spectrum of diseases. Rather than using the term "eosinophilic granuloma" for localized disease and LCH for multisystem disease, the terms localized or focal LCH and diffuse or multisystem LCH are preferred. The most frequently affected organs in adults with LCH include the lungs, the skeleton (especially skull and axial skeleton), the central nervous system (especially the hypothalamic region), and skin *(2)*. Pulmonary

involvement is common in adults with LCH and may occur either in isolation (around 85% of pulmonary LCH occurs in isolation) or in the setting of multiple organ involvement *(4)*.

Epidemiology and Demographics

The true incidence and prevalence of pulmonary LCH are unknown. An estimate obtained from a large series of 502 surgical lung biopsy specimens performed during evaluation of diffuse lung diseases identified pulmonary LCH in only 17 cases (or 3.4%) *(5)*. This probably represents an underestimate of the true prevalence since a proportion of pulmonary LCH patients have minimal symptoms and never undergo surgical lung biopsy. Pulmonary LCH afflicts predominantly Caucasians and seems to be very unusual in individuals of African or Asian descent. The disease occurs principally in young adults between the ages of 20 and 40 years, though it can present in any age group, and can be diagnosed in individuals >65 years of age *(4, 6)*. The reported relative sex distribution of pulmonary LCH varies, with earlier studies suggesting a male preponderance *(7)*. More recent studies suggest no gender predilection *(4, 8)*.

Several epidemiologic and clinicopathologic studies imply that isolated pulmonary LCH is a smoking-related lung disease *(4, 8–13)*. In adults, isolated pulmonary LCH is primarily a disease seen in young adult smokers; most studies report a smoking history in at least 90% of patients with isolated pulmonary LCH *(4, 8, 10)*. The role of smoking in the subgroup of patients who have pulmonary LCH in the context of multisystem disease is unclear. Pulmonary involvement occurring in multisystem LCH may be seen in approximately a third of all adult LCH cases, of whom at least half are non-smokers *(6)*. In a cohort of 314 adults with LCH, only 3 of 87 patients with isolated pulmonary LCH were non-smokers, whereas a history of tobacco exposure was not present in 155 of the remaining 227 patients *(12)*. Similar findings were reported in a more recent large observational cohort of 274 patients from several countries *(6)*.

Isolated pulmonary LCH is uncommon in children, despite the fact that multisystem LCH is about three times more prevalent than in the adult population *(14, 15)*. This probably reflects the importance of smoking in the pathogenesis of pulmonary LCH and suggests that in the absence of direct cigarette smoke exposure, pulmonary LCH is an exquisitely rare condition. The reported cases of cigarette smoking precipitating the onset of pulmonary LCH in adolescents who were in remission from non-pulmonary LCH in early childhood provide further evidence in favor of a direct role for smoking in the pathogenesis of pulmonary LCH *(9)*.

Despite the close association between cigarette smoking and the incidence of pulmonary LCH, there are no data that correlate the amount of daily cigarette consumption with the severity of the disease. Most patients with pulmonary LCH tend to be heavy smokers; however, the disease may also occur following relatively brief exposure to tobacco smoke (unpublished observations). The effects of second-hand smoking or recreational drugs on pulmonary LCH are unclear. Genetic factors probably do not play a prominent role in the development of pulmonary LCH as the overwhelming majority of LCH (pulmonary or otherwise) occurs in a sporadic fashion and only in exceptional circumstances has LCH been reported to affect more than one family member *(16)*.

Gross Pathology and Histologic Features

Gross inspection of the lung in advanced LCH typically demonstrates cysts on the surface, and upon sectioning, one may observe nodules varying in size from a few millimeters to 1.5 cm in diameter, although most are 1–5 mm in size *(17)*. In advanced cases, nodules may be absent, and the predominant finding may be that of a hyperinflated lung with advanced bullous and cystic destruction that may be difficult to differentiate from advanced emphysema. Varying degrees of honeycombing may also be present *(8, 17)*.

Traditionally, pulmonary LCH has been classified as one of the interstitial lung diseases. It is more appropriate to think of this disease as a small airway disease or inflammatory bronchiolitis, since the inflammatory early lesions consist of loosely formed nodules of immune cells that collect around small airways *(8, 17, 18)*. Varying degrees of interstitial infiltration may accompany the bronchiolocentric lesions, and in some patients, extensive alveolar macrophage infiltration may be found in the airspaces *(19)*. In addition to bronchiolar, interstitial, and varying degrees of alveolar involvement, some cases of pulmonary LCH are associated with extensive vascular infiltration, resulting in a proliferative vasculopathy that may be observed in both arteries and veins *(8, 20, 21)*. An appreciation of the varying pulmonary compartments that the disease involves is important, since the extent of airway, interstitial, and vascular involvement may vary greatly from one patient to another, and serves to partially explain the variability observed in lung function testing and clinical findings *(4)*.

The microscopic appearance on lung biopsy specimens varies depending on the stage of the disease process at the time of biopsy. The earliest lesion consists of loose cellular nodules adjacent to small airways and scattered throughout the lung parenchyma (forming loosely formed granulomas) *(8, 17, 22)*. These nodular collections are typically composed of a mixed population of inflammatory cells *(17)*. Langerhans' cells are usually abundant in early lesions. Varying degrees of T lymphocyte, macrophage, plasma cell, monocyte, and eosinophilic infiltration may also be seen *(8)*. Eosinophilic infiltration is often encountered and may be quite extensive, hence the former term "eosinophilic granulomas" *(17)*. However, eosinophils are not always present, and the inflammatory lesion does not always have the appearance of a granuloma. The relative proportions of the different inflammatory cell types vary greatly, even in adjacent nodules in the same patient. This variability has been described by some expert lung pathologists as being quite characteristic of pulmonary LCH *(17)*.

The bronchiolocentric lesions of pulmonary LCH typically form symmetric stellate lesions with central scarring *(17)*. In the adjacent airspace, varying degrees of pigmented alveolar macrophage accumulation may be seen *(17)*. This alveolar filling with pigmented macrophages results in so-called pseudo-desquamative interstitial pneumonia (pseudo-DIP) changes *(8, 17)*. The stage of evolution of the process has an important influence on the pathologic findings. In the early stages, numerous cells accumulate adjacent to terminal or respiratory bronchioles, resulting in destruction of the bronchiolar wall and the adjacent alveolar structures. In more advanced disease, cellularity may diminish considerably, and fibrotic changes predominate *(17)*.

The ultrastructural features of Langerhans' cells in these lesions are similar – although not identical – to those described for normal pulmonary Langerhans' cells. The lesional Langerhans' cells (formerly known as the Hx cell) have pale, eosinophilic cytoplasm and possess oblong or elongated nuclei with delicate folds and clefts *(17)*. In areas where lymphocytic infiltrates are present, close contact between Langerhans' cells and lymphocytes occurs, an observation that led some to

speculate that active presentation of antigen by the Langerhans' cells to T cells occurs in these regions *(23)*. Definitive identification of Langerhans' cells is possible by the recognition of Birbeck granules (pentalaminar rod-shaped intracellular structures) that may be visualized by electron microscopy or by immunohistochemical staining for the CD1a antigen on the cell surface, a characteristic not shared with macrophages *(23, 24)*. Identification of Langerhans' cells in biopsy specimens using monoclonal antibody staining to the CD1a surface antigen is recommended for definitive diagnosis, although expert pathologists may be able to make a confident diagnosis on the basis of morphological appearance *(17)*. S-100 staining is also frequently employed to detect the presence of Langerhans' cells in tissue specimens, but false positives may be seen with certain macrophage populations that react with S-100. Other immunohistochemical markers used to identify Langerhans cells include Langerin and DC-SIGN. Although the identification of Langerhans' cells is required to enable a definitive diagnosis, the mere presence of these cells in pulmonary lesions is not equivalent to pulmonary LCH, since Langerhans' cells may be identified in a variety of other lung pathologies, including lung cancer and certain interstitial lung diseases like idiopathic pulmonary fibrosis *(25, 26)*. Conversely, advanced pulmonary LCH nodules may be relativity pauci-cellular, and the presence of infiltrating Langerhans' cells may not be as striking as in early inflammatory lesions *(17)*.

The cystic lesions that form are not a direct consequence of necrosis of the nodular lesions *(18)*. Cystic lesions form as the peribronchial lesions destroy the cellular and connective tissue components of the bronchiolar walls, resulting in progressive dilatation of the lumina of small airways which are eventually surrounded by fibrous tissue *(17, 18)*. This sequence of events leads to the formation of bizarre-shaped, irregular parenchymal cystic lesions. In addition, as seen in other fibrotic disorders, traction emphysema of alveoli adjacent to the stellate scars and peribronchial fibrotic rings are commonly observed.

The histologic findings in pulmonary LCH may sometimes be confused with another smoking-related interstitial lung disease, desquamative interstitial pneumonia (DIP) *(27, 28)*. Cigarette smoking itself causes an increase in pulmonary macrophages, both in small airways and in the alveolar and interstitial spaces. In some patients with pulmonary LCH, accumulation of alveolar macrophages in the alveolar spaces may be quite striking, causing a DIP-like reaction to occur *(17, 19)*. In some instances, the extent of DIP changes may be so extensive as to overshadow the diagnostic lesion of pulmonary LCH *(19)*. Sampling error may create difficulty in the histologic diagnosis of pulmonary LCH. Sampling error is a significant problem with transbronchial biopsies due to the focal nature of the lesions, and even surgical lung biopsy specimens need to be carefully evaluated since lesions may not be found in every block.

Pathogenesis

Langerhans' cells – CD1a and Birbeck granule expressing epithelial-associated dendritic cells found in the airways, gut mucosa, and skin – play a central role in the pathogenesis of pulmonary LCH *(29, 30)*. Like dendritic cells, Langerhans' cells are potent antigen-presenting cells that regulate innate and acquired immune responses at mucosal surfaces *(31)*. Although Langerhans' cells are primary antigen-presenting cells in the airway mucosa, there are at least two other types of dendritic cells in the lungs: the plasmacytoid dendritic cell and CD1a-negative myeloid dendritic cells *(32)*. The

role of these other dendritic cell subtypes in the pathogenesis of LCH is unknown, and it is the Langerhans' cell that is considered the primary orchestrator of LCH pathobiology.

In the normal lung, Langerhans' cells serve a primary line of defense surveying antigens that reach the lower airways. Both in the lung and in the gut, mucosal Langerhans' cells send out cellular projections in between epithelial cells (similar to periscopes), enabling them to constantly sample the epithelial lining fluid and survey the landscape for any "danger" signals *(33, 34)*. Following exposure to antigens, toll receptor agonists, activation of the CD40 receptor, and a variety of other mechanisms, dendritic cells and Langerhans' cells undergo a process of maturation, which is associated with loss of the capacity to process new antigen, migration to regional lymphoid tissues, and upregulation of cell surface receptors (such as members of the B7 co-stimulatory family) that facilitate co-stimulation of T cells and B cells in secondary lymphoid structures *(35)*. Pathogens or exogenous antigens that breach the airway epithelial barrier will encounter and be sampled by the web-like network of projects that render the Langerhans cells their characteristic appearance (the term dendritic cell was originally used because these cells were originally presumed to be neuronal in origin due to their multiple cellular projections that resemble dendrites). In view of the constant exposure of the airway to inhaled antigens, Langerhans' cells play a critical role in orchestrating the immune response to a vast array of different antigenic challenges. The details of how Langerhans' cells co-ordinate the delicate balance that results in the induction of immunity to "danger" signals expressed by infectious pathogens, while inducing tolerance to "innocuous" antigens ubiquitous in the environment, are not fully appreciated. The influence of epithelial cells and resident macrophages on Langerhans or other pulmonary dendritic cell functions is also not entirely clear. It is, however, apparent that cytokines, chemokines, and other factors produced by both epithelial cells and macrophages can profoundly influence a variety of Langerhans and dendritic cell functions.

Cigarette smoke is the most important epidemiologic factor associated with the development of pulmonary LCH. Despite a clear epidemiologic association between smoking and pulmonary LCH, specific mechanisms by which smoking contributes to LCH pathogenesis are only partially understood. Cigarette smoking itself is a sufficient stimulus for recruitment of Langerhans' cells in the lung, as indicated by bronchoalveolar lavage studies conducted on smokers without overt lung disease *(36)*. This observation has now been reproduced in murine models of chronic cigarette smoke exposure, which demonstrate that even 6 weeks of exposure to high levels of tobacco smoke is sufficient to cause expansion of the resident lung dendritic cell pool *(37)*. At least in one study, mice chronically exposed to cigarette smoke developed loosely formed granulomatous lesions reminiscent of pulmonary LCH *(38)*. Unfortunately, the latter observation has never been reproduced, and to date there is no animal model of pulmonary LCH.

Accumulation of Langerhans' cells in a sub-epithelial distribution is the most likely early lesion of pulmonary LCH (Figure 17.1) *(39)*. Cigarette smoking may promote this through a number of potential mechanisms. In vitro studies demonstrate that whole cigarette smoke extracts, as well as individual constituents present in cigarette smoke, induce tumor necrosis factor-alpha (TNFα), granulocyte macrophage colony-stimulating factor (GM-CSF), and transforming growth factor-beta (TGFβ) from airway epithelial cells *(40, 41)*. Local generation and accumulation of these cytokines

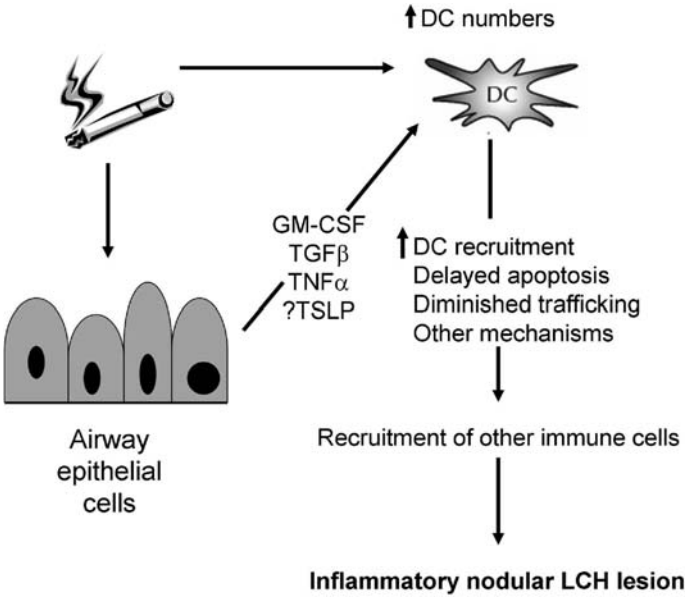

Figure 17.1 Simplified pathogenesis of early pulmonary LCH. Accumulation of Langerhans' cells in a sub-epithelial distribution is the most likely early lesion of pulmonary LCH. Cigarette smoking may promote this through a number of potential mechanisms, such as the induction of the cytokines TNFα, GM-CSF, TGFβ, and potentially others (such as thymic stromal lymphoprotein or TSLP) from airway epithelial cells. Local generation and accumulation of these cytokines in small airways may lead to sustained recruitment of Langerhans' cells precursors from the circulation, promote in situ differentiation of myeloid cells into functional Langerhans' cells, and possibly even enhance their survival and local retention. In addition to epithelial cells, alveolar macrophages, fibroblasts, and other cell types may provide substantial amounts of cytokines involved in sustaining Langerhans' cell expansion in the small airways of patients with pulmonary LCH. Sustained accumulation and persistence of activated Langerhans cells may subsequently lead to recruitment of other inflammatory cells such as monocytes, plasma cells, T lymphocytes, and eosinophils which further perpetuate the inflammatory milieu

in small airways may lead to recruitment of Langerhans' cells' precursors from the circulation, promote in situ differentiation of myeloid cells into functional Langerhans' cells, and possibly even enhance their survival and local retention *(30, 39, 42)*. These putative mechanisms of Langerhans cell accumulation in sub-epithelial regions are supported by immunohistochemical techniques that identify abundant GM-CSF protein expression in the epithelium of bronchioles affected by the inflammatory lesions of pulmonary LCH and co-localizes with the presence of numerous CD1a+ Langerhans' cells in proximity *(39)*. A pathogenic role for GM-CSF is further suggested by the observation that the expression of GM-CSF is substantially lower in bronchioles not affected by disease *(39)*. In parallel, certain cell types in pulmonary LCH lesions also demonstrate abundant expression of TGFβ, a cytokine critical for functional development of Langerhans' cells *(43, 44)*. In addition to epithelial cells, alveolar macrophages, fibroblasts, and other cell types may provide substantial amounts of cytokines involved in sustaining Langerhans' cell expansion in the small airways of patients with pulmonary LCH.

Cigarette smoke is a complex mixture of different chemicals, many of which have potential immune-modifying properties *(45, 46)*. A number of cigarette smoke constituents have been implicated in the pathogenesis of pulmonary LCH. Tobacco glycoprotein, a phenol-rich glycoprotein present in tobacco leaves, actives T lymphocytes, induces cytokine release from epithelial cells, and stimulates polyclonal B cell proliferation *(47, 48)*. Lymphocytes obtained from normal donors proliferate and produce interleukin-2 [IL-2] in the presence of tobacco glycoprotein. This contrasts with pulmonary LCH patients, in whom the response of peripheral lymphocytes to tobacco glycoprotein is with decreased proliferation and cytokine release (especially interleukin-2) *(49)*. Although Langerhans' cells are known to express the IL-2 receptor *(50)*, the precise functional consequence of IL-2 stimulation on Langerhans' cell function is not clear.

Although the link between smoking and pulmonary LCH appears strong, the disease occurs in a very small percentage of smokers, indicating that in addition to exogenous factors, there must be some inherent host genetic (or additional environmental) factors that lead to pulmonary LCH. However, extensive analyses of LCH tissue and patient specimens looking for evidence implicating viruses or genetic factors have failed to consistently identify any candidate genes or pathogenic viruses *(51, 52)*.

Pulmonary LCH – a Neoplastic or Reactive Process?

A key feature of a neoplasm is its clonal derivation from a single cell. Using X chromosome-linked DNA probes that detect clonal or polyclonal X chromosome inactivation patterns in female tissues, Willman and colleagues identified clonal Langerhans' cells in lesional tissues in each of 16 females affected with LCH *(53)*. The subjects included pediatric and adult patients, but none had isolated pulmonary LCH. This seminal observation sparked considerable discussion regarding the natural biology of LCH *(53, 54)*. This discussion was further instigated by the observation that clonal expansion of CD1a-positive Langerhans' cells occurs only in a minority of cases of pulmonary LCH, suggesting reactive polyclonal expansion in the majority of pulmonary LCH lesions *(55)*. The demonstration of polyclonality suggests a reactive process secondary to some inciting stimulus, potentially cigarette smoking. This suggests that pulmonary LCH is a distinctive reactive subtype of LCH. It also provides evidence for the contention that mechanisms of disease and treatments utilized in systemic childhood LCH may not apply for adults with pulmonary LCH.

Clinical Features

About two-thirds of patients are symptomatic at presentation, while a third are minimally symptomatic, and come to clinical attention following discovery of incidental abnormalities on chest radiography *(4)*. Non-specific symptoms such as cough, dyspnea on exertion, and fatigue are the most common symptoms at diagnosis. Chest pain and acute dyspnea due to spontaneous pneumothorax occur in 10–20% of patients and may be recurrent *(56)*. Constitutional symptoms with fever, sweats, and weight loss occur in 15–20% *(4)*. Hemoptysis is very uncommon and its occurrence should raise suspicion of a bronchogenic carcinoma or development of a fungus ball in a cystic LCH cavity.

Around 10–15% of patients with pulmonary LCH have disease in organ systems other than the lung *(4)*. Organs that may be involved include skin, bones (particularly the skull and axial skeleton), lymph nodes, and the hypothalamic region *(4)*.

Physical examination of the pulmonary system is generally unremarkable or non-specific. Finger clubbing is unusual, and auscultation of the chest may be normal, or reveal evidence of airflow limitation in more severe or advanced cases. There are no diagnostically useful serum biochemical or hematological tests, and most individuals with pulmonary LCH have normal hematological indices (including circulating eosinophil counts) and serum angiotensin-converting enzyme levels. A proportion of patients will have a modest elevation of the sedimentation rate (unpublished observation). Hypercalcemia does not occur.

Radiographic Findings

The chest radiograph (CXR) is frequently abnormal. In early disease, bilateral, poorly demarcated nodular and reticulonodular infiltrates predominate (Figure 17.2), while more advanced disease is usually associated with a prominent cystic component *(57)*. Contiguous cystic cavities (up to 2 cm in size) may occur in advanced stages of pulmonary LCH resulting in a radiographic appearance that is very similar to advanced emphysema. The finding of cystic change on the CXR should always prompt the clinician to think of pulmonary LCH as a cause of the radiologic abnormality. Unusual radiographic findings on CXR include discrete pulmonary nodules *(58–60)* and pleural effusions. Although abnormal in most cases, the CXR abnormalities are non-specific and necessitate further evaluation.

High-resolution CT scan (HRCT) of the chest is a useful and sensitive tool that should be obtained in every patient suspected of having pulmonary LCH. HRCT

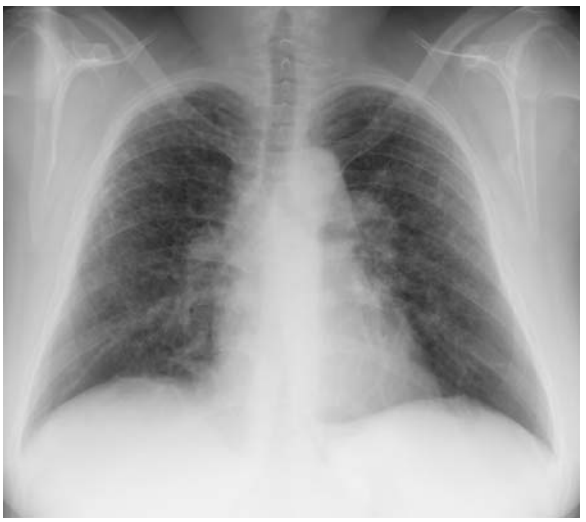

Figure 17.2 Chest X-ray findings in pulmonary LCH. Forty six-year-old smoker with biopsy-proven pulmonary LCH. Chest radiograph demonstrated diffuse bilateral nodular and reticular infiltrates with upper and middle lobe predominance

provides radiographic correlates of pathologic findings and provides information regarding the distribution of disease that should assist the surgeon in choosing an optimal site for lung biopsy. Several descriptive studies illustrate the utility of HRCT diagnostically for delineating the nodules and cysts that often have a characteristic appearance and distribution *(61–63)*. In the early stages of disease, nodules are very common. These generally range in size from a few millimeters to up to 2 cm in size and may show central cavitation *(61, 62)*. In more advanced disease, nodular changes become infrequent while lung cysts tend to predominate *(64)*. In a number of patients, the HRCT demonstrates both nodular and cystic changes, occurring predominantly in a middle and upper lobe distribution (Figure 17.3) with sparing of the lung bases. Although the combination of cystic and nodular changes is frequently described as the "classical" changes associated with pulmonary LCH, the most frequently encountered HCRT findings are lung cysts which are generally (but not always) less than 20 mm in size, have a thin (1 mm or less) wall, and are frequently bizarre in shape (unlike the more symmetric appearing cysts occurring in LAM) *(4, 63)*.

The pattern of abnormalities seen on the HRCT is very useful diagnostically, and in a substantial proportion of cases, the combination of cystic lesions and nodules with sparing of the lung bases results in a radiographic pattern that is so characteristic of pulmonary LCH that a presumptive diagnosis of pulmonary LCH may be established *(61)*. When the HRCT shows characteristic changes in an appropriate clinical setting, our practice is to avoid further invasive testing with lung biopsy unless a definitive diagnosis is required *(7)*.

In addition to lung cysts and nodules, other radiographic findings have been reported and are important to recognize since their presence may cause diagnostic confusion with other conditions. Ground-glass attenuation due to alveolar macrophage accumulation may occur and may be sufficiently extensive to render the radiographic picture

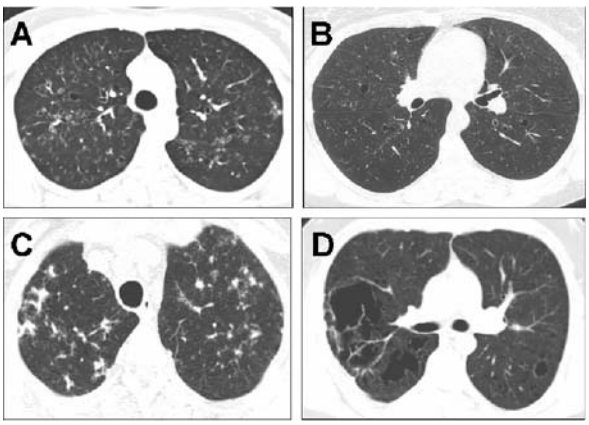

Figure 17.3 Chest CT findings in pulmonary LCH. (**a**) Chest CT demonstrating nodular and cystic lesions identified in both upper lobes in a smoker with biopsy-proven pulmonary LCH. (**b**) Chest HRCT demonstrating bilateral upper lobe thin-walled cystic lesions from a smoker with biopsy-proven pulmonary LCH. (**c**) Chest CT demonstrating bilateral upper lobe nodular lesions in a former smoker with biopsy-proven pulmonary LCH. (**d**) Chest CT showed performed in a former smoker with biopsy-proven pulmonary LCH demonstrating multiple thin-walled cystic lesions predominantly affecting the right middle lung field

similar to hypersensitivity pneumonitis *(19)*. Similarly, mild mediastinal or paratracheal adenopathy may be detected in a third and may present diagnostic confusion with sarcoidosis (unpublished observations).

Pulmonary Function Testing and Exercise Physiology

Unlike other interstitial lung diseases which are generally characterized by restriction, pulmonary LCH causes a variety of abnormalities in pulmonary function testing *(4, 8, 65)*. About 20% of patients have normal lung volumes, spirometry, and diffusing capacity at the time of diagnosis *(4)*. When abnormal, the most common lung function test abnormality is a reduction in diffusing capacity to carbon monoxide [DLCO], reported to occur in about two-thirds of patients *(4, 8)*. A reduction in the DLCO may be the sole abnormality at diagnosis or may accompany obstructive, restrictive, or mixed lung function abnormalities. Although in many of the interstitial lung diseases a reduction in DLCO generally signifies pathology in the interstitial compartment, abnormalities in the pulmonary vasculature of patients with pulmonary LCH are also an important factor *(20, 65)*.

The various patterns of lung function abnormalities are a manifestation of varying degrees of interstitial, airway, and vascular involvement that occurs in LCH. In early disease, restriction appears to be more prevalent, whereas obstruction is more common in advanced disease *(4)*. In a series of 23 patients with early disease, Crausman and colleagues reported either normal or restrictive physiology on lung function testing *(65)*. Similarly, in a study that included 102 adults with histologically confirmed pulmonary LCH, restriction was present in 37 out of 81 patients (46%) with pulmonary function testing documented at the time of diagnosis *(4)*. Others have reported a slight predominance of obstruction, potentially reflecting difference in the timing of lung function testing in those reports *(10)*.

As in other lung diseases, obstructive physiology is manifested by increased lung volumes (elevated total lung capacity – TLC), air trapping (elevated residual volume – RV and RV/TLC), and diminished forced expiratory volume in 1 s (FEV1) and is explained in part by the small airway disease and distal airspace enlargement, as well as co-existent emphysema which is common in advanced cases *(4, 19, 66)*. In some instances, patients will initially present with low lung volumes and subsequently develop normal or "pseudo-normalization" of the TLC as air trapping and hyperinflation develops. For this reason, the TLC alone should not be used to monitor response to therapy or render treatment decisions in pulmonary LCH.

Physiologic studies have identified important limitations in the exercise capacity. Crausman et al., studied lung mechanics and exercise physiology in 23 patients and found that exercise performance was severely limited in all patients, even those with relatively normal pulmonary function testing *(65)*. In that study, measurements of pulmonary vascular function such as the DLCO, baseline VD/VT, and exercise VD/VT correlated with overall exercise performance, implying that exercise impairment is a consequence of pulmonary vascular dysfunction, rather than ventilatory limitation, at least in early stages of disease. In patients with more advanced disease – which is often accompanied by development of cystic abnormalities – exercise limitation is likely to reflect a combination of pulmonary vascular dysfunction as well as ventilatory limitation *(65)*.

Diagnostic Evaluation of Individuals with Suspected Pulmonary LCH

The diagnosis of pulmonary LCH should be suspected in any smoker with diffuse lung infiltrates, particularly if nodular or cystic lesions are noted on radiography. From the initial clinical encounter, a number of clinical scenarios should alert the clinician to the possibility of pulmonary LCH: a history of a spontaneous pneumothorax, the presence of diffuse bilateral lung infiltrates in a smoker, the presence of cysts on the chest radiograph, bilateral lung infiltrates with evidence of obstruction on pulmonary function testing, a history of diabetes insipidus or skin rash in a patient with lung infiltrates, or "emphysema" occurring in a young adult.

All patients suspected of having pulmonary LCH should undergo HRCT of the chest. The HRCT may reveal imaging findings that are highly characteristic. Bronchoscopy with transbronchoscopic lung biopsy (TBLB) has a low diagnostic yield *(8, 28, 67)*. However, TBLB may provide alternative diagnosis such as sarcoidosis, hypersensitivity pneumonitis, fungal infection, or LAM. Bronchoscopy also provides an opportunity for sampling the cellular constituents of the distal airspaces with bronchoalveolar lavage (BAL). Non-specific changes – such as increased macrophage numbers indicative of cigarette smoking – are commonly found on BAL analysis of pulmonary LCH patients. A reduction in the CD4/CD8 ratio of T cells and an increase in eosinophil counts have also been reported, but these findings are inconsistent *(11)*. A more specific finding is the detection of abundant CD1a-positive Langerhans' cells *(68–72)*. If the percentage of CD1a-positive cells in the BAL is greater than 5, pulmonary LCH is highly likely and in the appropriate clinical scenario is sufficient to enable confident provisional diagnosis *(68)*. In many patients with histologically proven pulmonary LCH, an indeterminate elevation (2–5%) in CD1a-positive cells is found, which makes this test relatively insensitive when a 5% increase is taken a cut-off *(68)*. Surgical lung biopsy may be necessary if the HRCT scan or BAL/TBLB are non-diagnostic or if there is a need to establish a definitive diagnosis (for example, in the setting of transplant evaluation). In the patient with documented Langerhans' cell histiocytosis outside the lung (such as skin or bone), the diagnosis is usually established if HRCT shows features consistent with pulmonary LCH.

Management

To date, there has been no single therapeutic intervention shown prospectively to be effective in reducing mortality in this disease. Considering the strong association with cigarette smoking, it is recommended that smoking cessation be the first and central component of the management strategy for all patients. Abstinence from smoking leads to stabilization of symptoms in many patients and in a substantial proportion represents the only intervention required for improvement or stabilization *(13, 73)*. Not all individuals with pulmonary LCH improve or stabilize following smoking cessation, and some develop progressive disease in spite of smoking cessation. Effective pharmacologic therapies are urgently needed for this subgroup of patients with progressive disease.

Corticosteroids have been the primary pharmacological agent used in the management of pulmonary LCH. As in many other interstitial lung diseases, the use of corticosteroids is not supported by prospective or randomized studies. Corticosteroid use has been associated with improvement in symptoms in some retrospective studies and case reports *(10, 74–76)*. Unfortunately, the efficacy of corticosteroids reported in those studies is difficult to define due to the confounding effect of smoking cessation, which may in itself lead to symptomatic improvement. Due to the lack of prospective or controlled trials, clinicians are often unsure who should receive treatment with corticosteroids. A practical approach – based on the available evidence – is to prescribe a trial of corticosteroids only to patients who have objective evidence of progressive disease (as judged by pulmonary function testing). Since the FEV1 and DLCO are predictors of poor outcome *(4, 66)*, it is reasonable to use serial measurement of these parameters to detect patients at risk of developing progressive disease. Whether corticosteroids should be employed in active smokers with progressive decline in FEV1 or DLCO is a subject of controversy, but the inability to quit smoking should not be the sole reason for denying treatment. The doses of corticosteroids and the duration of treatment prescribed vary widely. This author's approach is to prescribe 0.5 mg/kg/day of prednisone to selected patients in whom progressive disease is present (defined by >15% longitudinal decline in either FEV1 or DLCO) and re-evaluate with full pulmonary function testing in 3 months. In the absence of an objective response, corticosteroids should be rapidly tapered.

A variety of chemotherapeutic agents including vinblastine, methotrexate, cyclophosphamide, etoposide, and chlorodeoxyadenosine have been employed empirically in patients with progressive disease unresponsive to corticosteroids or in which multisystem involvement was a predominant feature *(77–80)*. Due to limited data on their efficacy, these drugs should be reserved for patients with progressive disease. One agent that deserves more study is chlorodeoxyadenosine which has been demonstrated in a number of case reports and small series to be effective in inducing disease remission in both multisystem and occasional isolated pulmonary LCH *(78, 81–83)*. However, the long-term toxicity of chlorodeoxyadenosine in young patients with pulmonary LCH is not very well defined, and treatment trials with this agent should only be undertaken by centers with experience in both LCH and use of chlorodeoxyadenosine.

Pneumothoraces should be managed in a standard fashion, although pleurectomy is generally avoided in patients for whom lung transplantation is an option. Similarly, the development of cor pulmonale is managed in a standard fashion with diuretics. Pulmonary hypertension is an important complication and is under-recognized in many patients with this disease. It is this author's practice to screen all patients at the time of diagnosis and during follow-up for pulmonary hypertension, as many patients derive substantial benefit from vasodilator therapy with bosentan (an endothelin-1 receptor antagonist) or sildenafil (a phosphodiesterase inhibitor) and experience objective improvement in right heart mechanics as measured by 2-D echocardiography and functional measures of exercise tolerance such as the 6-minute walk test (unpublished observations). Although there is no ideal clinical screening test for pulmonary hypertension, echocardiography is a useful and non-invasive test that should be performed at the time of diagnosis and upon follow-up particularly in patients with dyspnea that seems out of proportion to measured pulmonary function, or patients with progressive decline in DLCO. In those patients with signs of possible pulmonary hypertension (evidence of diminished right heart systolic function or elevated right ventricular systolic pressure

>45 mmHg), it is prudent to consider cardiac catheterization with the goal of confirming the presence and defining the severity of pulmonary hypertension and objectively determining the hemodynamic response to a vasodilator trial *(21)*. In addition to appropriate trials of vasodilator therapy, patients with moderate to severe pulmonary hypertension may benefit from anticoagulation and supplemental oxygen.

Lung transplantation is an option for patients with severe respiratory impairment and should be considered in the patient with rapidly declining lung function or if there is severe limitation due to symptoms unresponsive to smoking cessation or a trial of immunosuppressive therapy *(84–87)*. It is imperative that patients stop smoking prior to lung transplantation, as the disease may recur in the transplanted lung if smoking is resumed *(88)*. There is also a report of recurrence in the transplanted lung, in spite of presumed abstinence from tobacco use *(80, 89)*.

Outcomes and Prognosis

Overall the prognosis of most patients with pulmonary LCH is relatively good, particularly if smoking cessation is achieved early in the course of disease before the development of significant lung function impairment. Patients who are asymptomatic at presentation seem to have the best long-term prognosis. Some retrospective studies imply that the majority of patients experience minimal progression over time *(10)*. Other studies suggest that the outcomes of adults with pulmonary LCH may be worse than previously suspected and demonstrate that a substantial proportion die prematurely from respiratory failure *(4)*. The role of smoking cessation or immunosuppressive therapy on the course of disease or outcomes has never been clearly defined.

A variety of factors have been associated with adverse clinical outcome including extremes of age, multisystem involvement, prolonged constitutional disturbance, extensive cysts and honeycombing on CXR, markedly reduced diffusing capacity, low FEV_1/FVC ratio, corticosteroid therapy at time of follow-up, and a high RV/TLC ratio *(4, 66)*. As alluded to previously, a proportion of patients develop progressive disease with respiratory failure. It is very difficult to determine how many patients with pulmonary LCH progress to respiratory failure since a proportion of these patients have associated emphysema due to long-standing tobacco abuse.

The effect of pregnancy on lung function of women with pulmonary LCH has never been reported. Anecdotally, complete remission of cutaneous and lymph node involvement by LCH during pregnancy has been reported *(90)*. Expert opinion seems to indicate that in the absence of significant respiratory impairment, pulmonary LCH is not a contraindication to pregnancy *(91)*.

Several case reports and series describe a variety of neoplasms in association with adult and childhood LCH, including lymphoma, multiple myeloma, myelodysplastic syndrome, adenocarcinoma of the lung, and a variety of solid organ cancers *(27, 92–101)*. The increased prevalence of malignant neoplasms in patients with LCH may be reflective of the heavy cigarette smoking that some of these patients are exposed to, use of chemotherapeutic agents to treat LCH, and inherent chromosomal or genetic abnormalities. Adults with pulmonary LCH have a substantially increased prevalence of malignant hematological cancers, especially myeloproliferative disorders *(4)*. These cancers may either predate or occur after the diagnosis of LCH. It is important for clinicians to recognize this association due to implications in follow-up and counseling of these patients.

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18

Sarcoidosis

Ralph J. Panos and Andrew P. Fontenot

Abstract Sarcoidosis is a chronic multisystemic disorder of unknown cause that is characterized histopathologically by noncaseating granulomas and aggregation of T lymphocytes. Although sarcoidosis may affect any organ system including skin, kidneys, heart, brain and nerves, eyes, and endocrine tissues, the lungs are most frequently involved. Approximately one-third of patients will experience extrapulmonary manifestations during the course of their disease. Despite significant insight into the cellular interactions and cytokines involved in the pathogenesis of sarcoidosis, the primary inciting event(s) remains elusive. Experiments of nature and biological therapies including the near-complete absence of sarcoid in individuals with HIV infection and reduced T helper cells and the profound increase in sarcoid prevalence during the immune reconstitution syndrome or during interferon therapy for hepatitis, and amelioration of disease by inhibitors of TNF-α confirm an essential role for various cells and cytokines in the development of sarcoid. The predominance of a limited number of Vβ-expressing T lymphocyte subsets in the lungs of certain sarcoidosis subjects suggests that the dominant immune response may be directed against only a few antigens or epitopes. Clinical observations suggest a hereditary predilection to the development of sarcoid and it is likely that multiple genes, rather than a single gene, comprise the genetic predisposition to disease.

Keywords: sarcoid, sarcoidosis, granuloma, lymphocyte, T-cell receptor

Introduction

Sarcoidosis is a chronic multisystemic disorder of unknown cause that is characterized histopathologically by noncaseating granulomas and aggregation of T lymphocytes. Although significant insight has been gained in the cellular interactions and cytokines involved in the pathogenesis of sarcoidosis, the primary inciting event(s) remains elusive. In this chapter we will review the clinical pulmonary manifestations of sarcoidosis, describe recently reported experiments of nature as well as unforeseen consequences

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of recently introduced therapeutic strategies that provide in vivo biological confirmation of the cells and cytokines that have been implicated experimentally in vitro in the pathogenic mechanisms causing sarcoidosis. The second part of this chapter will summarize recent basic science discoveries in the immunologic and cellular mechanisms causing sarcoidosis.

Epidemiology

The prevalence of sarcoidosis is generally estimated to be approximately 1–40 cases per 100,000 population *(1–3)*. Although sarcoidosis may affect any individual, racial, geographic, temporal, occupational, and familial clustering have been described *(4–7)*. Typically, the diagnosis of sarcoidosis is made in adults between 20 and 50 years of age *(3, 4)*. In Europe, sarcoidosis is slightly more prevalent in northern compared with southern countries. In the United States, sarcoidosis is more common in African-Americans than in whites with an estimated lifetime risk of 2.5% compared with 0.85%, respectively *(8, 9)*.

Clinical Manifestations

Sarcoidosis is a multisystemic disorder with protean manifestations. Sarcoid may affect any organ system including skin, renal, cardiovascular, neurologic, ocular, and endocrine. The lung is involved in approximately 90% of cases. Approximately one-third of patients will experience extrapulmonary manifestations during the course of their disease. This chapter will be limited strictly to the pulmonary manifestations of sarcoidosis.

Respiratory symptoms are the most frequent presenting complaints and include cough, dyspnea, and chest discomfort. Although not as well recognized, wheezing is a frequent respiratory symptom of individuals with sarcoid and may mimic asthma and chronic bronchitis *(10)*. Nonspecific constitutional symptoms such as fatigue, weight loss, weakness, and fever may occur alone or accompany the respiratory complaints. Löfgren's syndrome, fever, erythema nodosum, bilateral hilar adenopathy, and polyarthralgias, occurs frequently in the initial presentation of sarcoidosis *(4)*. In up to half of sarcoid cases, the disorder presents asymptomatically and is detected by incidental findings on chest imaging studies *(9)*.

Imaging

The chest X-ray is abnormal in over 90% of patients with sarcoidosis. Approximately 5–10% of patients will have normal chest X-rays at presentation. The chest radiographic abnormalities of sarcoidosis are classified into five stages: 0 – no radiographic abnormalities; I – bilateral hilar and/or mediastinal adenopathy with no pulmonary parenchymal abnormalities; II – hilar and/or mediastinal adenopathy with pulmonary parenchymal abnormalities; III – diffuse parenchymal disease with no lymph node enlargement; IV – end-stage pulmonary fibrosis with honeycomb changes. Approximately 50% of patients present with stage I radiographic abnormalities. Between 20

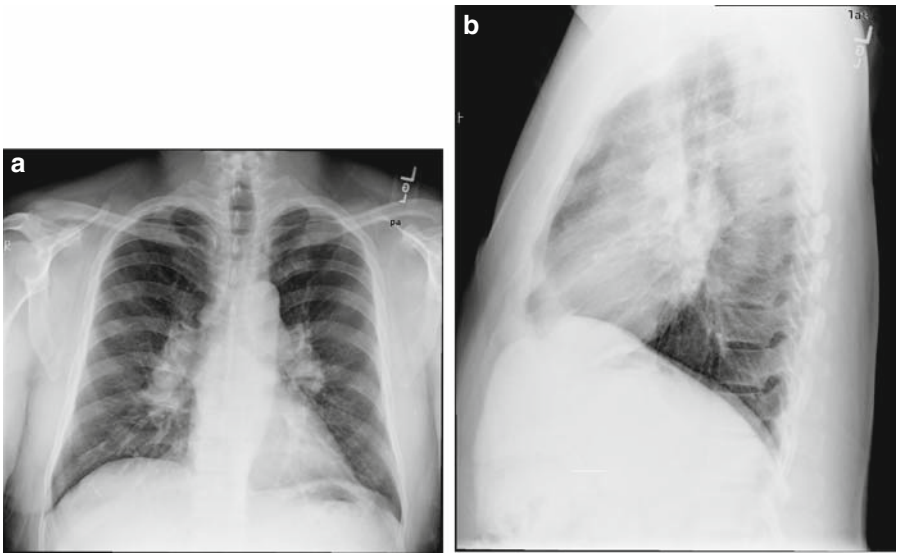

Figure 18.1 (**a** and **b**) Posterior–anterior and lateral chest X-rays demonstrating enlarged hilar and mediastinal adenopathy with normal appearing lung parenchyma. Transbronchial lung biopsies performed during bronchoscopy demonstrated noncaseating granulomas. The clinical, radiographic, and histopathologic data supported a diagnosis of stage I sarcoid

and 30% of patients present with stage II radiographic changes and 10–20% of patients initially have stage III abnormalities. Endobronchial involvement that is radiographically occult occurs in approximately 40% of stage I patients and 70% in stages II and III (Figure 18.1).

Chest CT imaging frequently shows bronchovascular bundles with widened intralobular septae and reticulonodular abnormalities accompanied by hilar and mediastinal adenopathy. Nodules occur within the interlobular septae, along the major fissures, and may cause irregularities in the bronchial walls. The reticulonodular opacifications are often in a mid- to upper lung zone predominant pattern. There are often patchy areas of ground glass opacification superimposed upon the reticulonodular pattern (Figure 18.2).

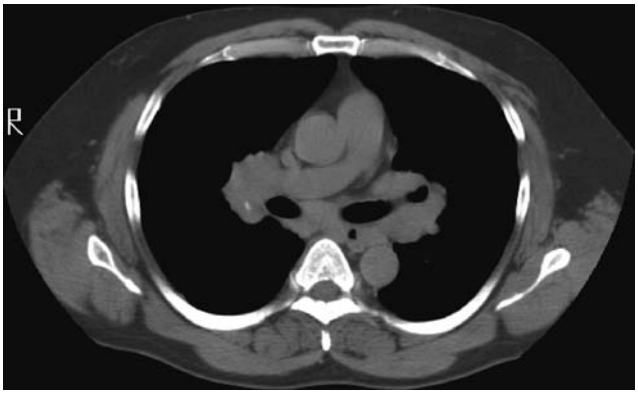

Figure 18.2 Chest CT scan corresponding to the chest X-rays presented in Figure 18.1 demonstrating hilar, mediastinal, and subcarinal adenopathy

Laboratory Abnormalities

Various non-specific laboratory abnormalities occur in patients with sarcoidosis including elevation of the serum angiotensin-converting enzyme (ACE) level and erythrocyte sedimentation rate. Other laboratory findings include anemia, lymphopenia, and hypergammaglobulinemia. Elevated ACE levels are neither sensitive nor specific for the diagnosis of sarcoidosis and the utility of ACE levels as a marker of disease activity remains controversial *(11–13)*.

Pulmonary Function Studies

Diminution in the diffusing capacity for carbon monoxide (DLCO), lung volumes, and forced vital capacity (FVC) are commonly found in patients with sarcoidosis. Airflow limitation may occur with endobronchial involvement *(10)*. Arterial blood gasses may demonstrate hypoxemia at rest or with exertion and occasionally hypocapnia due to hyperventilation. Pulmonary function studies are relatively insensitive and non-specific in the diagnosis of sarcoidosis. However, along with clinical symptoms and serial imaging studies, they provide a quantitative assessment of disease activity and clinical course in individual patients.

Diagnosis of Pulmonary Sarcoidosis

The diagnosis of pulmonary sarcoidosis is usually suggested by the clinical presentation, physiological findings on pulmonary function testing, and radiographic imaging. In a review of 189 patients enrolled in the Case Control Etiology of Sarcoidosis Study (ACCESS), Judson and colleagues *(14)* found that the diagnosis of sarcoidosis was established on the first physician visit in only 15.3% of cases. Patients with pulmonary symptoms had significantly more physician visits until the diagnosis was made than did individuals who did not have pulmonary symptoms, 4.84 + 0.38 (mean + SE) visits vs. 3.15 + 0.24 visits, respectively. Whereas, the presence of pulmonary symptoms was associated with a prolonged time to diagnosis, cutaneous findings led to a more rapid diagnosis. Interestingly, patients with more severe radiographic findings, stage III or IV disease, had a greater time to diagnosis than did those with stage 0, I, or II disease. In this study, a tissue biopsy was required for the diagnosis of sarcoid. However, the need for the histopathological demonstration of noncaseating granulomas in the diagnosis of sarcoid remains controversial, especially in asymptomatic individuals with bilateral hilar adenopathy or erythema nodosum *(15–17)*.

The usual method of lung biopsy is transbronchial lung biopsy (TBB) performed during bronchoscopy. The yield of bronchoscopic lung biopsy varies from 40 to 90% depending upon the number of biopsies and the degree of parenchymal involvement at the time of biopsy *(18–21)*. Although a bronchoalveolar lavage (BAL) demonstrating >12% lymphocytosis with a CD4:CD8 ratio >3.5 is specific for a diagnosis of sarcoidosis, this technique lacks sensitivity *(18, 22)*. Transbronchial needle aspiration (TBNA) and most recently endobronchial ultrasound-guided needle aspiration are useful in the diagnosis of sarcoid presenting with hilar or mediastinal adenopathy and minimal or no parenchymal involvement. TBNA is diagnostic in 40–80% of patients with sarcoid *(23–25)*. Combining endoscopic TBB, BAL, and TBNA may increase the diagnostic sensitivity to 100% *(18)*.

Pathology

The gross appearance of the lung in sarcoid is dependent upon the level of pulmonary involvement. The lung may appear grossly normal in minimally affected cases, whereas honeycombing and severe parenchymal derangement are present in stage IV sarcoid. In other cases, masses of granulomas ranging from barely visible to several centimeters in diameter are distributed along lymphatic channels within the pleura, septae, and bronchovascular bundles. Histopathologically, the characteristic finding in sarcoid is the noncaseating granuloma, tight, well-formed, nodal aggregates of epithelioid cells with no or very minimal evidence of fibrinoid necrosis. Multinucleated giant cells may be present and are known as asteroid bodies when they assume a star-like configuration. Although there may be occasional surrounding lymphocytes, there is usually no significant inflammation around the granuloma.

Although strongly suggestive of sarcoid, the histopathological presence of noncaseating granulomas does not establish the diagnosis because granulomas are a nonspecific finding and may be present in other lung diseases. Special stains as well as cultures for mycobacterial and fungal organisms should be performed. Hypersensitivity pneumonitis is also associated with granulomas that tend to be looser, less well formed, and scattered throughout the lung parenchyma. Also, the granulomas in hypersensitivity pneumonitis tend to be associated with more inflammation, especially within the bronchioles. The granulomas of berylliosis are indistinguishable from sarcoid and these two disorders must be differentiated based upon the clinical history as well as other immunologic tests.

Natural History of Pulmonary Sarcoidosis

In the vast majority of patients, pulmonary sarcoidosis has a very benign course and less than 10% develop significant fibrosis. Spontaneous remission occurs in approximately 60–80% of patients with stage I disease, 50–60% of those with stage II disease, and <30% of those with stage III disease *(26)*. The presence of Löfgren's syndrome portends a better prognosis. In the British Thoracic Society Sarcoidosis Study, 41 of 58 patients with parenchymal pulmonary findings (stage II, III, or IV disease) who remained stable during the initial 6-month observation period developed normal appearing chest X-rays with no treatment during the subsequent nearly 5 years of the study *(27)*. In contrast, only 15 of 58 similar patients who were treated with either long-term or selective steroid regimens regained normal radiographic appearances *(27)*. Thus, the natural history of pulmonary sarcoidosis is very variable and there are no known predictors of clinical course or definitive methods to assess disease activity besides clinical symptoms, physiological measurements, and radiographic findings.

Treatment

Corticosteroids are the mainstay of therapy for sarcoid. Glucocorticoids suppress inflammatory genes, such as IL-1 and TNF-α, adhesion molecules, and receptors and induce expression of anti-inflammatory genes *(28)*. The imbalance between type 1 and type 2 T helper cell cytokine production in sarcoid is rectified by corticosteroids *(29)*. Despite decades of use, no study has demonstrated that steroids improve

mortality in sarcoidosis. A Cochrane analysis *(30)* of 12 randomized controlled trials of steroids in sarcoidosis involving 1051 patients concluded that oral steroids improved the chest radiographs and global score of chest X-ray, symptoms, and spirometry over 3–24 months but there was no evidence for improvement in lung function. Patients with stage II or III disease and moderate-to-severe or progressive symptoms or radiographic changes were most likely to receive benefit from oral steroids. There was no conclusive evidence to recommend treatment beyond 2 years and there was little evidence for the efficacy of inhaled steroids. Although no data on potential side effects of steroids were available from the studies selected in the Cochrane analysis, a large meta-analysis by Reich *(31)* demonstrated that the mortality rate for sarcoidosis was 4.8% in referral settings and 0.5% in population-based settings and that referral patients were seven-fold more likely to receive steroids. After normalizing for disease stage to account for possible adverse selection, it was suggested that the increased use of corticosteroids might adversely affect outcome at the referral centers.

Methotrexate, chloroquine, cyclosporin A, and pentoxifylline have been used in the treatment of sarcoid but a Cochrane analysis concluded that there is only limited evidence supporting their use *(32)*. These agents have significant side effects. Methotrexate and pentoxifylline were associated with a mild steroid sparing effect.

Lessons from Experiments of Nature and Unforeseen Consequences of Recently Introduced Therapeutic Strategies

Prevalence of Sarcoid in HIV Infection: Decrease with Active Infection and Increase with Effective Treatment

HIV infection devastates the cellular immune system by affecting both CD4 and CD8 lymphocyte responses. Both circulating and tissue-specific CD4 lymphocytes are severely depleted during untreated HIV infections. The CD4 cell is hypothesized to be an essential component of the granulomatous response. These cells preferentially accumulate in areas of granulomatous inflammation and display a TH1 phenotype with elevated spontaneous production of interleukin-2 and interferon γ. HIV infection, thus, provides an experiment of nature to test the role of the CD4 lymphocyte in the pathogenesis of sarcoid.

Sarcoid is believed to occur very rarely in untreated HIV-infected individuals *(19, 33)*. Table 18.1 presents 16 individuals with untreated HIV infection and newly diagnosed sarcoid reported in the medical literature. Respiratory complaints or chest imaging abnormalities are the most common presentations. Chest radiographs reveal lymphadenopathy, chronic lung nodules, or reticular opacifications. CT scans demonstrate lymphadenopathy, chronic lung nodules, thickening of interlobular septa, as well as reticular and ground glass opacifications. The radiographic features of newly diagnosed sarcoidosis in HIV-infected individuals are very similar to the imaging findings of sarcoidosis in non-HIV-infected individuals. The CD4 count was greater than 200 in 75% of these patients. Thus, it appears that depletion of CD4 cells reduces the prevalence of sarcoid whereas the preservation of a CD4 count greater than 200 is required for the subsequent development of sarcoid.

The immune reconstitution inflammatory syndrome is an exuberant inflammatory reaction to both infectious and non-infectious antigens that develops after the initiation

Table 18.1 Reports of newly diagnosed sarcoid in individuals with untreated HIV infection.

	N	CD4 count
Morris et al. *(48)*	2	210 410
Haramate et al. *(49)*	6	250 253 390 25 194 60
Granieri et al. *(50)*	1	110
Amin et al. *(51)*	2[a]	900
Newman et al. *(52)*	1	310
Gauder et al. *(53)*	1	570
Lowry et al. *(54)*	1	388
Coots and Lazarus *(55)*	1	384
Ingram et al. *(56)*	1	329
Means ± SD	15	319 ± 217

[a]CD4 count only reported in one patient.
Adapted from Morris et al. *(48)*.

of highly active anti-retroviral therapy (HAART). The syndrome has also been called the immune reconstitution phenomenon, immune reconstitution syndrome, and immune restoration disease. Pathogenic mechanisms of the immune reconstitution inflammatory syndrome are unknown but appear to involve alterations in immune responses restored by successful anti-retroviral therapy. Restoration of numbers of CD4+ cells, CD8+ cells, the ratio of CD4+ to CD8+ cells, and their respective cytokine production as well as chemokine receptor expression have all been implicated in the pathogenesis of the immune reconstitution inflammatory syndrome. The incidence of the syndrome varies between 10 and 40% and risk factors for its development include a CD4+ cell count less than 200, end-stage AIDS, opportunistic infections, and a rapid response to HAART. The clinical manifestations of the immune reconstitution inflammatory syndrome vary depending upon the extent and the vigor of the inflammatory reaction against either a microbiologic pathogen or other antigens. The syndrome has been described with both typical and atypical mycobacterial infections, fungal infections caused by cryptococcus, histoplasmosis and aspergillosis, viral infections including herpes viruses, hepatitis B and C viruses, parasitic infections including toxoplasmosis, and malignancies. Autoimmune diseases have also been observed with this syndrome, including systemic lupus erythematosus, rheumatoid arthritis, and polymyositis. In addition, an increased incidence of sarcoidosis has been noted with the immune reconstitution inflammatory syndrome.

In patients developing new onset sarcoidosis after initiating HAART treatment for HIV infection, the lung appears to be the most common organ involved with granulomatous inflammation. Cutaneous, hepatic, renal, and gastrointestinal involvements have also been described. In a study from metropolitan Paris from 1996 to 2000, Foulon and colleagues *(34)* evaluated the epidemiology and clinical presentation of sarcoidosis in HIV-infected individuals. They estimated the incidence of HIV-associated sarcoidosis at 3.20–7.24 cases per 1,000 sarcoidosis-patient-years. The interval between HIV diagnosis and sarcoidosis diagnosis was 92 ± 46 months and the duration of HAART was 29 ± 16 months. The clinical, radiographic, and histopathologic features of HIV-associated sarcoid were indistinguishable from non-HIV-associated sarcoid. Bronchoalveolar lavage revealed a total cell count of 371 ± 215 cells per

microliter with 37.1 ± 18.5% lymphocytes. Approximately two-thirds of these lymphocytes were CD4+ and the CD4:CD8 ratio was 3.52 ± 2.5. Nearly one-quarter of the patients showed clinical or radiographic resolution of their sarcoid with continued HAART, one-half showed improvement or stability, and one-quarter required corticosteroid therapy for clinical, pulmonary function, or radiographic deterioration.

Thus, HIV infection with a reduction in CD4 cells and altered immune response capability reduces the incidence of sarcoid, whereas restoration of the CD4 count and immune response with HAART increases the incidence of sarcoid. It appears that a circulating CD4+ lymphocyte count greater than 200 is required for the development of sarcoid. The increased diagnosis of sarcoid after the initiation of HAART and restoration of the immune system strongly implicates an intact and functional CD4+ lymphocyte in the pathogenesis of sarcoidosis.

Sarcoid Associated with Interferon Therapy and Hepatitis C

Sarcoid was first described after interferon therapy for renal cell cancer in 1987 *(35)*. Subsequently, in 1993 Blum and colleagues *(36)* described cutaneous sarcoidosis in a patient with hepatitis C treated with interferon-α. There have been numerous subsequent reports of sarcoid associated with interferon therapy for multiple underlying diseases including multiple sclerosis, leukemia, lymphoma, melanoma, and Kaposi's sarcoma. Three patterns of sarcoidosis have been recognized in individuals with hepatitis C: (1) de novo onset of sarcoid after interferon therapy, (2) reactivation of sarcoid with initiation of interferon therapy, and (3) development of sarcoid in treatment naive hepatitis C patients.

The prevalence of sarcoid in hepatitis C patients treated with antiviral therapy appears to be approximately 1,000–2,000 cases per 100,000 hepatitis C patients, which is significantly greater than the estimated prevalence of sarcoid in the general population, 1–40 per 100,000 population *(37–39)*. Further, Ramos-Casals and Colleagues *(37)* estimate, based upon a four-fold increase in the number of reported cases of sarcoid associated with interferon α combination therapy with ribavirin, that ribavirin may augment the development of interferon-associated sarcoidosis.

Sarcoidosis associated with interferon α therapy in patients with hepatitis C usually occurs within the first 6 months of therapy. Although sarcoid may rarely occur after therapy, it nearly always occurs within 3 months of the completion of interferon treatment.

The clinical manifestations of sarcoid associated with hepatitis C and interferon therapy are slightly different than usual sarcoid (Table 18.2). Although pulmonary symptoms remain the most common manifestation, the percentage of lung involvement is slightly less, 76%, compared with usual sarcoidosis, 90%. There is an approximately two- to three-fold increase in cutaneous manifestations compared with usual sarcoid. Sarcoid associated with hepatitis C and interferon therapy is generally less severe with less pulmonary fibrosis and has a lower incidence of cardiac and neurologic involvement.

Improvement or spontaneous remission occurs with discontinuation of antiviral therapy in approximately 85% of patients with sarcoid associated with hepatitis C and interferon therapy *(37)*. Approximately one-third of the patients require treatment with systemic corticosteroids. In patients with mild cutaneous or pulmonary involvement, sarcoid may resolve spontaneously despite continuation of antiviral therapy.

Table 18.2 Clinical manifestations of sarcoid associated with IFN therapy for HCV compared with usual sarcoid.

	HCV + IFN sarcoid	Usual sarcoid
Pulmonary	76%	90%
Cutaneous	60%	25%
Severe (Fibrosis, cardiac, neurologic)	<5%	10%

Adapted from Ramos-Casals et al. *(37)*.

TNF-α Inhibition

TNF-α is a TH-2 T lymphocyte cytokine that has been implicated in the pathogenesis of sarcoid. It is spontaneously produced at high levels by lymphocytes from individuals with sarcoid and BAL from these individuals contains elevated levels of TNF-α. The effect of TNF-α can be blocked by pentoxifylline, phosphodiesterase inhibitors, and thalidomide. Humanized and chimeric neutralizing antibodies to TNF-α have been developed and approved for the treatment of rheumatoid arthritis and Crohn's disease. Of these antibodies, etanercept has been shown not to be effective for the treatment of stage II and III progressive pulmonary as well as ocular sarcoid *(40, 41)*. Interestingly, etanercept has also been shown to be ineffective in other granulomatous diseases. Both adalimumab and infliximab have been demonstrated to be effective for the treatment of pulmonary sarcoid in case reports and small case series *(42–46)*. A large multicenter, randomized, placebo-controlled trial demonstrated that infliximab may be mildly effective in the treatment of pulmonary sarcoid *(47)*. Patients were treated with infliximab for 24 weeks. An increase in FVC was noted at 26 weeks and the 6-min walk distance was increased at 52 weeks. No significant changes were found in the SGRQ, Borg's dyspnea scale, or lupus pernio assessment. Pneumonia was more common in the infliximab group compared with the placebo group, 6.6% vs. 2.3%, respectively, and one patient developed a sarcoma. Thus, it appears that inhibition of TNF-α by infliximab and adalimumab may, at least partially, improve the manifestations of pulmonary sarcoid and hence implicates TNF-α in the pathogenesis of sarcoidosis.

These experiments of nature and biological therapies confirm an essential role for various cells and cytokines in the development of sarcoid. The near-complete absence of sarcoid in individuals with HIV infection and reduced T helper cells and the profound increase in sarcoid prevalence during the immune reconstitution syndrome dramatically implicate functioning helper T cells in the pathogenesis of sarcoidosis. The increased prevalence of sarcoid in individuals with hepatitis C treated with interferon α strongly suggests a critical role for interferon α in the pathogenesis of sarcoid. Lastly, improvement in the clinical course of patients with sarcoid treated with inhibitors of TNF-α implicates this cytokine in the immunologic and cellular mechanisms leading to sarcoid. Despite these insights into the pathogenetic mechanisms, the primary inciting event(s) remains elusive.

Pathogenesis of Sarcoidosis

Although the cause(s) of sarcoidosis is unknown, evidence suggests that the immune response occurs after a specific environmental exposure in a genetically susceptible

individual. Whether an infectious or a non-infectious environmental agent is responsible for the initiation of the inflammatory response remains an active area of investigation. Furthermore, several epidemiologic studies of disease clusters support the existence of a shared environmental exposure. These include a case–control study of a sarcoidosis cluster on the Isle of Man, which showed that a significantly greater percentage of cases as compared with control subjects had a previous contact with a sarcoidosis patient. In addition, clusters of disease have been found among nurses, a group of firefighters, and certain individuals exposed to pine pollen.

Immune Basis of the Pathogenesis of Sarcoidosis

In general, activated T cells evolve into at least three major subsets of T helper cells that are distinguished by the profile of cytokines that they produce (Figure 18.1) *(57–59)*. These cytokines play a pivotal role in the initiation and eventual resolution of the immune response. Type 1 helper T (Th1) cells mainly synthesize interleukin-2 (IL-2), interferon-gamma (IFN-γ), and tumor necrosis factor-α (TNF-α), while Type 2 helper T (Th2) cells primarily are distinguished by their secretion of IL-4, IL-5, IL-10, and IL-13. Type 17 helper T (Th17) cells represent a distinct lineage of T cells from either Th1 or Th2 cells, whose role is at the interface between the innate and adaptive immune response *(60, 61)*. These major types of T helper cells have different functions. Th1 cells primarily enhance cell-mediated immune responses such as delayed-type hypersensitivity reactions (e.g., granulomatous inflammation). Conversely, Th2 cells mainly provide help for B cells by promoting class switching and enhancing the production of certain IgG isotypes and production of IgE. Th17 cells secrete IL-17, a proinflammatory cytokine that is critical for the clearance of several pathogens *(61–63)*. IL-17 expression by memory $CD4^+$ T cells is strongly induced by IL-23 *(64, 65)* and results in the recruitment of neutrophils through the induction of granulocyte colony-stimulating factor (G-CSF) and IL-8 *(66, 67)*. The overexpression of IL-17 leads to autoimmunity *(68–70)*. However, the role of IL-17 in sarcoidosis is currently unknown. In addition, the cytokines produced by Th1, Th2, and Th17 subsets cross-regulate each other's development and function (Figure 18.3). For example, IFN-γ produced by Th1 cells inhibits the development of Th2 and Th17 cells. On the other hand, IL-4 and IL-10 produced by Th2 cells inhibit Th1 and Th17 development and activation as well as macrophage activation by Th1 cytokines. Recently, other T helper cell populations such as Th3 and FoxP3-expressing T regulatory cells have been shown to be increasingly important in controlling the immune response.

The inflammatory response in sarcoidosis is characterized by the accumulation of activated $CD4^+$ T lymphocytes, mononuclear phagocytes, and noncaseating granulomas in involved organs (Figure 18.3) (reviewed in *(4)*. This $CD4^+$ T-cell alveolitis likely represents the earliest event in the generation of the noncaseating granulomatous response. These $CD4^+$ T cells secrete Th1-type cytokines, such as IL-2, TNF-α, and IFN-γ, while the antigen-presenting cells express IL-12, IFN-γ, and TNF-α. The production of both has been shown to be essential for the development of granulomatous inflammation. Especially in the lung, the mononuclear cell infiltration and associated fibrosis can lead to progressive organ dysfunction, thereby accounting for the majority of associated morbidity and mortality. Several lines of evidence suggest that disease-specific T cells are enriched in the BAL of patients with sarcoidosis and play a central role in initiating and perpetuating the disease process *(71–79)*. For example, lymphocytes in

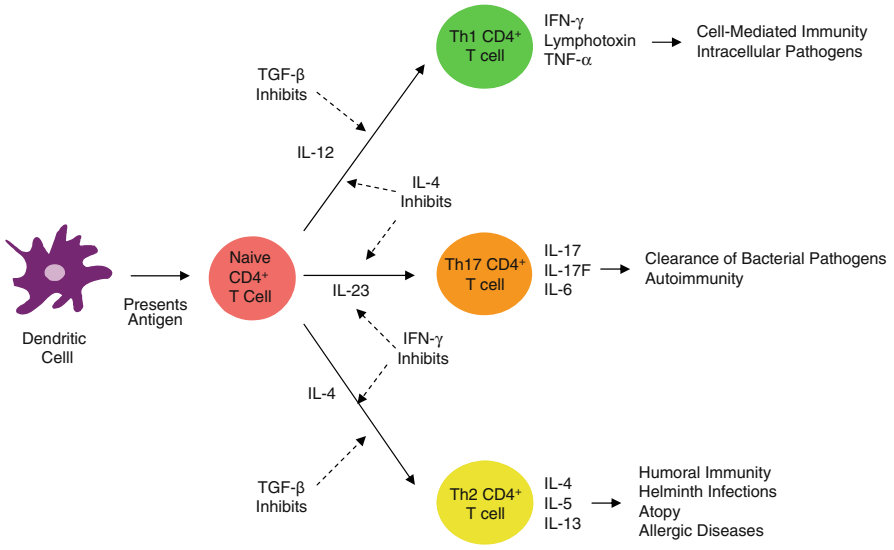

Figure 18.3 Model of Th1, Th17, and Th2 lineage development. Naive T cells are activated by antigen-presenting cells, and the cytokine environment in which the T cell resides determines the terminal lineage commitment. IL-12 potentiates IFN-γ expression, through a STAT-1 and T-bet-dependent mechanism; IL-4 increases IL-4 production in a STAT-4 and GATA-3-dependent fashion. Retinoid-related orphan receptor γt (RORγt) is the critical transcription factor involved in Th17 differentiation. IFN-γ inhibits Th2 and Th17 development, while IL-4 inhibits Th1 and Th17 development. TGF-β inhibits Th1 and Th2 differentiation, but is necessary for initiation of Th17 differentiation.

the BAL of sarcoidosis patients have a higher percentage of $CD4^+$ T cells compared to peripheral blood lymphocytes or similarly obtained lung lymphocytes from healthy individuals *(71, 72, 76–78)*. Furthermore, in contrast to BAL lymphocytes from healthy subjects, a subset of pulmonary $CD4^+$ T cells from sarcoidosis patients express surface markers of cellular activation and proliferate in vitro in culture medium supplemented with IL-2 *(72, 74, 76, 77, 79, 80)*. Freshly isolated BAL cells from these patients also spontaneously secrete IL-2 and other cytokines that recruit and aggregate mononuclear phagocytes *(73–77, 79)*.

$CD4^+$ T cells recognize antigen presented by major histocompatibility complex (MHC) class II molecules via a surface TCR composed of an α- and β-chain linked by disulfide bonds *(81)* (Figure 18.4). Functional TCR α-chain (TCRA) and β-chain (TCRB) genes are formed through somatic rearrangement of germ line gene segments, similar to rearrangement of immunoglobulin genes. Expressed TCRB genes having high antigen-binding diversity are generated from the rearrangement of variable (V) to diversity (D) to junctional (J) region gene segments. Further diversification is created by random nucleotide additions and deletions at the V-to-D and D-to-J joining points. The TCRA genes rearrange in a similar fashion with the exception of the absence of the diversity region. The highly variable Vβ-Dβ-Jβ and Vα-Jα junctional regions form the complementarity determining region 3 (CDR3), which is critically involved in the TCR's interaction with conventional antigens by making direct contact with the peptide presented by the appropriate MHC molecule (Figure 18.5). When considering the

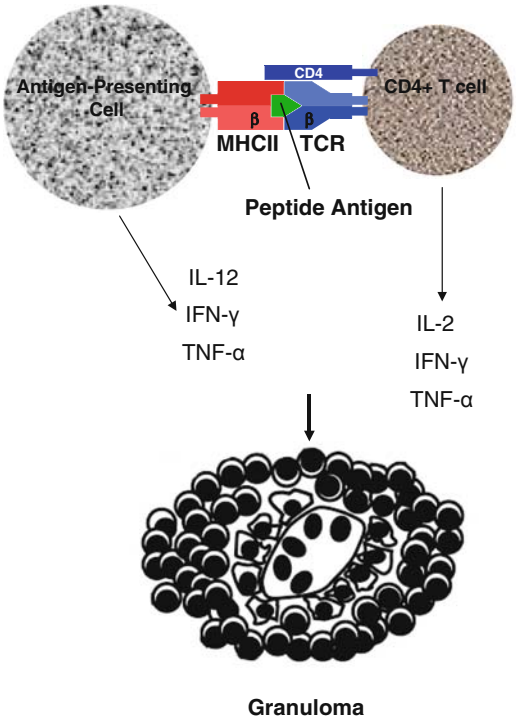

Figure 18.4 Immunopathogenesis of sarcoidosis. Following inhalation of the etiologic sarcoidosis antigen, the antigen is ingested by antigen-presenting cells (APC) where it is digested and presented as peptide epitopes to CD4+ T cells. The expression of IL-12 by the APC polarizes the lymphocyte toward to TH1 phenotype, resulting in the secretion of IL-2, IFN-γ, and TNF-α. Both IFN-γ and TNF-α are involved in the generation of the granulomatous response

combinatorial and junctional mechanisms involved in TCR diversification, even after positive and negative selection, the potential αβ TCR repertoire is enormous. Recent studies have suggested that among the 10^{12} T cells in the adult human body, there are at least 2.5×10^7 different TCR specificities *(82)*. Thus, in any given population of T cells that is being studied, the presence of multiple clones expressing an identical TCR indicates clonal expansion in response to conventional antigen. Furthermore, there is little to no chance that two or more T-cell clones should express nearly identical TCRs unless they were selected to express homologous TCRs through a response to the same antigen/MHC complex.

In this regard, studies have shown that the TCR repertoire of BAL T cells is altered in sarcoidosis. For example, a subset of patients with increased expression of T-cell receptor (TCR) Vβ2, Vβ8, or Vα2.3 has been described *(83–86)*. Sequencing of the TCR junctional regions of expanded TCR V regions has demonstrated that these T-cell populations are oligoclonal, suggesting their accumulation in response to conventional antigen stimulation *(86–90)*. The $V\alpha2.3^+$ oligoclonal expansions present in the lungs of patients with acute sarcoidosis (Löfgren's syndrome) are of particular significance because of their almost absolute correlation with the expression of HLA-DR3. These T-cell populations are compartmentalized to the lung and disappear with disease

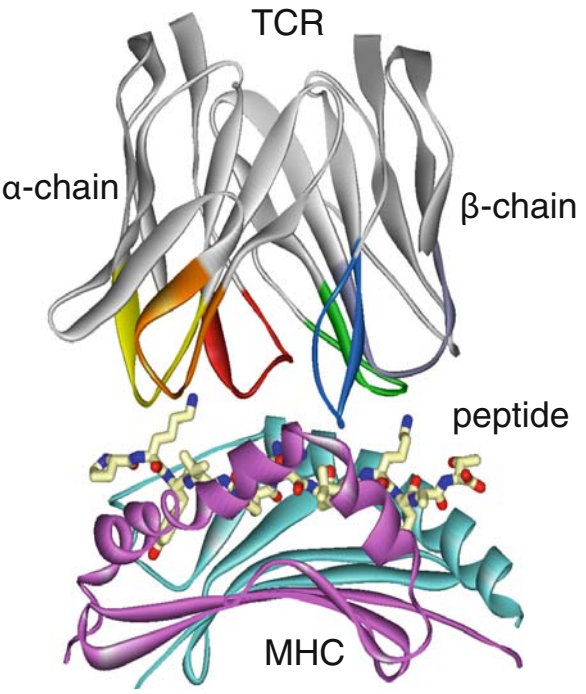

Figure 18.5 The α- and β-chains of the TCR are shown. The hemagglutinin peptide (306,307,308,309,310,311,312,313,314,315,316,317,318) is depicted as a stick structure bound to HLA-DR1. Adapted from Hennecke et al. *(101)*

remission, again suggesting their importance in the generation of the disease process *(91–93)*. The specificity of the association of a T-cell phenotype for a particular MHC class II molecule suggests that the immune response in this subset of sarcoidosis patients is triggered by a single antigen (peptide) bound to the HLA-DR3 molecule on the surface of antigen-presenting cells and recognized by antigen-specific $CD4^+$ T cells expressing the Vα2.3 α-chain. Taken together, the predominance of a limited number of Vβ-expressing subsets in the lungs of certain sarcoidosis subjects suggests that the dominant immune response may be directed against only a few antigens or epitopes.

Another population of $CD4^+$ T cells, naturally occurring regulatory T cells, has recently been shown to play a key role in the pathogenesis of sarcoidosis. These $CD4^+$ T cells expressing the transcription factor, FoxP3, accumulate at the periphery of the sarcoid granuloma and exhibit potent antiproliferative activity *(94)*. In contrast, these cells did not completely suppress effector cytokine (e.g., IFN-γ and TNF-α) secretion. These findings suggest that these FoxP3-expressing cells may regulate, although incompletely, the inflammatory response in the lung of sarcoidosis patients.

Genetic Susceptibility to Sarcoidosis

The variation in incidence, severity, and manifestations of disease among different racial and ethnic groups suggests a genetic predisposition to disease development. Other

findings supporting a genetic contribution include observations of familial clustering of disease. For example, sarcoidosis occurs two to four times more frequently in monozygotic than in dizygotic twins. Previous studies have shown that up to 19% of affected African-American families and 5% of Caucasian families have more than one affected family member. Results of a case–control etiologic study of sarcoidosis (ACCESS) further support these observations. This study enrolled over 700 case–control pairs in the United States matched on age, gender, race, and ethnicity. An analysis of nearly 11,000 first- and over 17,000 second-degree relatives of these cases and controls showed an overall adjusted familial relative risk of developing sarcoidosis of 4.7 (95% CI = 2.3–9.7) *(95)*. Interestingly, in this study Caucasian cases had a much higher familial relative risk as compared with African-American cases (18.0 vs. 2.8; $p = 0.098$). Given these observations, it is likely that multiple genes, rather than a single gene, comprise the genetic predisposition to disease. For example, the butyrophilin-like 2 (*BTNL2*) gene has recently been associated with sarcoidosis *(96)*. This disease-associated allele (polymorphism) is characterized by a G→A transition, leading to the use of a cryptic splice site and a premature stop codon in the spliced mRNA *(96)*. The resulting protein lacks a C-terminal IgC domain and transmembrane helix, thereby disrupting its membrane localization. *BTNL2* is a member of the immunoglobulin superfamily and has been implicated as a costimulatory molecule involved in T-cell activation based on its homology to B7-1 (CD80). Its engagement is thought to down-modulate T-cell activation, similar to CTLA-4. After antigen-specific T-cell activation, the lack of T-cell down-regulation resulting from *BTNL2* gene dysfunction could contribute to an exaggerated immune response that is compatible with the clinical immunology of sarcoidosis, a disease characterized by dysregulated helper T-cell activation *(97)*.

Studies of polymorphisms in the HLA family of genes have yielded inconsistent results. Associations between specific HLA-DR, HLA-DQ, and HLA-DP alleles and the presence of sarcoidosis or specific characteristics of the disease have been identified, and the associated alleles have differed based on race and ethnicity. The ACCESS study results demonstrate a significant association between sarcoidosis and HLA-DRB1*1101 across the entire patient cohort, including African-Americans and Caucasians *(98)*. Despite these and other associations of genetic polymorphisms with disease, the exact nature of the genetic predisposition to sarcoidosis remains unclear.

Potential Etiologic Antigens in Sarcoidosis

Despite these recent advances in our understanding of the genetic susceptibility and immunopathogenesis of sarcoidosis, the etiology and stimulating antigens of this disease have remained elusive. A major step forward in our understanding of the immunopathogenesis of sarcoidosis would be the identification of specific antigens responsible for the activation and recruitment of $CD4^+$ T cells to the lung and subsequent granuloma formation. Numerous infectious and noninfectious agents have been linked to the etiology of sarcoidosis although definitive proof that these agents are causative is lacking *(4)*. Recent studies have again raised the possibility that mycobacterial infection may be involved in the pathogenesis of sarcoidosis. For example, Song et al. *(99)* identified mycobacterial catalase-peroxidase (mKatG) as a target of the adaptive immune response in sarcoidosis, and Drake et al. *(100)* recently found IFN-γ-secreting cells in the blood of sarcoidosis subjects in response to two mycobacterial proteins, mKatG and ESTAT-6. However, whether these proteins are

involved in the generation of sarcoidosis or simply reflect previous infection remains unknown.

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19

Scleroderma Lung Disease

Brent W. Kinder

Abstract The hallmarks of systemic sclerosis (scleroderma) are autoimmunity and inflammation, widespread vasculopathy affecting many vascular beds, and progressive interstitial and perivascular fibrosis. The most commonly used classification system divides the disorders based on the extent of skin involvement into limited or diffuse. The diffuse form of the disease is regularly accompanied by internal organ involvement including the lungs, with prevalence estimates of up to 80%. Diffuse interstitial lung disease is the most widespread pulmonary manifestation followed by pulmonary hypertension. These two manifestations can occur in isolation or together. In general the degree of pulmonary involvement by scleroderma is not correlated with the extent of extra-pulmonary involvement, and early pulmonary involvement is often asymptomatic. The prognostic significance of lung involvement in scleroderma is illustrated by the fact that it is now the leading cause of death in this patient population. Treatment is typically directed at suppression of the immune system and efficacy has been demonstrated in scleroderma-associated interstitial lung disease.

Keywords: scleroderma, systemic sclerosis, interstitial lung disease, epidemiology, pulmonary, autoimmunity

Introduction

Scleroderma is a term used to describe a skin condition characterized by pathologically thickened skin. There are a diverse group of diseases that share the common clinical feature of scleroderma *(1)*. The hallmarks of systemic sclerosis are autoimmunity and inflammation, widespread vasculopathy affecting many vascular beds, and progressive interstitial and perivascular fibrosis. These disorders have been organized in a variety of different schema. However, the most commonly used classification system divides the disorders based on the extent of skin involvement into limited or diffuse. These diseases are often accompanied by microvascular abnormalities and Raynaud's phenomenon. The diffuse form of the disease is regularly accompanied by internal organ involvement

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and may also be referred to as systemic sclerosis. The most common internal organs implicated are the esophagus and lungs *(2)*. In fact, lung involvement is quite common in this disease with prevalence estimates of up to 80% *(2)*. Diffuse interstitial lung disease (ILD) is the most widespread pulmonary manifestation followed by pulmonary hypertension. These two manifestations can occur in isolation or together. Other less common pulmonary manifestations include pleural effusions, aspiration pneumonia, spontaneous pneumothorax, bronchiectasis, drug-induced pneumonitis, and lung cancer *(3–7)*.

As ILD is the most common pulmonary manifestation in scleroderma, the primary focus of this chapter will be on this disease. In general the degree of pulmonary involvement by scleroderma is not correlated with the extent of extra-pulmonary involvement *(8)*, and early pulmonary involvement is often asymptomatic. The prognostic significance of lung involvement in scleroderma is illustrated by the fact that it is now the leading cause of death in this patient population *(2)*.

Epidemiology

Scleroderma is a rare disorder, with an annual incidence rate of 1–2 cases per 100,000 population and a US prevalence rate of 26 cases per 100,000 population *(9)*. Although females are more frequently diagnosed with scleroderma than males (female-to-male ratio 6–8:1), the discrepancy in extensive disease is less pronounced (female-to-male ratio 3:1 for diffuse disease) *(2, 10)*. Overall, patients with scleroderma have an increased mortality risk compared with population-matched controls *(10)*. In general, limited cutaneous scleroderma has been shown to have a significantly better prognosis and less internal organ involvement than diffuse disease *(2, 11, 12)*. The 10-year cumulative incidence of mortality for patients with diffuse cutaneous sclerosis ranges from 38 to 79% *(10)*.

Genetic Basis and Molecular Pathogenesis

The pathogenesis of systemic sclerosis is incompletely understood *(13)*, although immunological inflammation seems to be a central factor. Primary abnormalities in cells of the adaptive immune system (particularly B and T cells), fibroblasts, and endothelial cells are thought to be involved in the development of the clinical and pathological manifestations of the disease *(14)*. Derangements in these cell lines lead to characteristic pathological changes in systemic sclerosis: the production of numerous autoantibodies, chronic mononuclear cell infiltration of affected organs, and dysregulation of lymphokine and growth factor production. These abnormalities lead to progressive cutaneous and visceral fibrosis, as well as obliteration of the lumen of small arteries and arterioles *(14)*. The initiating event or how exactly they interconnect to cause the progressive fibrotic process in systemic sclerosis is unknown. At the cellular level, tissues from scleroderma subjects demonstrate oxidative stress associated with the buildup of large amounts of reactive oxygen species (ROS) in fibroblasts *(15)*. It was recently observed that a pathway linking the signaling proteins Ha-Ras, growth factor – activated extracellular-signal – regulated kinases 1 and 2 (ERK1/2), and ROS is augmented in fibroblasts from patients with scleroderma *(16)*.

Approximately 94% of patients with systemic sclerosis demonstrate antinuclear antibodies in their serum *(17)*. These auto-antibodies tend to be mutually exclusive and define distinct clinical subsets of disease *(18, 19)*. In particular, the presence of anti-topoisomerases (anti-Scl-70 antibodies) has been strongly associated with the development of pulmonary fibrosis (odds ratio of 17) *(18)*. Interestingly, anti-topoisomerase positivity was also strongly associated with carriage of HLA-DRB1*11 alleles (previously included as part of HLADR5) with an OR of 14 *(18)*. A recent case–control study demonstrated the presence of stimulatory auto-antibodies to platelet-derived growth factor(PDGF) exclusively in subjects with systemic sclerosis and not in any of the idiopathic pulmonary fibrosis control patients, suggesting a different pathogenesis *(15)*. Furthermore, these stimulatory antibodies initiated a cascade of reactive oxygen species within cultured mouse fibroblasts activating collagen-gene expression.

Since the early stages of pathogenesis of systemic sclerosis are believed to involve a T-cell-mediated response to an antigenic stimulus (for example, epitopes of DNA topoisomerase I), the described HLA associations have focused on the major histocompatibility complex (MHC) region on chromosome 6 for the identification of predisposing genetic factors for this disease *(14)*. The contribution of genetic determinants of disease has been further suggested by the observation of familial clustering of the disease, the high frequency of autoimmune disorders and auto-antibodies in family members of patients with systemic sclerosis, and differences in prevalence and clinical manifestations among different ethnic groups *(13, 17)*. In addition to the MHC-based genes, genes encoding pro-/anti-inflammatory cytokines and chemokines, and those involved in fibroblast and endothelial cell functioning, are potential candidates for a role in the genetic basis of systemic sclerosis. Recently, a polymorphism (G-945C) in the promoter of the connective tissue growth factor (CTGF) gene was found to be highly associated with susceptibility to systemic sclerosis in a cohort that represented 10% of the scleroderma population in the United Kingdom *(20)*. This polymorphism was found to result in reduced transcription of CTGF, demonstrating its in vivo functional relevance. Furthermore, this polymorphism was strongly associated with presence of anti-topoisomerase I antibodies and scleroderma interstitial lung disease. A number of other genetic polymorphisms have been investigated for association with pulmonary fibrosis in systemic sclerosis with few consistent and reproducible associations. To date most of these studies have been based on a candidate gene approach. With evolving microarray technology and decreasing cost of whole genome association studies, there is promise in the near future for identification of novel pathways in disease pathogenesis.

Animal Models

A variety of animal models have been investigated as spontaneous or inducible models for scleroderma. Although none of them reproduce all pathogenetic mechanisms of the disease, some models do demonstrate selected phenotypic features. The tight skin (*Tsk1*/+) mouse phenotypically demonstrates extensive thickening of the skin *(21)*. Although mice homozygous for the *Tsk1* mutation expire in utero at 8–10 days of gestation, heterozygous (*Tsk1*/+) mice are viable and develop tight skin that is firmly bound to the underlying subcutaneous tissue. Contrary to human systemic sclerosis, which is characterized by thickening and sclerosis of the dermis, *Tsk1*/+ mice manifest hyperplasia of the subcutaneous tissue but the dermis is unaltered *(22)*. In addition, *Tsk1*/+

mice develop emphysema-like lung pathology instead of interstitial fibrosis, and vasculopathy is not present. The *Tsk1* mutation is a tandem duplication in the gene encoding fibrillin-1, a microfibrillar connective tissue protein *(23)*. It has been suggested that the *Tsk1* mouse phenotype represents tissue fibrosis due to deregulated TGF-beta activation and enhanced profibrotic signaling by this cytokine *(24)*.

Bleomycin has been injected into the subcutaneous tissue of mice to produce a phenotype that closely mimics the skin changes in systemic sclerosis. The sequence of histopathological changes is characterized by early mononuclear cell accumulation and upregulated TGF-beta and chemokine expression followed by dermal fibrosis with accumulation of α-SMA-expressing myofibroblasts *(25, 26)*. In addition, these mice demonstrate pulmonary and renal fibrosis. This model has several features that have led to its use with increasing regularity to explore the roles of specific gene products in scleroderma-like disease, including reproducibility, relative strain independence, and ease of induction *(27)*.

Murine sclerodermatous graft-vs.-host disease (Scl GVHD) from injection of MHC-mismatched bone marrow cells models human scleroderma, with prominent skin thickening, lung fibrosis, and up-regulation of cutaneous collagen mRNA *(28)*. A GVHD mice model induced by the injection of splenic cells into sublethally irradiated recipient mice demonstrated dermal thickening, particularly in the extremities, progressive fibrosis of internal organs, vasoconstriction and altered expression of vascular markers in skin and internal organs, early immune activation, inflammation in skin and internal organs, and auto-antibody generation *(29)*. These models have been used to investigate specific contributions of imputed effector molecules such as TGF-beta. Early elevated cutaneous mRNA expression of TGF-beta1, but not TGF-beta2 or TGF-beta3, and elevated C–C chemokines macrophage chemoattractant protein-1 and macrophage inflammatory protein-1alpha precede subsequent skin and lung fibrosis *(28)*. Antibodies directed against TGF-beta prevented Scl GVHD by effectively blocking the entry of monocyte/macrophages and T cells into skin and by preventing up-regulation of TGF-beta1, thereby preventing new collagen synthesis *(28)*.

Clinical Presentation

Symptoms and Physical Exam

Interstitial lung disease in scleroderma usually presents as dyspnea, initially only with exertion and later also at rest. Patients often will limit physical activity and thus dyspnea may be denied *(10)*. A dry cough may be present and is often one of the most troublesome symptoms for patients *(30)*. Hemoptysis, pleurisy, and fever are much less common. Physical examination may reveal bilateral basilar fine inspiratory crackles (i.e., "Velcro" rales). Clubbing, common in other interstitial lung diseases like idiopathic pulmonary fibrosis *(31)*, is uncommon in scleroderma because of the cutaneous restriction and reduction of digital blood flow *(32, 33)*. When ILD has progressed to end-stage fibrosis, signs of cor pulmonale will often appear including peripheral edema, jugular venous distention, and hepatojugular reflux. Pulmonary hypertension with or without pulmonary fibrosis and, when present, is often the cause of right-sided heart failure *(34)*.

Physiologic Findings

The typical pulmonary physiologic abnormalities include a restrictive ventilatory defect with impairment of gas exchange. Expiratory flow rates during spirometry are conserved or reduced proportionate with the low lung volumes. Early ILD cannot be excluded by normal spirometry. The static compliance of the lung is reduced and is not a result of skin tightening of the chest wall *(35–37)*. Abnormalities of the diffusing capacity of carbon monoxide (DLCO) are sensitive indicators of underlying pathology even in the absence of volume restriction or imaging changes *(38–40)*. The percent predicted DLCO is a reliable surrogate for the extent of parenchymal disease on high-resolution-computed tomography imaging *(41)*. Arterial blood gases demonstrate normal or reduced oxygen and carbon dioxide tensions at rest, widening of the alveolar–arterial oxygen gradient, and arterial desaturation during exercise *(42–45)*. Exercise capacity is impaired in scleroderma and is accompanied by an abnormally high ventilatory response to exercise *(45)*.Occult pulmonary impairment is best recognized during formal cardiopulmonary exercise testing *(46)*.

Imaging

Early in the course of ILD the chest radiograph may be normal or show diffuse hazy opacities and linear densities in the lower lung zones. As the disease advances, the radiograph typically shows diffuse symmetric reticular opacities. Although either reticular or nodular densities may be seen in both CREST syndrome and diffuse scleroderma, a mixed reticular and nodular pattern is more common. The ILD tends to be basilar in location with sparing of the apices. Areas of cyst formation (honeycombing) are a less common feature.

The specific features of the ILD are best characterized with high-resolution-computed tomography (HRCT) imaging. The earliest HRCT finding is usually an ill-defined, subpleural hazy opacity in the posterior segments of both lower lobes (see Figure 19.1). As with the plain radiograph in progressive disease, the opacities have a reticulonodular appearance and may be associated with honeycomb cysts. On tomography, these cysts are multiple and range in size from a few millimeters up to 2.0 cm *(47)*. Serial HRCT scans may be the best means to evaluate the disease course in scleroderma *(48)*.

Histopathology

The predominant histopathological pattern seen in scleroderma lung disease is that of non-specific interstitial pneumonia (NSIP). In the largest published case series of 80 scleroderma ILD patients with lung biopsies at the Brompton Hospital in London, 78% of patients had an NSIP pattern *(49)*.This pattern demonstrates a relatively uniform appearance at low magnification due to a cellular interstitial infiltrate of mononuclear inflammatory cells associated with varying degrees of interstitial fibrosis. Focal areas of organizing pneumonia (OP) resembling the changes seen in the syndrome of cryptogenic organizing pneumonia, otherwise known as bronchiolitis organizing pneumonia (BOOP), are a common finding. The NSIP pattern differs from classical BOOP, however, in that "BOOP-like" areas represent less than 10% of the cross-sectional area of the tissue and are overshadowed by the interstitial pneumonia *(50)*. Other reported histopathological patterns attributed to scleroderma include usual interstitial pneumonia

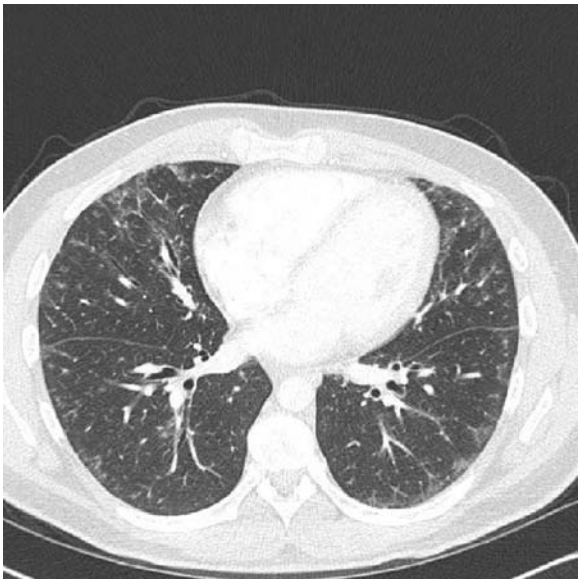

Figure 19.1 High-resolution computed tomography image from a patient with early scleroderma lung disease. The key feature is bilateral predominantly subpleural ground-glass opacification

(UIP) and end-stage fibrosis. It is unclear if the histopathological pattern has prognostic significance, although limited data suggest that in scleroderma lung disease it does not *(49)*.

Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) is a technique used to sample the alveolar compartment through the instillation and withdrawal of normal saline. BAL with determination of white blood cell differentials demonstrated that scleroderma patients have an increased percentage of neutrophils and eosinophils compared with normal controls *(51)*. It had been postulated that BAL neutrophilia or eosinophilia could predict which patients would be most at risk for progression or death *(52)*. A recently completed large randomized controlled trial in scleroderma lung disease used BAL neutrophil (>3%) or eosinophil (>2%) percentage as indicators of active alveolitis and one of the entry criteria *(53)*. In post hoc analysis, BAL neutrophil or eosinophil percentage did not affect the change in FVC at 1 year when included in their multivariate model *(54)*. However, longitudinal follow-up of these patients is on-going and the effects of BAL cellular content on survival may be available in the future. More recently in a European study, BAL neutrophilia has been shown to predict early mortality but not necessarily through an intermediary step of decreased lung function *(55)*. It is unclear how BAL neutrophilia is related to mortality and whether serial BAL with determination of white cell differential is more useful than baseline levels alone. Consequently, experts disagree about the utility of BAL white blood cell differential determination in the routine care of scleroderma ILD patients.

Diagnostic Approach

The initial evaluation of patients with scleroderma-associated ILD includes high-resolution computed tomography (HRCT) and pulmonary function testing, including diffusing capacity of carbon monoxide (DLCO) (see Figure 19.2). These tests are used to determine the extent and severity of disease, and the magnitude of impairment in lung function. It is important to establish a baseline for these radiographic and functional parameters prior to initiating therapy. Bronchoalveolar lavage is controversial and not necessarily regularly warranted except to rule out infection. At the present time, we do not routinely recommend lung biopsy unless an alternative diagnosis is seriously considered. Decrements in serial pulmonary function tests, particularly FVC or DLCO, are likely the best indicators of progressive disease and a worse prognosis.

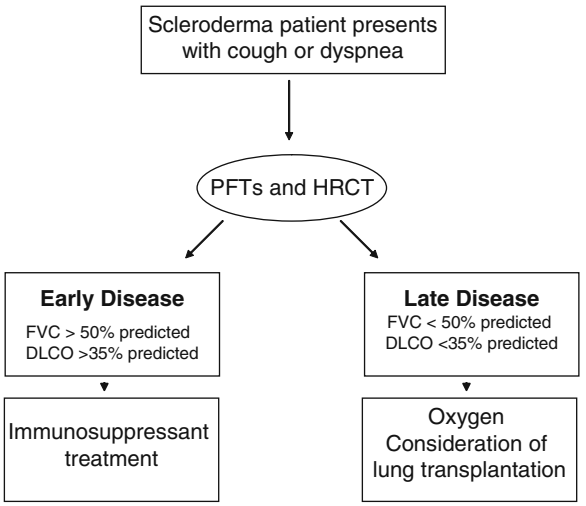

Figure 19.2 Schematic of the evaluation and management of patients with scleroderma lung disease

Conventional Management and Treatment

Given the prognostic implications of interstitial lung disease in this patient population as outlined above, many therapeutic agents have been used in scleroderma. Unfortunately, most of the studies that have been performed are of low quality and did not use randomized, double-blind, placebo-controlled protocols. The medications that are most frequently used include cyclophosphamide, corticosteroids, azathioprine, and more recently mycophenolate mofetil. Based on one large multi-center randomized placebo-controlled trial *(53)*, many experts recommend daily oral cyclophosphamide at 1–2 mg/kg with or without low-dose prednisone (<10 mg) as first-line therapy.

The trial of 162 patients with early scleroderma-associated ILD (defined by the presence of ground-glass opacities on HRCT or BAL fluid with elevated neutrophils or eosinophils) to receive either oral cyclophosphamide (initial dose of 1 mg/kg/day increased to a maximum of 2 mg/kg/day as tolerated) or placebo. The concurrent use of

glucocorticoids (up to 10 mg/day prednisone) was permitted. At the end of 12 months of therapy, the mean change in forced vital capacity (FVC), the primary outcome measure, showed a significantly smaller decline in patients who received cyclophosphamide compared to those on placebo (–1.4 vs. –3.2%). There were more adverse events (hematuria, leukopenia, neutropenia, and pneumonia) in the cyclophosphamide-treated group. The improvement in pulmonary function parameters and dyspnea score persisted off therapy for approximately 6 months before regressing back to the level of placebo by the second year since randomization *(56)*. There are concerns about the long-term adverse events in the cyclophosphamide-treated group such as bladder malignancy that may not become clinically evident until years after treatment. The side effect profile and potential increase in long-term risk of malignancy coupled with the modest clinical benefit of the intervention are problematic. Consequently, some experts recommend regimen of prednisone with azathioprine or mycophenolate mofetil as alternatives despite the absence of rigorously performed clinical trials to support their use *(57, 58)*.

Given the toxicity associated with the above-described agents, many experts reserve their use for patients most likely to receive a benefit. Experts with longstanding clinical experience note that patients with end-stage fibrosis, or honeycomb lung, are unlikely to respond to immunosuppressive therapy. Consequently, we often treat those patients with preserved lung function more aggressively than those with more advanced disease (see Figure 19.1). However, one should note that the thresholds provided herein should serve as a guideline as they are not based on hard evidence and thus should not be rigidly applied to any given individual patient. Patients who are not candidates for aggressive immunosuppressant therapy should be considered for palliative efforts or lung transplantation. Oxygen serves as a useful adjunct in patients with hypoxemia either at rest or with exertion. Increasingly, patients with moderate-to-severe ILD are being referred for enrollment in pulmonary rehabilitation programs. The limited data available suggest that these patients may have an improvement in quality-of-life measures *(59)*.

Future Therapeutic Targets and Directions

As insights into the molecular underpinnings of scleroderma come forward there is hope for the emergence of targeted, less toxic therapeutic modalities. The recent identification of stimulatory auto-antibodies to PDGF as a potentially critical step in the pathway leading to tissue fibrosis is an intriguing development *(15)*. This discovery coincides with the availability of a FDA-approved class of drugs that target specific molecular pathways that have been demonstrated to be abnormal in patients with fibrotic lung disease such as transforming growth factor (TGF)-beta and PDGF receptors. Imatinib antagonizes specific tyrosine kinases that mediate fibrotic pathways involved in the pathogenesis of systemic sclerosis, including c-Abl, a downstream mediator of transforming growth factor (TGF)-beta, and platelet-derived growth factor (PDGF) receptors. Imatinib has been approved by the FDA for the treatment of newly diagnosed adult patients with CML (newly diagnosed adult patients and for the treatment of patients with an accelerated phase), and for patients with a certain type of gastrointestinal cancer (called stromal tumors) but it has not been approved to treat systemic sclerosis. There are currently several on-going clinical trials evaluating its efficacy in scleroderma lung disease. Other novel agents, gefitinib and erlotinib, are potent tyrosine kinase inhibitors of EGFR. Pre-clinical studies in mice have suggested that gefitinib prevents

bleomycin-induced fibrosis *(60)*. Clinical studies of these agents in fibrotic lung disorders are in the early enrollment of Phase I/II trials.

The drug mycophenolate mofetil limits the expansion rapidly dividing B cells and thus may be able to attenuate the production of auto-antibodies. If the above-described stimulatory PDGR auto-antibodies are an important mediator of tissue damage, then mycophenolate mofetil could be effective in blunting their production, albeit in a relatively less targeted manner. Several observations support the role of activated T cells in both the blood and lungs of affected patients with scleroderma. Abatacept, a recombinant fusion protein that blocks T-cell activation, has recently been approved by the FDA for rheumatoid arthritis. It is possible that inhibition of T-cell activation with abatacept may be efficacious in limiting the tissue damage in these patients. There is currently a pilot trial investigating this therapy in scleroderma patients.

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