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Heterogeneity in Asthma

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Heterogeneity in Asthma

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Preface

Application of Translational Science to the Clinical Problem of Asthma

The Problem

Asthma is a chronic relapsing airways disease that represents one of the most common chronic diseases worldwide (Busse et al. 2001; Masoli et al. 2004). Currently it is estimated that over 300 M people suffer from asthma, and its prevalence is increasing in both adult and pediatric populations. Asthma therefore represents a major public health problem and is worthy of attention to develop cost-effective prevention and management approaches.

Clinically asthma is a syndrome characterized by episodic, reversible obstructive airways obstruction that variably presents as a myriad of symptoms from cough to wheezing, shortness of breath, or chest tightness. The presence of bronchial muscular hypertrophy, mucous hypersecretion, tissue remodeling, and a T-lymphocyte predominant inflammation are pathogenic signatures of this disease (Busse et al. 2001; Lemanske et al. 2010). Asthma is a disease significantly modified by environmental interactions. Characteristic of asthma is intermittent exacerbations provoked by airway mucosal exposure to proinflammatory stimuli. Here, common cold (RNA viral infections) or inhaled allergens are two of the most common precipitants (Lemanske et al. 2010; Montalbano et al. 2002).

It is widely appreciated that asthma is different from one individual to the next; this heterogeneity is manifested in onset, exacerbating stimuli, severity, and treatment response. Presently, asthma classification methods are largely descriptive, focus on a single aspect or dimension of the disease, and do not lead to actionable intervention. Specifically, the current standard of care treatment for asthma is a stepped-care model; for those with persistent symptoms, anti-inflammatory treatment with inhaled corticosteroids (ICS) remains the first-line treatment. Yet efficacy studies indicate up to 30 % of subjects do not have a response to ICS. Currently, no reliable biomarker has been validated that identify an ICS response (Szefler 2002).

A robust, objective method for diagnosis and measurement of the efficacy for treatment interventions is, therefore, sorely needed.

Molecular Profiling and Personalized Medicine

With the completion of the human genome project, we are now in the era of postgenomic medicine where sensitive measurements of 100s–1,000s of proteins, metabolites, and genes in a single patient can be routinely performed (Hamburg et al. 2010). Properly conducted and interpreted, these multidimensional measurements, a process known as molecular profiling, can identify subtle differences in the pathophysiology of the disease at a level of precision that is not otherwise possible using physiological or clinical measurements. As a result the application of molecular profiling has revolutionized the field of medicine because it identifies robust objective, measurable, and quantitative features associated with disease phenotypes.

The ultimate goal of molecular profiling lies in its translational application to personalized medicine. Personalized medicine relies on measurements of an individual patient's molecular profiles to identify specific tailored interventions that will result in the greatest efficacy and safety, rather than apply generic therapies developed for a large population. The promise of personalized medicine is to individualize the prevention, diagnosis, and treatment of human disease. This individualized approach will result in better treatment responses, thereby reducing morbidity, lowering the rate of adverse drug reactions, enhancing efficacy, promoting better compliance, and reducing healthcare costs (Offit 2011). From a clinical investigative standpoint, the use of molecular profiling can be used to speed the development of therapies in select subgroups of asthmatics. Nevertheless, despite this promise, the full realization of personalized medicine faces significant challenges that will require innovation in basic scientific, regulatory, and translational spheres (Hamburg et al. 2010).

Motivation

We contend that asthma as a complex, heterogeneous and multifaceted disease is well poised for applications of personalized medicine. Recent exciting findings indicate molecular profiling can be applied to identify subclinical phenotypes of asthma that are not readily apparent by conventional assessment (Brasier et al. 2008, 2010). Moreover, these molecular profiles can be used to predict subtypes of asthma that differ in response to pharmacological therapy, cellular inflammatory phenotype, or response to bronchial provocation (Brasier et al. 2010). Upon further validation, these profiling and predictive techniques are ready for meaningful application to the improved prevention and treatment of asthma.

This book is organized into discrete sections edited by established investigators in the field. Part I will introduce asthma as a clinical disease and discuss methods for diagnosis, its epidemiology, environmental interactions, and current therapeutic approaches. Part II will reveal how genetics, epigenetics, and genomics approaches reveal asthma subtypes and etiologies. Part III will focus on methods for molecular profiling asthma, including exciting new developments in protein profiling, quantitative measurements of signaling pathways, and examination of functional responses of airway cells to immunomodulators. Part IV will discuss how complex datasets can be combined and related to disease manifestations and or subclinical phenotypes [or "endotypes," (Anderson 2008)]. Part V will consider how complex and surprising biobehavioral determinants influence the perception and manifestations of asthma. We will conclude with a discussion of next steps and implications.

Readers of this work will acquire advanced understanding how to collect, measure, and visualize multidimensional profiling data in asthma facilitate the understanding of disease modifiers and/or therapeutic intervention. We stand at the door of a revolution in asthma through the application of personalized medicine.

Acknowledgments This book is the result of a multidisciplinary effort of MTTs at the UTMB. This research was supported by grants from the NIHNIAID, AI062885, NIH-NHLBI-HHSN268201000037C NHLBI Proteomics Center for Airway Inflammation (ARB), NIEHS P30 ES006676, and the Clinical and Translational Science Award (UL1TR000071) from the National Center for Advancing Translational Sciences, NIH (ARB).

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Part I
The Spectrum of Asthma: An Introduction

William J. Calhoun

Asthma is a syndromic disorder, that is, it is defined by a collection of clinical signs, symptoms, and findings, rather than a single definitive test. Hyperglycemia, osteopenia, obesity, and many other clinical disorders can be defined using quantifiable criteria, the performance characteristics of which can be measured. Asthma is associated with three principal characteristics: (1) variable airways obstruction, (2) airway hyperresponsiveness, and (3) airway inflammation. In turn, each of these characteristics may be variably, and sometimes inconsistently, defined leading to considerable variability in the diagnosis of asthma. Accordingly it is not surprising that there is a spectrum of clinical presentations encompassed under the broad umbrella "asthma."

Were there to be uniformity across the spectrum of asthma of clinical outcomes, responsiveness to therapy, rate of progression, impact on quality of life, and other parameters, a discussion of heterogeneity would be of academic interest only. However, patients with asthma vary considerably across these and other measures. For example, severe asthma is associated with dramatically increased healthcare utilization and costs, with resistance to corticosteroids, and persistently impaired lung function, despite the fact that usual clinical measurements do not individually separate severe asthma from those patients with less severe forms of the disease (Moore et al. 2007). Combinatorial analytics, in which a panel of measures is used, appear to have some promise in distinguishing subtypes of asthma (Pillai et al. 2012; Bhavnani et al. 2011) (See Part IV), but there is no current consensus on the features which should be included in a diagnostic panel.

The literature of asthma is replete with attempts to classify this heterogeneous disorder. In the mid-twentieth century, the concept of intrinsic (nonallergic) and extrinsic (allergic) asthma was commonly held, but lost currency when reductions in lung physiology, airway inflammation, and responsiveness to therapy did not align with the extrinsic/intrinsic distinction. Likewise, clinical phenotypes, derived

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from directly observable characteristics such as degree of airway obstruction, atopy, presence of sputum production, frequency of exacerbations, etc., have with rare exception failed to predict clinically important outcomes of response to therapy and rate of progression of disease. Using a small panel of characteristics, the US Severe Asthma Research Program (SARP) could identify clustering of characteristics that defined clinically more homogeneous groups, but these clusters provided no real mechanistic insights into the pathogenesis of asthma (Moore et al. 2010).

More recently, the concept of endotype has been advanced. In its purest form, an endotype represents a pathophysiologic mechanism that underlies expression of clinical asthma, and in that form, is a useful concept. However, the term "endotype" has been misapplied to subsets of asthma that are more properly termed a "clinical phenotype": early onset vs. late onset asthma, asthma in cross-country skiers, etc. (Lötvall et al. 2011). Accordingly, we and others prefer the term "molecular phenotype" (Brasier et al. 2008, 2009; Woodruff et al. 2009; Pillai et al. 2012) as it connotes pathophysiologic meaning to the classification of asthma.

In this eight-chapter section, *The Spectrum of Asthma*, we review asthma heterogeneity from a number of important perspectives. There is significant variability in the expression of asthma which can broadly be classified as clinical and phenotypic classification, epidemiology, initiating and triggering factors, and responsiveness to interventions, both pharmacologic and sociologic. In addition, a wide range of physiologic tests can be profitably employed to gain understanding of the range of objective abnormalities in lung function that underlie asthma. The overall purpose of each of the chapters in this section is to provide a road map of the often complex and sometimes confusing landscape of asthma heterogeneity in order to clarify the scientific approaches that must be used to dissect distinct molecular mechanisms that account for the pathogenesis of asthma.

In Chap. 1, Pillai and Calhoun provide an overview of the field of asthma, including decades-old concepts of heterogeneity in the clinical expression of asthma. These older clinical phenotypes still have utility, but do not appear to inform the molecular mechanisms of asthma. Approaches to developing more informative phenotyping are reviewed, and the relative strengths and limitations are articulated.

Chapters 2 and 3 review the epidemiologic aspects of asthma, including the important ramifications of expression of asthma from a public health perspective. Divekar and Croissant develop the concepts of risk associated with the urban environment, with identifiable demographic characteristics, and with deficiency of Vitamin D.

Sokol, Sur, and Ameredes provide a detailed review of environmental toxicants and allergen exposures that can modify the expression of asthma. Asthma is expressed in the setting of a permissive genetic background, on which the cumulative effects of various environmental exposures (viruses, allergens, and toxicants) are expressed. The timing, consistency, and dose of environmental factors also play a role in asthma expression. It is then unsurprising that considerable heterogeneity of expression of clinical asthma exists, because of the multiplicity of genetic and environmental factors involved, and the plethora of ways that these factors interact. In fact, some environmental exposures can act as initiating factors (producing first

manifestation of asthma), others act as triggers (producing worsening of disease in patient who already have had asthma), and still others may affect the response to therapeutic agents. Consequently, a detailed understanding of the role of environmental factors is critical for understanding the heterogeneity of asthma expression.

The current diagnostic armamentarium for asthma and other obstructive respiratory diseases is reviewed by Shaw and Sharma in Chap. 5. Although some of these tests have been available for decades, others, such as impedance oscillometry and mannitol challenges, are more recent and offer potential advantages to patients and physicians who manage asthma. Advantages and limitations of various testing methods are important considerations.

In Chap. 6, Reddy and Gupta comprehensively review the current standard guidelines for asthma management, both from the USA and international sources. Guidelines provide diagnostic and management approaches that are appropriate for most patients, but necessarily group patients into categories which may not accurately reflect the expressed phenotype of a given individual. Hence, there is operational latitude and other options expressly incorporated into guideline documents, to accommodate phenotypic heterogeneity. Prospectively identifying such heterogeneity and using that information better to inform diagnosis and management is one of the purposes of this monograph.

Petronella summarizes community intervention in asthma in Chap. 7, which can be applied in a group manner or individualized based on expressed clinical phenotype. These approaches clearly have implication for other diseases, and their potential application is quiet broad.

In Chap. 8, Calhoun reviews the evidence for heterogeneity in therapeutic responsiveness in asthma. The understanding that therapeutic responsiveness is heterogeneous in asthma is relatively recent, gaining currency in the past 15 years. Heterogeneity in response to all classes of asthma therapies has been demonstrated, and the underlying mechanisms have been elucidated in some, but not all, of those pathways. Predictors of response to therapy, if simple, noninvasive, rapid, and inexpensive, would have perhaps the greatest impact on the clinical management of asthma of any advance of the past 20 years.

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Chapter 1
Introduction to Asthma and Phenotyping

Regina A. Pillai and William J. Calhoun

Keywords Asthma • Heterogeneity • Clinical phenotypes • Clusters • Physiology • Treatment • Molecular phenotype • Induced phenotype

1.1 Introduction and Primer on Asthma and Its Heterogeneity

Asthma is a heterogeneous disease process that is characterized by three cardinal features: (1) chronic inflammation of the airways leading to (2) variable airflow obstruction and (3) airway hyperresponsiveness (NHLBI 2007; Balzar et al. 2011). Over the past two decades, there has been a rise in asthma prevalence, such that about 8 % of the population in 2010 and more than 25 million Americans are affected (Akinbami et al. 2012). The clinical presentation varies in degree of severity, but common symptoms include wheezing, shortness of breath, and cough (NHLBI 2007). There has been a steady increase in asthma prevalence from 2001 to 2010 which is particularly marked in the pediatric population; children have required increased emergency room visits and hospitalizations (Akinbami et al. 2012). Despite the overwhelming prevalence of asthma worldwide, extensive healthcare costs and immense economic burden, a detailed understanding of the underlying pathophysiology of asthma, particularly those features leading to variable expression of disease (recognized as clinical phenotypes) remains to be

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developed (Bousquet and Khaltaev 2007). Asthma affects industrialized nations as well underdeveloped countries, and similar phenotypes have been recognized across these borders (Weiss et al. 1992). Recent investigations have noted distinct clinical asthma phenotypes, but the characteristics that separate these phenotypes overlap significantly, including those factors that contribute to the difficulty in clinical management of these patients (Calhoun et al. 2003).

As noted elsewhere (Chap. 6), current management of asthma rests on short-acting beta-2 bronchodilators, inhaled corticosteroids, long-acting bronchodilators, and a small number of other agents. Heterogeneity in response to therapy is a key management issue, reviewed in Chap. 8. The heterogeneity of asthma extends beyond the clinical symptoms and response to therapy, to variable ages of onset, duration of disease process, extent of airway obstruction (NHLBI 2007), nature and sensitivity to triggering agents, characteristics of the airway inflammatory process, and perhaps even the fundamental nature of the immune response.

Clinicians and researchers have used a variety of approaches to categorize, to understand, and ultimately to utilize heterogeneity of expression of asthma to inform clinical management, improve patient care, and strengthen experimental designs. To date, most of these approaches have had limited penetration because of invasive testing (bronchoscopy), overlap among identified groups, and lack of predictive power. In fact, empiric therapeutic trials of various agents, guided by the USA and other international guidelines (NHLBI 2007; GINA 2011), are the norm for clinical care of asthma patients because predictive testing is not available.

Accordingly, classifying a heterogeneous disease like asthma into several smaller, more homogeneous subgroups ("phenotypes") offers the possibility of clearer delineation of asthma mechanisms; more effective, mechanism-based therapy; and improved prediction of disease course.

1.2 Phenotyping Strategies

Phenotypes are groups of patients that are definable based on observable characteristics. In this brief review, we will focus on two complementary phenotyping strategies: clinical phenotyping and molecular phenotyping, with brief mention of cellular phenotyping. Although there are several approaches to asthma phenotyping in the literature, clinical phenotyping and molecular phenotyping remain the two most productive approaches (Wenzel 2012). The goals of phenotyping are multifold, including forecasting the clinical course, predicting response to therapy, identifying subgroups at risk for adverse events and other complications, and reducing clinical heterogeneity in clinical trials (Pillai et al. 2012).

Clinical phenotypes have been defined in asthma for decades: intrinsic (i.e., non-allergic) vs. extrinsic (i.e., allergic) asthma is one such example. Clinical phenotypes attempt to provide reproducible groupings of patients based observed signs and symptoms, combined with testing carried out as part of usual clinical care, and as such is most commonly an observational approach. Clinical phenotyping focuses

on the observed clinical presentation including response to therapy, known inherent characteristics such as weight and sex, and outcomes of diagnostics tests to classify patients into subgroups (Pillai et al. 2012). In the clinical arena, a patient's "phenotype" describes prominent observed characteristics that arise from gene expression and, importantly, can be influenced by environmental elements. Several environmental factors can influence the phenotype including noninfectious allergens, infection mediators such as viruses, and medications that are often chronically administered (Wenzel 2004). These latter phenotypes, dependent on a specific environmental interaction, can legitimately be considered to be induced phenotypes (vide infra).

In contrast, molecular phenotyping looks beyond the visualized presentation and categorizes patients at a molecular level with an emphasis on the genetic makeup, inflammatory pathways, and signaling mechanisms (Pillai et al. 2012; Wenzel et al. 1999). Using these approaches, researchers have made much progress in understanding the mechanistic pathways in asthma that may govern the expression of asthma in heterogeneous groups of patients. Molecular phenotyping attempts to produce reproducible groups based on a molecular signal or signature, which may be protein based, mRNA based, DNA based, or conceivably metabolome based. Fundamental to understanding molecular phenotyping are the concepts that molecular methodologies may provide information that enhances the understanding of pathogenic pathways and that no single measurement or marker is likely to provide sufficient discriminant function to classify unambiguously a heterogeneous group of patients. Hence, many molecular phenotyping approaches employ panels, or groups of analytes, to enhance the performance characteristics of the approach.

In the last decade, technological advances have supported asthma research in both the clinical and molecular arena in an attempt to combine both these phenotype approaches to optimize asthma management and contribute to the field of personalized medicine (Wenzel 2012).

1.3 Application of Clinical Asthma Phenotypes

Clinical phenotypes use features that clinicians are able to observe. Patients with common characteristics are grouped together in an attempt to guide therapy and management. Asthma is a syndromic disease resulting from airway inflammation, a largely unmeasured feature; hence, phenotyping on the basis of clinical characteristics is hampered by the lack of a "gold standard" against which to test the phenotype. Further, the borders between adjoining subgroups of asthma overlap, reducing the discriminant value of the approach (Busse et al. 1993; Horwitz and Busse 1995). Despite these limitations, there are several prominent clinical phenotypes that are noteworthy, which have assisted clinicians over the decades, including the concept of allergic vs. nonallergic asthma, the syndrome of exercise-induced bronchospasm, and the association of difficult-to-control asthma with obesity and with smoking.

When patients with asthma and positive IgE reactions to commonly encountered aeroallergens are evaluated in the clinic setting, the descriptive term allergic asthma is often utilized. This term is more than shorthand, as allergic asthma is well described and has assisted clinicians over the years in recognizing and managing patients who have exaggerated responses to various stimuli. Moreover, the term "allergic asthma" became commonly recognized in lay society and so has external validity as well. Finally, the term conveyed an understanding of the putative etiology of symptoms for these patients (vide infra).

Exercise-induced bronchospasm is another entity that is well known to practitioners and has distinct features in clinical presentation and therapeutic management that sets it apart. Although exercise can be a trigger for many kinds of asthma, the entity of exercise-induced bronchospasm (EIB) per se occurs solely in the context of exercise, and not in other situations (nocturnal, seasonal, etc.), and the timing of onset of symptoms following cessation of exercise is characteristic. Its management generally relies on prophylactic use of inhaled short-acting beta-agonists prior to onset of exercise, as the degree of airway inflammation in EIB is generally less than that seen in other forms of the disease. Recognizing this clinical phenotype then is helpful to the clinician in formulating therapeutic management plans.

1.3.1 US Severe Asthma Research Program

More recently the US Severe Asthma Research Program (SARP) has analyzed an extensive dataset of patients with severe and non-severe asthma, in an attempt to identify and describe robust subgroups of asthma patients with distinct features (Jarjour et al. 2012), which possibly could be, but as yet has not been shown to be, an informative guide to personalized therapy. The approach used is termed clustering and by use of a variety of statistical analyses interrogates the dataset to identify characteristics that most accurately distinguish among subgroups within the study population.

The prevalence of asthma and health-care costs from asthma-related complications continues to grow despite the aggressive research in many academic centers (Weiss et al. 1992). Hence, the National Heart, Lung, and Blood Institute (NHLBI) established the Severe Asthma Research Program (SARP) made up of eight academic institutions to further investigate this group of patients (Jarjour et al. 2012). SARP has provided much clinical advancement in the field of asthma research. Specifically, in the area of clinical phenotyping, analysis of the SARP population, that incorporated more than 1,500 asthma patients, more than 500 of whom were severe, led to the identification of five "clusters," or clinical phenotypes (Walker et al. 1992). The clustering approach did not presuppose that any particular metric would be predictive. This paper was one of the first to apply the methodology to a large dataset of asthma patients with a wide range of severity.

SARP defined severe asthma as fulfilling at least one major criterion (daily use of high-dose inhaled corticosteroids or use of systemic corticosteroids) and at least two

of seven minor criteria proposed from the ATS guidelines on refractory asthma (ATS 2000). Patients in this study had extensive biologic and physiologic characterization and were followed for approximately 2 years. The five discrete subgroups of asthma patients emerged by agglomerative cluster analysis. Cluster 1, the mild allergic asthma cluster, was characterized by history of atopy and an early onset of the disease. It is interesting to note that these patients had essentially normal lung function and doctor's visits were minimal. Cluster 2 is a prominent group with the largest population with mild-to-moderate allergic asthma. This group like cluster 1 had history of atopy and early onset; however, strikingly this group had borderline low FEV1 values. The next group, cluster 3, was quite different, with an older age range, late onset symptoms, and higher BMI. Cluster 3 patients had a reduced frequency of atopy, but still required frequent systemic corticosteroids and greater than three controller medications. Clusters 4 and 5 had an increased duration of illness and high healthcare utilization and comprised together a third of the patients. Cluster 4, severe variable allergic asthma, had longer duration symptoms, minimal reduction in FEV1, and almost normal reversibility with bronchodilators. In contrast, cluster 5 patients showed more marked reduction in FEV1 and less marked bronchodilator response. Cluster 5 is characterized by fixed airway obstruction which was associated with increased utilization of health-care resources (Jarjour et al. 2012).

1.3.2 Allergic Asthma

As noted, the term "allergic asthma" is used by many clinicians and patients because the term, and associated clinical phenotype, provides physicians with a basic mechanistic understanding of the disease and gives patients an understanding of the cause of their symptoms. Allergic reactions to otherwise innocuous agents remain the hallmark of allergic asthma. These responses are dependent on initial exposure to allergen and subsequent development of IgE antibody that reacts to that allergen, a process known as sensitization. IgE is bound to mucosal mast cells that express the high-affinity receptor for IgE, (FcεR1). Later exposure of those mast cells to the sensitizing allergen results in mast cell activation, inflammatory mediator and cytokine release, and the development of characteristic eosinophilic airway inflammation and airway hyperresponsiveness (NHLBI 2008). Allergic asthma patients thus exhibit airway eosinophilia, mast cell recruitment, positive skin test responses to aeroallergens, and elevated serum immunoglobulin E (IgE) (NHLBI 2008; Abbas et al. 1996; Walker et al. 1992). The concept of Th1–Th2 lymphocyte imbalance is now decades old, and the evidence for Th2 bias in the development of allergic asthma is compelling (Abbas et al. 1996). T-cell differentiation to the Th2 cellular pathway leads to the production of prototypical cytokines, specifically interleukin 4 (IL-4), interleukin 5 (IL-5), and interleukin 13 (IL-13) (Wills-Karp et al. 1998). The role of IL-13 in airway hyperresponsiveness appears to be of particular note, because there are mechanistic, diagnostic, and potential therapeutic implications of this finding (Gauvreau et al. 2011).

1.3.3 Exercise-Induced Asthma

Exercise-induced bronchospasm (EIB) refers to airway constriction that occurs following exercise. The specific underlying mechanisms remain controversial (McFadden 1995). Asthma symptoms, commonly, shortness of breath and wheezing, occur well after the onset of exercise (McFadden 1995) and typically are experienced upon cessation of exercise for up to 10 min after exercise. Recovery time varies, but most patients return to baseline respiratory status within 60 min of exercise cessation (Edmunds et al. 1978). There are two prominent theories underlying the mechanism of EIB: heat loss and hyperosmolality. During and after exercise, the airway mucosa is exposed to rapid, large volumes of air which may be inadequately warmed, not fully humidified, or both (Randolph 1997; Anderson and Daviskas 2000). Either heat loss or water loss (and osmotic change) is thought to trigger release of inflammatory mediators (McFadden and Gilbert 1999; Anderson and Daviskas 1999). Regardless of the initiating mechanism, the clinical presentation of EIB is well described, and aggravation of bronchospasm may be seen in almost half of asthma patients (Hallstrand et al. 2002). This clinical phenotype is useful, because therapeutic use of short-acting beta2 agonists prophylactically prior to exercise can blunt or eliminate the development of symptoms.

1.3.4 Induced Phenotype

The abovementioned clinical phenotypes in asthma give the physicians and researchers a basic structure to categorize patients to understand the disease process and improve clinical management. Infections with bacteria (sinusitis) and viruses and other allergic or environmental exposures can also increase the severity of presentation. In contrast, the administration of effective controller medications can reduce the apparent severity of disease presentation, reducing symptoms, inflammation, and exacerbations. Inhaled corticosteroids remain the mainstay of chronic therapy for asthma; however, relative corticosteroid insensitivity has been noted, particularly in severe disease, and response to ICS is inconsistent (Bhavsar et al. 2008). One can consider a phenotype that is dependent on a specific environmental factor (allergen, virus, controller medication) to be an "induced phenotype": that is, a phenotype not expressed unless the environmental factor is present. This "induced phenotype" is important to recognize as it represents the clinical phenotype in the presence of therapy or other modifying factors (Pillai et al. 2012). The degree to which the clinical-induced phenotypes may inform mechanistic understanding and predict therapeutic responses is not currently known, but is an area of active research at present.

1.4 Cellular Phenotyping

The type and quantity of inflammatory cells in the airway of asthma has been used as a means to categorize the disease. Cellular phenotypes in asthma have been correlated with important features of clinical presentation, with neutrophilic disease being associated with acute severe asthma (Sur et al. 1993), and with steroid resistant asthma (Wenzel). These differences in cellular phenotype may also be associated with altered responsiveness to other therapeutic agents (Fahy et al. 1997; Pearce et al. 1999). Hence, it is plausible to suggest that the nature of cellular inflammation asthma might predict other important clinical outcomes.

Bousquet et al. (1990) demonstrated a correlation with the presence of eosinophils in blood and BAL fluid with asthma severity. Later, Wenzel et al. identified eosinophilic and noneosinophilic cellular subtypes in a group of severe asthma patients and noted a thickening of the subepithelial layer in the eosinophilic phenotype (Wenzel et al. 1997, 1999). The concept of a neutrophilic cellular phenotype was supported by studies that associated increased airway obstruction with neutrophilic infiltration (Shaw et al. 2007; Green et al. 2002). The eosinophilic phenotype is associated with a significant benefit from corticosteroids (Berry et al. 2007), while the neutrophilic phenotype is associated with a poor steroid response (Berry et al. 2007). The neutrophilic phenotype associates with acute severe asthma exacerbations in fatal and nonfatal status asthmaticus (Fahy et al. 1995; Sur et al. 1993). However, cellular phenotypes overlap, and a mixed eosinophilic and neutrophilic phenotype has been associated with severe respiratory symptoms (Hastie et al. 2010).

1.5 Molecular Phenotyping

Identifying, analyzing, and validating the presumed links between specific clinical presentations of asthma (“clinical phenotypes”) and the associated underlying molecular pathways may offer further insight in tailoring therapy to a specific patient or patient populations. In essence, uncovering the fundamental mechanisms and pathways will establish the basis of variable clinical presentations. The term molecular phenotyping implies the use of quantitation of specific molecular species to develop mechanistically similar subgroups of asthma. Molecules of interest and potential utility include nucleic acids, proteins, posttranslational modifications, and metabolites. Small molecules are easily analyzed using high-throughput analytics and are therefore attractive candidates for the development of biomarkers. We will review briefly two approaches to molecular phenotyping: gene-expression-based and protein-expression-based methods. Because genetics are essentially static throughout life, genetic associations in genomic DNA may inform presentation and course of disease, response to therapy, and associated clinical features, but do not

well address the clinical temporal variability of asthma, which is punctuated by exacerbation and remissions. To address these questions, an understanding of the regulated elements (mRNA, gene expression; protein types and quantities, protein expression; and end products, metabolites) is necessary. The field of metabolomics in asthma is in its infancy; this review will focus on gene- and protein-expression-based approaches. These brief considerations are exemplary, not exhaustive in scope. A more comprehensive discussion of Genetics, Genomics, and Epigenetics can be found in Sect. 2 (Chaps. 9, 10, 11).

1.5.1 Molecular Phenotyping Based on Gene Expression

In the last decade technological advances have permitted addressing scientific questions regarding the relationship between gene expression and clinical presentation of asthma. A seminal example of this approach was that of Woodruff and colleagues who studied gene-expression profiling of airway epithelial cells in asthma. They identified a group of genes the expression of which was enhanced by IL-13 (Woodruff et al. 2007, 2009). Further, there was variability in IL-13 expression, and subgroups of asthmatics were identified with both high and low levels of IL-13, indicating high and low levels of activation of Th2-like pathways, despite similar symptoms. CLCA1, periostin, and serpinB2 are downstream of IL-13 and were increased by exposure to IL-13. Corticosteroid treatment suppressed expression of these genes (Woodruff et al. 2009). Furthermore, the classification of asthmatic patients into “Th2 high” and “Th2 low” correlated with different degrees of airway inflammation and with corticosteroid responsiveness (Woodruff et al. 2007, 2009). Hence, this approach to molecular phenotyping provided both important mechanistic information and also a potential predictive analytic for inhaled steroid responsiveness.

1.5.2 Molecular Phenotyping Based on Protein Expression

An alternative and complementary approach to molecular phenotyping is based on the expression of proteins in the organ of interest, in this case the airway. Using the sample bank from the US Severe Asthma Research Program, Brasier and colleagues measured a panel of cytokines in BAL fluid from asthma patients with severe asthma and compared them with asthma patients with mild-to-moderate disease (Brasier et al. 2008). Protein expression of a broad panel of inducible proteins was measured, including markers of innate immune activation, Th1 activation, Th2 activation, and relevant chemokines. Using unsupervised cluster analysis, four cytokine expression clusters were identified, one of which was highly enriched with patients who had severe asthma. Further analysis of this protein-expression dataset demonstrated that clinically important intermediate phenotypes could be predicted

solely by protein-expression data and that these intermediate phenotypes in turn mapped to the clinical syndromes of severe asthma and mild-to-moderate asthma (Brasier et al. 2010). These findings suggest that protein-expression data may be a useful platform from which predictive biomarkers for therapeutic response and other clinically important outcomes could be based.

Additional advanced analytics yielded additional insights. Using network analysis and visual analytics, Bhavnani and colleagues examined this same protein-expression dataset (Bhavnani et al. 2011). Cytokine expression clustered by pathway: Th2-like cytokines clustered together, and innate immunity cytokines clutered together, but in a different space than the Th2 cytokines. Further, patients with mild-to-moderate asthma tended to cluster with the Th2 cytokines, whereas the patients with severe asthma tended to cluster with markers of innate immune activation. This analysis then provided important mechanistic insights in both severe and non-severe asthma. Accordingly, the promise of molecular phenotyping for a variety of important predictive analytics is great.

1.6 Summary

Asthma is an inflammatory disorder characterized by airway obstruction, airway hyperresponsiveness, and airway inflammation, all of which are variable among patients and variable in time within any specific patient. Understanding the mechanism that underlies this observed variability, and using that understanding to advance the science of asthma and the care of asthmatic patients, is an essential purpose of developing phenotypes. Clinical phenotypes have been used for decades, but overlap each other, and do not map cleanly to either pathophysiologic mechanism or with therapeutic response. Molecular phenotyping, although as yet only partially developed, offers the promise of dissecting the mechanistic underpinnings of the variability of asthma and of providing predictive therapeutics for the benefit of patients with this common and troubling disease.

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Chapter 2
Epidemiology of Asthma: Prevalence and Burden of Disease

Sharon Croisant

Abstract While clinical guidelines clearly define mechanisms for asthma diagnosis based upon history, lung function testing, symptoms, and physical examination, surveillance for asthma is much less straightforward. Epidemiologists have long debated the best means of assessing the scope and burden of asthma, seeking to reduce the potential for confounding introduced by differential means of diagnosis and even slight differences in surveillance questions, both of which can bias surveillance results such that we over- or undercount cases. This chapter will provide an overview of asthma epidemiology in the USA and internationally, as well as review of the data and findings from the major surveillance systems, a discussion of a networked approach to the science and evaluation of therapeutic treatments using the exemplar of the Inner-City Asthma Network, and assessment of public health implications.

Keywords Clinical guidelines • Epidemiology • Surveillance • Asthma prevalence • Public health

2.1 Introduction to Asthma Epidemiology

While clinical guidelines clearly define mechanisms for asthma diagnosis based upon history, lung function testing, symptoms, and physical examination, surveillance for asthma is much less straightforward. Given that asthma is a non-reportable disease in the USA, we rely upon a surprisingly complex,

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multifaceted system that differs from agency to agency and often from state to state. This is even more problematic for global surveillance where diagnostic practices may differ even further among countries and cultures depending upon patients' access to medical care and an adequate, well-trained health professions workforce. Epidemiologists have thus long debated the best means of assessing the scope and burden of asthma, seeking to reduce the potential for confounding introduced by differential means of diagnosis and even slight differences in surveillance questions, both of which can bias surveillance results such that we over- or undercount cases. Regardless, however, there is little disagreement that even with data from such a wide variety of different systems, asthma has increased markedly since the 1970s, despite a lack of consensus regarding causes for new onset disease. Virtually all surveillance systems and programs, both nationally and internationally, indicate an increasing burden of disease in terms of both prevalence (the proportion of existing cases in a population at a given time point) and incidence (onset of new cases within a specified period of time).

This chapter will provide an overview of asthma epidemiology in the USA and internationally. From a translational standpoint that finds the importance of the data in the use to which it is put, it includes a review of the data and findings from the major surveillance systems, a discussion of a networked approach to the science and evaluation of therapeutic treatments using the exemplar of the Inner-City Asthma Network, and assessment of public health implications. If one subscribes to the view that most cases of asthma are treatable and controllable, then it serves to follow that through an integrated approach to treatment and education, even greater reductions in morbidity and mortality can be achieved, especially in Emergency Department visits and unnecessary hospitalizations.

2.2 Sources of Data and Methods of Surveillance

2.2.1 USA

The United States Centers for Disease Control and Prevention carry out a wide variety of surveillance activities to determine the scope of asthma and the burden of disease, including prevalence of asthma, number of emergency department visits with a primary diagnosis of asthma, and both hospitalizations and deaths due to asthma. They also track other more indirect effects of asthma morbidity, including the limitations on activities of daily living, the number of days of work and/or school lost due to asthma, the use of medications including both rescue and control drugs, the proportion of patients who receive asthma self-management education, and the number of physician visits. Much data is collected through administration of the National Center for Health Statistics (NCHS) surveys and the Vital Statistics System. State-level data are provided from administration of the Behavioral Risk Factor Surveillance System (BRFSS) and its periodic Asthma Call-back Survey

(ACBS). While many other sources of data are readily available, the following CDC surveys (CDC 2012) largely drive development of public health programs and development of policies and guidelines, including:

- BRFSS: a random-digit-dialing telephone surveillance program designed to monitor the prevalence of adult asthma, morbidity, and mortality.
- ACBS: a comprehensive asthma survey carried out with BRFSS survey respondents reporting a previous diagnosis of asthma.
- National Health Interview Survey: a multistage probability sample survey carried out via interview with representative US households that includes questions related to both health and demographic information.
- The National Ambulatory Medical Care Survey: a survey conducted with non-federally employed office-based physicians to provide information on provision and use of ambulatory medical care services.
- The National Hospital Ambulatory Medical Care Survey: a survey conducted with Emergency Departments (ED) and outpatient departments of noninstitutional general and short-stay hospitals to provide information on utilization and provision of ambulatory care services in ED and outpatient clinics.
- The National Hospital Discharge Survey (NHDS) and The National Hospital Care Survey (NHCS): The NHDS, a national probability survey, was carried out yearly from 1965 to 2010 to collect inpatient data related to hospitalization stays for patients who had been discharged from non-federal short-stay US hospitals. The NHCS integrated the NHDS surveillance methodology with new survey questions that expanded the study to include ED, outpatient clinic, and ambulatory surgery center data collected as a part of the National Hospital Ambulatory Medical Care Survey. Combining data from these two surveys, along with inclusion of personal identifiers, now makes it possible to link findings to both the National Death Index and Medicaid and Medicare data, an important step in allowing a more comprehensive analysis of patient morbidity and trends in patient outcomes related to provision of care in both inpatient and outpatient settings.
- The National Environmental Public Health Tracking Network: a system of integrated health, exposure, and hazard information and data from a variety of national, state, and city sources.

2.2.2 Global Surveillance

Historically, the two largest global studies of asthma are the European Community Respiratory Health Survey (ECRHS), which involved surveillance of adults and the International Study of Asthma and Allergies in Children (ISAAC). Phase I of the ECRHS was initiated in the 1980s in response to the increasing concern related to a global increase in asthma prevalence. This was an important initiative, particularly since it was the first study of its kind to carry out a multi-country study of allergic

disease and asthma using a standardized protocol. This two-stage study, carried out primarily in Europe, enrolled approximately 140,000 participants in a survey, with 26,000 enrolled in a clinical component. The purpose of the study was to investigate variation in the prevalence of asthma and asthma-like disorders, to better assess factors associated with risk for asthma, and describe variations in therapeutic asthma treatments in European countries. A follow-up study was carried out from 1998 to 2002, with a second follow-up now in the planning stages. The ECRHS was and continues to be important in providing data related to asthma causation. The first phase provided some of the first epidemiologic data identifying the importance of the effect of environmental exposures on asthma (ECRHS 2012).

Phase I of the ISAAC, rolled out in 1991, focused on investigating asthma, allergic rhinitis, and eczema. Like the ECRHS, a major emphasis was to carry out a large epidemiological investigation in multiple countries and study sites utilizing a uniform—and for the time period, unique—protocol. Phase I of the study utilized standard, age-appropriate questionnaires, combined with a videotape that provided children with illustrative examples of asthma signs and symptoms, in an effort to reduce confounding associated with lack of understanding of the disease and its manifestations. Phase II was built upon the knowledge gleaned in Phase I, including more in-depth studies in a smaller number of centers. Phase III was a follow-up of Phase I that investigated childhood asthma, rhino-conjunctivitis, and eczema worldwide. It also provided us with a world map of asthma prevalence. Phase IV, the current study, includes developing and expanding the ISAAC website, largely to provide resources for investigators and practitioners from low- and middle-income countries. An important addition is the inclusion of asthma management plans and other resources that have been documented as improving patient outcomes. Since initiation in 1991, the ISAAC has been carried out in 306 centers in 105 countries with nearly two million children (ISAAC 2012).

The 2004 publication of the Global Burden of Asthma (Masoli et al. 2004) combined data from the Phase I ISAAC and the ECRHS in an attempt to more comprehensively generate global estimates of asthma prevalence and disease burden. Prevalence varied tremendously from country to country, from as low as 0.7 % in children in Macau to 18.4 % in Scotland. Of real concern was the sheer number of cases at 300 million, with expected increases in developing countries undergoing rapid urbanization. This report, while important, was nonetheless a compendium of surveillance efforts that utilized different instruments and methodologies, thus limited (To et al. 2012).

To deal with the inconsistencies, in 2002–2003, the World Health Organization (WHO) developed and conducted the World Health Survey (BedirhanÜstün et al. 2003), which used a standardized protocol that would enable collection of data that allowed both between and within country comparisons. As a cross-sectional survey, the WHS was conducted within 70 of the 192 WHO member states, thus comprising the largest multi-country asthma surveillance of adults ever conducted. To et al. utilized this data to estimate and compare both the worldwide and country-specific burden of asthma, utilizing a stratified probability sampling design, where the sampling frame covered 100 % of the country's eligible adults ≥18 years of age in each

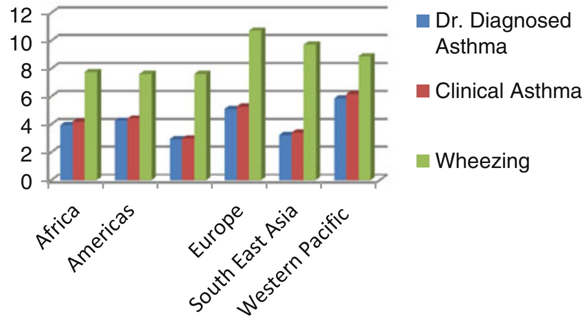

Fig. 2.1 Global asthma prevalence. Data abstracted from Stanojevic S, Moores G, Gershon AS, Bateman ED, Cruz AA, Boulet L-P (2012) Global asthma prevalence in adults: findings from the cross-sectional world health survey. BMC Public Health 12:204

of the countries. Of real importance was the effort to directly deal with issues of misdiagnosis and underdiagnosis by including questions related to previous diagnosis (doctor-diagnosed asthma), reported treatment for asthma either with or without a previous diagnosis (clinical asthma), and reported active wheezing/whistling breath in the past 12 months (symptoms of asthma) (To et al. 2012). Figure 2.1 depicts prevalence findings by region utilizing data from this report.

2.2.3 Limitations of Surveillance Methods

Although differences in surveillance methodology make it difficult to paint a comprehensive picture of the scope of asthma and the burden of disease, these fairly recent efforts to "marry" data sets and to launch new initiatives to standardize protocols have increased our understanding of national and global trends. Increasing interest is also focused on improving the evaluation of such surveillance systems, which are integral in driving many of our public health outreach efforts, interventions, and policies. In 2001, the CDC developed and published "Updated Guidelines for Evaluating Public Health Surveillance Systems" (CDC 2001), which provides six specific steps to evaluating surveillance programs:

1. Engage stakeholders
2. Describe system
3. Focus evaluation design
4. Gather evidence of system's performance
5. State conclusions and make recommendations
6. Ensure use of findings and share lessons learned

Of particular importance in gathering evidence of the system's performance is determining if the program was derived to include simplicity, flexibility, data quality, acceptability, sensitivity, positive predictive value, representativeness, timeliness, and stability. This is problematic for many of the surveillance systems in place, including several of the large-scale studies described. If criteria for a

clinical diagnosis of asthma are based upon multiple factors including history, symptoms, genetic predisposition, etc., then asthma surveillance in the form of a cross-sectional questionnaire must be carefully derived to be both reliable and valid. Many use differing means of determining whether the patient has ever had asthma or has current asthma. Few studies include a potential or probable diagnosis of asthma. Thus in the absence of a uniform case definition for asthma, multiple surveillance systems developed to characterize asthma are not only not generalizable to one another but may actually provide conflicting data that cannot easily be interpreted (Petronella and Ellis 2003). This is clearly indicated in the 2002 report of the Global Initiative for Asthma,

> Despite hundreds of reports on the prevalence and mortality of asthma in widely differing populations, the lack of precise definitions of asthma makes reliable comparison of reported prevalence from different parts of the world problematic. . . .because no epidemiological definition of asthma is emerging from current data, important components of epidemiological studies for asthma continue to include questionnaires, tests of airway hyper-responsiveness, and documentation of putative etiologic factors including atopic status.
> (GINA 2002, p. 13)

The NHIS survey, for example, assesses asthma prevalence by use of two questions: "Have you ever been told by a doctor or other health professional that you/your child had asthma?" and "Do you/your child still have asthma?" This was found to be a significant problem in school-based surveillance of childhood asthma which utilized these standard questions but also included diagnostic questions provided by physician asthma specialists. This particular study revealed that respondents who denied having received a previous diagnosis for asthma nonetheless reported morbidity due to asthma, including hospitalizations and ED visits. Among this group, Hispanics appeared to have been overrepresented, suggesting an underreporting of cases (Petronella et al. 2006). Still other surveillance questions for the purpose of identifying asthma cases are often based upon self-reported symptoms of wheezing and frequency of symptoms. Clearly, not all episodes of wheezing are due to asthma, and many patients may present with other symptoms such as a persistent cough in the absence of a cold. It is also well known that surveillance based upon personal recall that is not able to be validated by objective measure introduces the possibility for bias that in turn reduces our confidence in findings from surveillance studies. This potential for error is recognized, and as is demonstrated in the following sections, surveillance systems at the national and international levels are beginning to reassess the way we carry out and evaluate surveillance studies and systems.

2.3 Prevalence of Asthma: USA

In the past several decades, asthma prevalence has dramatically increased both nationwide and globally, sparking concern over what has been referred to as an "asthma epidemic." According to a 2012 report published by the CDC utilizing data from the National Health Interview surveys, rates have continued to rise.

In 2001, 7.3 % of Americans of all ages (20.3 million persons) had been diagnosed with asthma, compared to 8.2 % (24.6 million persons) in 2009, a 12.3 % increase (Schiller et al. 2012; Zahran et al. 2011). Of these, 17.5 million were over the age of 18, and 7.1 million children ages 0–17 (Akinbami et al. 2012a, b). Prevalence among children was 9.6 % and highest among low-SES (13.5 %) and non-Hispanic black children (17 %). Prevalence among adults (7.0 %) was revealed to be higher in women (9.7 %), and similar to children, among low-SES adults (10.6 %). As might be expected, those without health insurance reported not being able to purchase prescribed asthma medication (40.3 %), and only 58.6 % indicated they were able to afford to see a primary care physician about their asthma. This differs sharply from those with insurance, of whom only 11.5 % reported not being able to afford their medications and 85.6 % reported being able to afford to see a PCP (Zahran et al. 2011).

Significant disparities are observed among minority groups with asthma. More than one in four black adults and one in five Hispanic adults report not being able to afford their prescriptions, while one in four black adults and one in seven Hispanic adults report not being able to afford routine doctor's visits (CDC, National Asthma Control 2012). In 2007–2009, black Americans were found to be far more likely to die of asthma than whites, and asthma death rates were found to increase with age for both blacks and whites (Gorina 2012). Poverty and race/ethnicity considered together reveal even sharper disparities. While among the poor, non-Hispanic whites (12.5 %) and blacks (12.2 %) have similar prevalence rates, Puerto Rican Hispanics and the multiracial were found to have significantly higher prevalence of disease at 22.4 % and 20.5 %, respectively. Similar trends were reported for the near poor and the nonpoor (Moorman et al. 2011).

Trends revealed increases in asthma across all demographic groups. From 2001 to 2009 childhood asthma increased from 8.7 to 9.6 %. Among adults, prevalence increased from 6.3 % to 7.1 % in males and from 8.3 % to 9.2 % in females. Increases were also found for each ethnic group: whites, up from 7.2 % to 7.8 %; blacks, up from 8.4 % to 10.8 %; and Hispanics, up from 5.8 % to 6.4 %. Significant differences were found for age, gender, and race/ethnicity across the time frame as well. Among non-Hispanic black children, the increase was pronounced, up from 11.4 % to 17.0 %. Increases were also found for non-Hispanic white women (up from 8.9 % to 10.1 %) and non-Hispanic black men (4.7 % vs. 6.4 %) (Zahran et al. 2011).

A separate study evaluating NHIS data from 2005 to 2009 revealed similar results, with significant health disparities among ethnic groups and socioeconomic classes. Adult females were found to have a higher prevalence rate of current asthma (9.3 %) than males (7.0 %); however, the reverse was true for children, with boys at significantly increased risk (11.3 %) compared to girls (7.9 %). Prevalence was found to be highest among the black population (11.1 %) and lowest in the Asian population (5.3 %). While Hispanics had lower prevalence (6.3 %) than other ethnic groups, prevalence was lower in those of Mexican heritage (4.9 %) and significantly higher among Puerto Ricans (16.6 %). A pronounced increase in prevalence was seen by socioeconomic group, with those at 200 % of the poverty rate and above at

7.3 %, those at 100 % but less than 200 % of the poverty rate at 8.5 %, and those below the poverty rate at 11.6 %. Little differences were found between metropolitan dwellers (8.1 %) and non-metropolitan (8.7 %) (Akinbami et al. 2012a, b). Asthma death rates per 1,000 persons revealed 30 % higher rates for females than males, with elevated rates for blacks as well (Akinbami et al. 2012a, b). Asthma was linked to 3,447 deaths (approximately 9/day) in 2007 alone (CDC 2011). It should be noted that ethnic differences in asthma morbidity and mortality have been found to be linked to conditions such as poverty, inner-city environments, poor air quality both indoors and outdoors, and lack of patient education and access to appropriate medical treatment (Asthma and Allergy Foundation of America 2012).

The burden of asthma is significant, both in terms of financial expenses and in lost productivity. Asthma costs increased from $53 billion in 2007 to approximately $56 billion in 2009 (CDC 2011).

According to the Asthma and Allergy Foundation of America (AAFA), every day in the USA, the burden of morbidity associated with asthma is monumental. Over 40,000 people will have an asthma exacerbation, while 36,000 children will miss school and 27,000 adults will miss work due to asthma. Almost 5,000 will visit the ED and 1,200 will be admitted to the hospital for treatment of asthma. Asthma is also the number one cause of school absenteeism in the USA, accounting for more than 13 million missed days of school annually (Asthma and Allergy Foundation of America 2012).

2.4 Prevalence of Asthma: Global

The prevalence of asthma in different countries throughout the world varies a great deal. Previously, the rates were higher in more developed countries, but the gaps are now closing, given the rise in prevalence in low- and middle-income countries and plateauing in high-income countries. Prevalence has been associated with adoption of a modern, urban lifestyle; however, it is unclear what specific factors are causal in nature. Increased prevalence of asthma is also strongly correlated with increases in allergy, a trend observed over the past 40 years. Globally, approximately 300 million people have asthma, with 250,000 annual deaths attributed to the disease—most of which are preventable. Mortality rates do not appear to be correlated with prevalence but rather are highest in countries where access to appropriate medications is lacking, specifically, controller meds (World Health Organization 2007). Worldwide, as in the USA, asthma accounts for many days of lost productivity in terms of missed school and work days. Disability-adjusted life years attributed to asthma are estimated at 15.3, ranking asthma at 22nd worldwide, similar in impact to other chronic diseases such as diabetes or Alzheimer disease (Masoli et al. 2004). An important risk factor in low- and middle-income countries is smoking and exposure to secondhand smoke. Prevalence of active smoking in adults with asthma in these countries is approximately 25 %, which places them at increased risk of more severe asthma symptoms, decline in lung function, and reduced response to corticosteroid therapy. Based upon existing data,

the World Health Organization estimates that by 2025, the number of people with asthma will increase by 100 million, suggesting that marked increases in interventions be accelerated to minimize morbidity and its associated human and financial costs (World Health Organization 2007).

The World Health Survey described previously found great disparities between countries for doctor-diagnosed and clinical asthma as well as reported symptoms. Worldwide, the prevalence of doctor-diagnosed adult asthma in adults was reported as 4.3 % (95 % CI: 4.2; 4.4). Among the 70 countries included in the study, doctor-diagnosed asthma ranged from virtually no asthma in China (0.2 %) to a high of 21.0 % in Australia. Similar results were found for clinical asthma at a worldwide prevalence of 4.5 % (95 % CI: 4.4; 4.6), again with great disparities observed between and among countries, from a low of 1.0 % in Vietnam to 21.5 % in Australia. In rank order, the five participating countries with the highest prevalence of clinical asthma were Australia at 21.5 %, Sweden at 20.2 %, the UK at 18.2 %, the Netherlands at 15.3 %, and Brazil at 13.0 %. Reported asthma symptoms including wheezing or whistling in the chest in the past 12 months were reported as 8.6 % (95 % CI: 8.5; 8.7). The same five countries were found to have the highest prevalence of reported symptoms, albeit in slightly different rank order, with Australia topping the list at 27.4 %, the Netherlands at 22.7 %, the UK and Brazil tied at 22.6 %, and Sweden at 21.6 %. As observed in Fig. 2.1, which utilizes To and colleagues' data, differences in doctor-diagnosed and clinical asthma are negligible within regions, but with marked differences in prevalence of reported symptoms. While in general the differences in doctor-diagnosed and clinical asthma are not remarkable, the Western Pacific region has the highest prevalence, driven primarily by the high prevalence of asthma in Australia. Similar observations are made for Europe. For those who reported clinical asthma, 49.7 % also reported current symptoms, the highest proportion of whom are from Southeast Asia at 57.9 %. It should be noted, however, that this may be a manifestation of lack of treatment since many indicated that they had never been professionally treated for asthma. This might also be driven by prevalence of smoking since in Europe and Southeast Asia, more than one third of participants indicated being current smokers (To et al. 2012).

2.5 From Bench to Bedside: Inner-City Asthma Consortium

For decades it has been known that childhood asthma in the inner-city population tends to be increased, if not complicated by a host of factors which range from exposure to environmental pollutants including poor indoor and outdoor air quality and allergens as well as social factors including socioeconomic level, access to health care and prescription medications, appropriate follow-up, and even stress. While it is not clearly understood if mechanisms of asthma in the inner-city population actually differ from asthma in those who do not reside in this environment, by the 1990s it was becoming increasingly clear that this was an important scientific issue, especially as it related to guiding development of evidence-based, effective medical and public health interventions to reduce morbidity and increase quality of

life for inner-city children with asthma. To address this, the National Institute of Allergy and Infectious Diseases began what could now be described as one of the first comprehensive translational research programs by establishing the Inner-City Asthma Network Program in 1991, with a goal of improving outcomes for at-risk children in urban environments (Busse 2010).

The first of the networks established was to carry out the National Cooperative Inner-City Asthma Study over the period 1991–1997. This important study was focused on identifying environmental factors that might be causal factors in increased prevalence of childhood asthma and development of intervention strategies based upon these findings. While a number of key factors emerged indicating increased morbidity was associated with access to care and consistent treatment regimens, several environmental observations were made, namely, that sensitization to cockroach, house dust mite, and mouse was associated with increased hospitalizations, wheezing, and emergent medical visits. The second network, the Inner-City Asthma Study, was carried out from 1994 to 2001. This study was to design and conduct multicenter intervention trials to reduce asthma morbidity among inner-city children with asthma. This intervention study consisted of remediating exposure of enrolled children to cockroaches, dust mites, and rodents in addition to other environmental contaminants including pets, secondhand smoke, and molds. Those children in the intervention group were documented to have significant reductions in average maximal number of days with asthma symptoms and reduced number of emergent healthcare use for treatment of asthma; however, increases in lung function were not observed (Busse 2010).

The third NIAID-sponsored network, carried out from 2002 to 2009, was the Inner-City Asthma Consortium I. During this time, the Consortium focused on establishing a birth cohort, completing and publishing the results of the Asthma Control Evaluation trial, and beginning to investigate the use of immunotherapy treatment with cockroach antigen for children with asthma. A second phase of the Consortium work, scheduled to be conducted from 2009 to 2014, will involve investigating the birth cohort for immunologic causes of asthma, as well as designing, developing, and implementing clinical intervention protocols to evaluate immune-based therapies. Importantly, this phase will now be more team based than ever before. With a strong infrastructure for clinical trial management including a statistical and clinical coordinating center and the addition of two basic science projects focusing on epigenetics and microbiota, the networks have and continue to be strong examples of effective translational research (Busse 2010).

2.6 From Bench to Curbside: Public Health Implications

The experiences of the Inner-City Asthma Networks clearly demonstrate the potential for a translational approach to science to yield important findings that in turn are driving development of effective treatments and protocols for treatments for inner-city

children with asthma. The next logical step is to expand efforts to adopt a T4 or public health approach to positively impact both children and adults with asthma.

With appropriate access to health care and medications and avoidance of asthma triggers, most people with asthma can control their disease, thus greatly reducing the number of asthma ED visits and hospitalizations and largely enjoying symptom-free lives. However, in 2008, less than half of people surveyed in the USA reported receiving education on how to avoid triggers. Of those who did report receiving patient education, only 48 % followed prescriptive advice in the absence of a personalized asthma care plan. Public health education programs, geared toward both providers and patients, are needed to increase appropriate patient training and the likelihood that each provider/patient pair collectively creates a personalized action plan. This is an important component of the National Asthma Education and Prevention Program guidelines, which call for assessing and monitoring, patient education, control of factors contributing to asthma severity, and appropriate medical treatment (NHLBI 2007; Zahran et al. 2011). Intervention programs, including patient education programs, have been demonstrated to be successful in reducing both hospitalizations and deaths from the disease (Szefler et al. 2010). One study of 3,748 low-income, primarily minority children in the USA revealed marked decreases in hospitalizations (35 %), a 27 % decrease in asthma-related visits to an ED (World Health Organization 2007; Cloutier et al. 2005). This can and should be an important initiative for public health practitioners and organizations as well as the asthma coalitions that have been formed at both state and national levels.

Additional public health measures should include a more integrated tracking infrastructure for asthma rates and the effectiveness of control measures. Doing so will enable improvement of prevention efforts and thus reduce both morbidity and costs of asthma care. Preventive measures can include improvement in influenza and pneumonia vaccine rates for patients with asthma and promotion of healthy indoor and outdoor air quality through policies geared toward smoke-free environments as well as healthy schools and workplaces (CDC 2011).

2.7 Summary

While asthma has no cure, with combined efforts in environmental controls to minimize exposure to allergens and irritants, appropriate pharmacologic therapy, and patient and healthcare provider education, the disease can be successfully managed to reduce morbidity, mortality, and financial costs. Efforts must also be increased to ensure access to appropriate health care and effective case management, which are essential to any comprehensive plan to address asthma as a public health problem. A multidisciplinary approach spanning T1 through T4 translational research coupled with such a public health plan is promising and has already demonstrated success in reducing the burden of disease.

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Chapter 3
Heterogeneity of Asthma in Society

Rohit Divekar and William J. Calhoun

Abstract There has been an increased interest in studying other factors that affect asthma pathogenesis and cause heterogeneity in prevalence and incidence of asthma. The reason there are such varied expression patterns of disease in asthmatics is because of multiple variables that affect the pathogenesis of asthma. As an exemplar of an epidemiologic variable, we will discuss geographical location, obesity and vitamin D status of the individual, and their effects on asthma burden in humans. There is varying data regarding the prevalence or severity of asthma in urban versus rural setting which is likely related to the difference of the populations studied, complexity of causal variables involved, and local geographic factors. In addition to cross-sectional and cohort studies in humans, animal models and studies have established a link between asthma and obesity by investigating the mechanisms affecting both disease processes. The complicated interrelationship between obesity and asthma is an active area of epidemiological and experimental research with new insights being discovered at a rapid pace. Finally, vitamin D, an important immunomodulator thought to be important in pathogenesis of asthma, has both mechanistic and therapeutic implications in treatment of asthma. The influences of these factors on the clinical expression of asthma are discussed below.

Keywords Rural • Urbanization • Obesity • Population differences in asthma burden • Vitamin D • Public health and asthma • Heterogeneous expression of asthma in population

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3.1 Key Epidemiologic Factors and Influences on Asthma

The prevalence of asthma is diverse and the penetrance of the disease in the population is variable. Since there are multiple pathogenetic factors (Fig. 3.1) that are responsible for evolution of the disease process including but not limited to environmental factors (air pollution, pollens, mold and other aeroallergens, and weather), host factors (geographical location, BMI, smoking status, nutritional factors), agent factors (infectious agents like respiratory syncytial virus RSV, rhinovirus, etc.), and genetic factors (asthma susceptibility loci on genes involved in pathogenesis of the disease), the burden of asthma in the society is also mixed. Some factors have a direct input on the expression of the disease state like exposure to infectious asthmogenic viruses or exposure to allergens, while other factors act in more subtle ways. In the section that follows, we will discuss the effect of three factors as an exemplar of direct or indirect effect on the expression of the disease state in the population and will cover urban–rural differences in asthma prevalence, obesity and its link to asthma and vitamin D status, and its role in pathogenesis of asthma. Examples of triggers that act indirectly include obesity and nutritional state of the individual and have a broad influence on immunological or local (respiratory epithelium or bronchial smooth muscle) physiological mechanisms that lead to development of asthma. Some factors like geographical location act indirectly or directly because there are many things that influence the asthmogenic triggers like local weather, environmental pollutants, prevalent aeroallergens, and effects of local ecosystems.

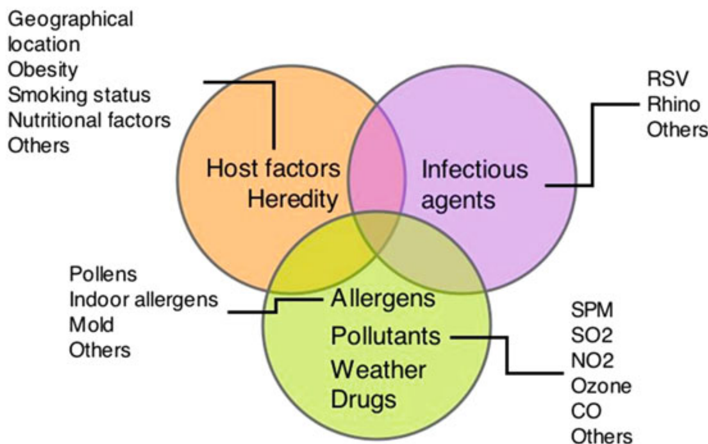

Fig. 3.1 Multiple causal factors in expression of asthma in society

3.2 Role of Urbanization

Asthma has a genetic component in pathogenesis, but the effect of the environment is also extremely important, and the increased exposure to air pollutants and inhalants may intuitively suggest an increase in respiratory-related disease in adults and children. Outdoors air pollution including ozone, diesel fumes, smoke, and small particulate matter is thought to contribute to the pathogenesis of airway hyperresponsiveness and has been reviewed in Chap. 3. ISAAC study has demonstrated that globally for affluent nations, the combined odds ratio for association of atopic sensitization (positive skin prick test) and asthma symptoms (wheezing) was approximately twice compared to nonaffluent nations (Weinmayr et al. 2007). The authors observed a link between improving economic development and atopic sensitization and asthma symptoms.

3.2.1 Increased Asthma in Urban Setting: Case for Local Factors

It may be inferred that since economic progress shares some basis in urban-industrial development, urbanization directly or indirectly may be responsible for higher rates of asthma. This has been demonstrated to be true at least in few instances where prevalence of asthma increased with increasing level of urbanization (Rodriguez et al. 2011). Specific epidemiologic studies to address the question of urban–rural disparity in asthma prevalence have been conducted using the National Health Survey (1988), which showed significant increase in asthma reported from urban areas (7.1 %) as compared to rural areas (5.7 %) (Gergen et al. 1988). The finding that urban areas have higher prevalence of asthma than rural areas was also documented in Canada, where investigators looked at age-defined cross-sectional data and found that asthma had lower prevalence in rural areas (Lawson et al. 2011). These differences have been validated by direct bronchoprovocation challenge studies between rural and urban residents with higher hyperreactivity in urban children with asthma (Turner et al. 1986). Whether increase in asthma secondary to urbanization was a direct consequence of human development or due to increase in atopy from urbanization in the population is still being investigated. In a study by Bibi et al. of the 448 asthmatic children tested, those living in urban areas demonstrated more allergic response to indoor and outdoor allergens (Bibi et al. 2002). There are also clues indicating that urban environment affects asthma prevalence without affecting atopy. Environmental air pollution is thought to have a direct influence on asthma, especially in previously sensitized individuals, where it made preexisting symptoms worse (Corbo et al. 1993). GABRIEL advance studies have shown a protective effect of farm exposure in asthma independent of atopic status (Fuchs et al. 2012).

3.2.2 Conflicting Results: Rural Versus Urban Asthma Debate

As early as 1978, Bouhuys et al. published that there was no difference in lung function tests between lifetime residents in a rural area and those in a small industrial town in Connecticut, USA (Bouhuys et al. 1978). The incongruence between air pollution and airway responsiveness was mirrored when bronchial hyperresponsiveness and asthma in young students in Guangzhou, China, were studied, and there was no significant difference between urban and rural population (Zhong et al. 1990). This lack of difference in lung function between long-standing residents of urban versus rural residents was surprising and may reflect the local environmental effects or protective genetic factors in populations studied. The importance of location-specific results is demonstrated by studies that show that overall prevalence of asthma is not different between urban and rural residence areas (Morrison et al. 2009) or that the difference is in increased morbidity from asthma in rural areas as opposed to prevalence (Pesek et al. 2010).

NHANES II found that residents of urban areas reported reduced prevalence of asthma as compared to residents of rural areas (Turkeltaub and Gergen 1991). In a study based in Tennessee, USA, rural children had increased asthma prevalence but similar asthma morbidity compared to urban children (Valet et al. 2011). The conflicting data regarding the different results of prevalence of asthma in urban setting is likely related to the heterogeneity of the populations studied, complexity of causal variables involved, and local geographic factors. Each defined geographic location has a distinct and unique set of factors that may be contributing to pathogenesis of asthma.

3.3 Obesity

Asthma manifests with dyspnea and wheezing which can also be seen in obese individuals. There has been a tremendous interest in obesity-related diseases including asthma in the recent time. In addition to the cross-sectional and cohort studies in humans showing a modest increase in asthma with increasing prevalence of obesity, animal models and studies have also established a link between asthma and obesity by investigating the mechanisms affecting both disease processes (Beuther et al. 2006).

3.3.1 Correlative Studies of Obesity and Asthma

In children with single diagnosis of asthma, the prevalence of being overweight was significantly higher in those with moderate to severe asthma (Luder et al. 1998). Though the population here was predominantly black and Hispanic children from

inner cities, this was also reported to be an issue in women. In a prospective cohort after controlling for the confounding factors, relative risk for asthma increased as BMI increased. Moreover, women who gained weight after age 18 were at increased risk of developing asthma (Camargo et al. 1999). This propensity to develop asthma after weight gain was not limited to adult-onset obesity but was also documented in preadolescent girls who became obese between 6 and 11 years of age (Castro-Rodríguez et al. 2001). Thus, the risk for asthma shows an increase with increase in BMI since childhood. While data seem definitive in women (Guerra et al. 2002), a meta-analysis showed a similar increase in odds ratio of incident asthma due to increasing weight in men as well (Beuther and Sutherland 2007). Correlation with asthma is not universal with all morphometric types of obesity; central obesity was significantly associated with increased risk of nonatopic asthma (Appleton et al. 2006).

3.3.2 Mechanisms of Effect of Obesity on Asthma

While it is instinctive that increased weight may have a restrictive effect on lung function, there are, in addition, bronchospastic and non-bronchospastic mechanisms in place. While adult men with lowest and highest BMI were more likely to develop airway hyperresponsiveness (Litonjua et al. 2002) demonstrating a nonlinear relationship, each increase in BMI of five units in children was associated with a decrease in FEV1/FVC of over 1 % with mild-to-moderate asthma (Tantisira et al. 2003). The distinction between airflow limitation and dynamic airway hyperresponsiveness is important in obese individuals, and it may be that not all obese individuals have wheezing as a consequence of increase airway hyperreactivity.

Leptin and adiponectin are adipocyte-derived hormones, demonstrating immunomodulatory effects, and have been studied for their effect on asthma in human subjects. Adiponectin levels have been shown to be inversely related to asthma symptoms and positively with FEV1/FVC ratio in male subjects independent of body size (Kattan et al. 2010). Increased levels of leptin correlated with obesity status of children, and higher levels of leptin were associated with bronchial hyperresponsiveness induced by exercise challenge (Baek et al. 2011). Although associative studies do not offer insights into the causal role of the factors being studied, the direct role of adipokines in asthma has been elucidated in the laboratory using animal models of asthma. Leptin infusion augmented methacholine hyperresponsiveness and serum IgE response in mice alluding to a possible role of serum leptin in pathogenesis of asthma (Shore et al. 2005). In contrast to effect of leptin, adiponectin treatment has shown reduced OVA-induced allergic airway responses in mice indicating a protective effect of this hormone on pathogenesis of asthma (Shore et al. 2006).

In addition to airway hyperresponsiveness as a cause of increased asthma, other immune mechanisms have been investigated for their role in asthma with mixed

results. While obese difficult-to-treat asthmatics demonstrated an inverse relationship to sputum eosinophils and FeNO with increasing body mass (Van Veen et al. 2008), there was no association between BMI and airway inflammation (including eosinophils) as measured by sputum cell counts (Todd et al. 2007). Results of studies attempting to find associations between systemic inflammation and asthma have been unclear as well. The question whether asthma develops in parallel to obesity or precedes or follows obesity is a difficult one to answer in human subjects. Using a cohort of patients from the NHEFS (nutrition evaluation survey epidemiologic follow-up study), increasing BMI was associated with increased asthma prevalence, but during observation period there was no increased incidence of asthma with increasing BMI (Stanley et al. 2005).

3.3.3 Treatment Considerations in Obese Asthmatics

Obese adults had poorer asthma control and higher likelihood of asthma-related hospitalizations (Mosen et al. 2008). In addition to the pathologic aspects of disease itself, there was also a synergistic influence of obesity on asthma. Compared to normal children as controls, quality of life was poorer in children with obesity and asthma, compared to children with asthma and normal weight and only overweight children (Van Gent et al. 2007). Besides physical impediments to asthma care and issues with symptom perception (Lessard et al. 2008), elevated BMI has effects on response to medications on a pharmacological level. Higher BMI was associated with blunted in vitro response to dexamethasone in obese patients with asthma (Sutherland et al. 2008). Obese asthmatics demonstrated less improvement with inhaled corticosteroids as measured by lung function and exhaled feNO than lean asthma patients (Sutherland et al. 2009). This reduction in benefit was also seen with combination controller therapy in obese asthmatics compared to normal asthmatics (Camargo et al. 2010). These differences could be related to the difference in distribution of the drug, metabolism, and receptor function in obese individuals resulting in a distinctive response to medications. This complicated interrelationship between obesity and asthma is an active area of epidemiological and experimental research with new results being discovered at a rapid pace.

3.4 Vitamin D

Airway inflammation in asthma plays an important role in disease progression. Early observations suggesting a pivotal role of vitamin D modulation of immune system and the immunological basis of airway inflammation alludes to effect of vitamin D on asthma (Mora et al. 2008).

3.4.1 Relationship of Vitamin D and Pediatric Asthma

Insufficient vitamin D levels were associated with higher odds of exacerbation of asthma in children (Brehm et al. 2010). The magnitude of association of vitamin D deficiency and severity of asthma exacerbation was greater in children with non-atopic disease (Brehm et al. 2012). The relationship between vitamin D-deficient state and asthma was not only quantitative but also temporal because vitamin D levels at younger age were predictive of asthma–allergy phenotype at later ages (Hollams et al. 2011). Several studies have also looked at the role of maternal vitamin D levels and the outcome in offspring of those mothers. Surprisingly, both low and high levels of maternal vitamin D were associated with increased total and specific IgE through age 5 years, but this study did not find an association between vitamin D levels in cord blood and asthma in children (Rothers et al. 2011). While some studies found that higher maternal vitamin D levels were associated with decreased odds of asthma (Carroll et al. 2011), other investigators have not found a significant association between maternal late-pregnancy 25-hydroxyvitamin D status, and either asthma or wheeze at age 6 years (Pike et al. 2012) indicating that the role of maternal vitamin D levels in development of asthma in offspring is complex.

3.4.2 Adult Vitamin D and Asthma

There is increasing evidence regarding the role of vitamin D in adult asthma, and results from studies in non-pediatric segments of the population point towards an important role of vitamin D. Each 10 ng/ml decrease in vitamin D was associated with 8 % greater odds of asthma (Keet et al. 2011). A correlation between the latitude, sun exposure, and adult asthma prevalence has also been reported. Each 10-degree change in geographical latitude from southern to northern regions of eastern seaboard in the USA was associated with 2 % increase in adult asthma prevalence (Krstic 2011). This effect of vitamin D on the pathogenesis of asthma seems disease specific because baseline vitamin D levels had no relationship to rates of acute exacerbation of COPD or time to first exacerbation of COPD (Kunisaki et al. 2012).

3.4.3 Mechanisms of Vitamin D Modulation of Asthma

There is pathologic basis for poor asthma control and propensity to worsening in populations that have lower levels of vitamin D. Children with severe treatment-resistant asthma with lower vitamin D were associated with higher airway smooth muscle mass likely contributing to a worse lung function and poor asthma control (Gupta et al. 2011). Low vitamin D was associated with higher levels of serum IgE

in pediatric asthmatic patients studied (Goleva et al. 2012). In offspring of vitamin D-deficient mice, increase in proliferation and cytokine production was seen in airway draining lymph nodes in response to OVA-induced asthma signifying an activated immune phenotype (Gorman et al. 2012). Vitamin D has a myriad of biologic effects, which have been shown to orchestrate essential roles in immune activation including downregulation of co-stimulators CD80/86, RANTES, CD40, MMPs, and PDGF while upregulating immunomodulators like IL-10 (Sandhu and Casale 2010). In addition to its effect in cell surface markers and biomolecules, levels of vitamin D were found to be positively correlated with Treg numbers. Regulatory T cells (Treg) are important in damping immune responses from effector T and help suppress inflammatory states. In addition to affecting immune system, vitamin D deficiency also has been shown to affect the physical properties of the lung in animal models including demonstration of reduction in lung volume in mice (Zosky et al. 2011). Thus, it is evident vitamin D has broad and overreaching influence on the genesis and evolution of asthma.

3.4.4 Treatment Considerations

The implications of vitamin D deficiency in asthma are not only mechanistic but also have therapeutic consequences. In animal studies, administration of vitamin D3 in ovalbumin (OVA)-induced asthma model of mice inhibited bronchial hyperresponsiveness, OVA-specific IgE, airway eosinophilia, and Th2-related cytokines in conjunction with OVA immunotherapy (Taher et al. 2008). Studies in humans have supported the role of vitamin D in asthma as well with reduced vitamin D being associated with impaired lung function, increased AHR, and reduced response to glucocorticoids in nonsmoking asthmatic patients (Sutherland et al. 2010). Levels of vitamin D have been demonstrated to have strong inverse correlation to the use of inhaled steroids or oral steroids in children with asthma (Searing et al. 2010), and children treated with inhaled budesonide and supplemented with vitamin D had fewer asthma exacerbations (Majak et al. 2011). These results point to an important role of vitamin D in treatment of asthma. At the present time, there is little evidence that treating vitamin D deficiency improves asthma, but there are several prospective clinical trials underway testing the hypothesis. While it may be premature to measure vitamin D levels in all asthmatics, it may be worthwhile considering levels of vitamin D in difficult-to-control asthmatics or those with severe asthma.

3.5 Summary

It is important to realize the variegated clinical presentation of asthma and the heterogeneous expression of the disease in the population share some of the causal factors. Recognition of these expression patterns of disease and appropriate identification of triggers will help clarify pathogenic mechanisms and allow for a patient-specific treatment regimen.

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Chapter 4
Inhaled Environmental Allergens and Toxicants as Determinants of the Asthma Phenotype

Kristin Sokol, Sanjiv Sur, and Bill T. Ameredes

Abstract The driving environmental factors behind the development of the asthma phenotype remain incompletely studied and understood. Here, we present an overview of inhaled allergic/atopic and mainly nonallergic/nonatopic or toxicant shapers of the asthma phenotype, which are present in both the indoor and outdoor environment around us. The inhaled allergic/atopic factors include fungus, mold, animal dander, cockroach, dust mites, and pollen; these allergic triggers and shapers of the asthma phenotype are considered in the context of their ability to drive the immunologic IgE response and potentially induce interactions between the innate and adaptive immune responses, with special emphasis on the NADPH-dependent reactive oxygen-species-associated mechanism of pollen-associated allergy induction. The inhaled nonallergic/nonatopic, toxicant factors include gaseous and volatile agents, such as sulfur dioxide, ozone, acrolein, and butadiene, as well as particulate agents, such as rubber tire breakdown particles, and diesel exhaust particles. These toxicants are reviewed in terms of their relevant chemical characteristics and hazard potential, ability to induce airway dysfunction, and potential for driving the asthma phenotype. Special emphasis is placed on their interactive nature with other triggers and drivers, with regard to driving the asthma phenotype.

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Overall, both allergic and nonallergic environmental factors can interact to acutely exacerbate the asthma phenotype; some may also promote its development over prolonged periods of untreated exposure, or possibly indirectly through effects on the genome. Further therapeutic considerations should be given to these environmental factors when determining the best course of personalized medicine for individuals with asthma.

Keywords Acrolein • Adaptive immunity • Butadiene • Dust mites • Tire breakdown particles • Diesel exhaust particles • Innate immunity • NADPH oxidase • Ozone • Ragweed pollen • Reactive oxygen species • Sulfur dioxide

4.1 The Relationship of Allergen Exposure to Asthma Prevalence, Triggers, and Phenotypes

4.1.1 Seasonal Asthma and Allergen Prevalence: The Seasonal Asthmatic Phenotype

There has been a long-standing association between asthma and allergy in both children and adults. The link between asthma and certain indoor allergens, such as house-dust mite, animal dander, and cockroach, and the outdoor fungus *Alternaria*, has been extensively studied and is well recognized. However, those who are sensitized to outdoor aeroallergens carry less risk for asthma. Yet, once development of aeroallergen sensitivity has been established, IgE-mediated reactions are a major contributor both to acute asthmatic symptoms and chronic airway inflammation (Lemanske and Busse 2010). Although sensitization to outdoor allergens poses less of a risk for asthma, studies have shown exposure to outdoor allergens such as grass and ragweed pollen has been associated with seasonal asthma. There are seasonal variations in asthma, with symptoms improving in summer, and symptoms worsening in fall and winter in the USA. This seasonal allergen exposure and subsequent sensitization has been implicated in asthma exacerbations and even in sudden asthma-related deaths (Sykes and Johnston 2008).

The NHANES (National Health and Nutrition Examination Survey), one of the landmark cohorts to study asthma, provided data on the importance of reactivity to certain aeroallergens and the subsequent effect on respiratory disease. The NHANES II, published in 1992, specifically presented which allergens are directly associated with asthma (Gergen and Turkeltaub 1992). The number of positive allergen skin prick tests correlated with the risk of asthma. Reactivity to any one allergen increased the risk of asthma by two to three times, except for *Alternaria* where the increased risk was almost five times. Although sensitization to *Alternaria* and house dust were the two allergens that provided the highest risk of developing asthma, there was also a positive correlation with ryegrass, ragweed, and oak (Gergen and Turkeltaub 1992). The NHANES III data, which resulted in 2007, revealed that often allergens tested

for, only cat, *Alternaria*, and white oak showed significant, positive associations with asthma after adjustment by the subject characteristics and all other allergens (Arbes et al. 2005). Although the NHANES II and III data concluded that many cases of asthma are attributable to atopy, the study did not discuss the seasonal impact of allergens on risk of asthma.

The Childhood Asthma Management Program (CAMP) is another landmark cohort that provided years of data on children with asthma. One purpose of the CAMP was to evaluate the relationship between sensitivity and exposure to inhalant allergens and pulmonary function and bronchial responsiveness in sensitized asthmatic children (Nelson et al. 1999). Using allergy skin testing, house dust mite collection, and determination of allergen exposure, the researchers concluded that only sensitivity to the indoor allergens dog and cat dander and the outdoor fungus *Alternaria* demonstrated significant increases in bronchial hypersensitivity (Nelson et al. 1999).

In addition to the NHANES and CAMP data, there have been many more studies in the USA that have examined the seasonal effect of allergens on asthma. One such study looked at the median weekly asthma admissions by age group to a hospital in Maryland from 1986 to 1999. The researchers concluded that asthma admissions increase four- to eightfold in the fall compared to the summer (Blaisdell et al. 2002). A study from 1986 established that the largest number of asthma admissions to a hospital in California occurred during the grass-pollen season (Reid et al. 1986). Recently, a study examined the effect of temperature and season on adult emergency department visits for asthma in North Carolina. This study found that the number of ER visits for asthma peaked in February (when daily temperatures were coldest) and were lowest in July (when daily temperatures were warmest) (Buckley and Richardson 2012).

International studies have also revealed the same seasonal phenomenon. For example, one study looked at the hospital admissions for asthma in Malta. Analysis revealed a peak in January and a trough in August for both pediatric and adult hospital admissions for acute asthma exacerbations (Grech et al. 2002). Another study in the Netherlands concluded that there was a decline in asthma symptoms and asthma medication use during the summer period and a peak during autumn to spring in pediatric patients over a 1-year time period (Koster et al. 2011). Another study revealed that in Taiwanese children aged 6–8, asthma and rhinitis peaked in winter, especially in December. However, they also found that children aged 13–15 had two peaks (winter and summer) for asthma and rhinitis (Kao et al. 2005). In another study done in Taiwan, school-aged children had a sharp increase in the number of asthma admissions in September and March that synchronized with school reopenings (Xirasagar et al. 2006). An additional study from Taiwan revealed differences among adult and childhood asthma seasonality. Although the asthma-related hospital admission for adults remained low in summer and increased in winter, the researchers concluded that adult asthma hospitalizations were highest in spring and significantly correlated with air pollution and climate (Chen et al. 2006). In Australia, researchers have recently found that there is a clear relationship between increased risk of childhood asthma emergency room visits and increased levels of ambient

grass pollen (Erbas et al. 2012). In Canada, a study using a national data set compared asthma hospital admission rates in cities with significantly different climates. They found that independent of season and air pollution levels, a doubling in basidiomycetes spore and pollen levels and grass pollen levels was associated with increases in daily asthma hospital admission rates (Dales et al. 2004).

In addition to the many studies revealing a seasonal effect on asthma exacerbations, there is evidence that treatment of seasonal allergic rhinitis improves asthma symptoms (Grembiale et al. 2000; Jacobsen et al. 1997; Johnstone and Dutton 1968; Novembre et al. 2004; Roberts et al. 2006). Since outdoor allergen avoidance is challenging, specific immunotherapy (SIT) is used as a strategy for the prevention of development of allergic rhinoconjunctivitis and asthma. The Preventive Allergy Treatment study (PAT) tested whether SIT could prevent the development of asthma. This study also examined whether the clinical effects of immunotherapy persist in children suffering from seasonal allergic rhinoconjunctivitis caused by allergy to birch and/or grass pollen as these children grow up (Jacobsen et al. 2007). It was demonstrated that SIT reduced the risk of developing asthma in children suffering from allergic rhinoconjunctivitis. Results were validated at termination of treatment with SIT. Results were also seen at 2 years, 7 years, and 10 years after termination of treatment, indicating a long-lasting benefit of SIT as the children grow up.

Daily oral antihistamine therapy is another treatment modality for asthma associated with seasonal allergic rhinitis. Cetirizine is described to inhibit the recruitment of inflammatory cells (mainly eosinophils) in the bronchoalveolar lavage induced by bronchial allergen inhalation challenge (Redier et al. 1992). Data has shown a protective effect of antihistamine against bronchial hyperresponsiveness. A randomized, placebo-controlled study concluded that the use of cetirizine on a daily basis is a safe and effective method to relieve upper and lower tract airway symptoms in patients with allergic rhinitis and concomitant asthma (Grant et al. 1995). Another study suggests that cetirizine may be useful in patients with asthma associated with allergic rhinitis due to the fact that this antihistamine produces a significant protective effect against allergen-induced late-phase response (Aubier et al. 2001).

It is clear that there is a seasonal effect on the prevalence of asthma exacerbations, both in the USA and abroad. Research has revealed an increase in asthma exacerbations during the fall, winter, and spring and has shown a decrease in asthma exacerbations during the summer. The causes of higher rates of asthma in the fall, winter, and spring include increased incidence of viral infection, increased prevalence of aeroallergens, and increased prevalence of air pollution. Interestingly, deaths from asthma that occur in the summer months have been hypothesized to be associated with higher levels of aeroallergens (Weiss 1990). And as stated above, there is substantiation that treatment of allergic rhinitis improves asthma symptoms.

It is difficult to elucidate whether the most significant factor affecting the seasonal differences in asthma exacerbations is aeroallergen sensitization. Thus, much research is now being conducted examining the pathophysiology of allergic asthma. Mechanisms of IgE-mediated allergic responses within airways and respiratory epithelium can help further explain the seasonal asthmatic phenotype.

4.1.2 Mechanisms of IgE-Mediated Allergic Responses as an Asthma Phenotype Determinant

Asthma associated with allergic responses, referred to as allergic asthma, is characterized by eosinophilic inflammation, airway hyperresponsiveness, and mucus hypersecretion (Suarez et al. 2008). It is a type I hypersensitivity reaction to an environmental protein such as pollen, dust mite excreta, or animal dander. The early response in asthma is immediate, occurring minutes to hours after an exposure, and clinical manifestations can include wheezing, breathlessness, chest tightness, bronchoconstriction, and nasal congestion (Verstraelen et al. 2008). The late-phase asthmatic reaction occurs 4–6 h after the early-phase reaction and is characterized by chronic airway inflammation caused by ongoing airway constriction, increased vascular permeability, enhanced airway hyperresponsiveness, and increased mucus secretion.

There is a strong causal relationship between asthma and allergens. It is now known that patients with asthma have higher serum-specific IgE antibodies and total IgE levels (Platts-Mills 2001). There are several early studies reporting the close correlation between serum IgE levels and asthma (Johansson and Bennich 1969; Kumar et al. 1971; Saha et al. 1975; Bryant et al. 1975; Burrows et al. 1989; Sears et al. 1991; Sunyer et al. 1995). In 1989, Burrows and colleagues reported a close link between serum IgE levels and asthma, concluding that "asthma is almost always associated with some sort of IgE-related reaction and therefore has an allergic basis" (Burrows et al. 1989). As stated above, many other studies have revealed elevated IgE levels correlating with both physician diagnosed asthma and physiologic evidence of bronchial hyperresponsiveness. As a result, allergen-specific IgE testing in clinical practice is used to diagnosis asthma and to guide therapy.

The early asthmatic response is initiated when IgE binds to the high-affinity mast cell surface FcεI receptor, triggering mast cell and/or basophil degranulation thus resulting in the clinical manifestations of allergic disease, including narrowing of the airway. Thus, it is well known that elevated allergen-specific IgE levels in serum are a hallmark of allergic asthma. However, new data look beyond the simple causal relationship between allergy and asthma and suggest that it may be far more complex than originally thought. Several recent studies have suggested that antigen-specific memory T cells, especially CD^{4+} T cells, are vital in the late-phase asthmatic response. CD^{4+} T cells promote airway hyperresponsiveness and inflammation via secretion of Th2-type cytokines such as IL-4, IL-5, and IL-13 (Mizutani et al. 2012). This exacerbated T helper type 2 cytokine production provides the immunologic basis of atopic sensitization and response to allergens (Langier et al. 2012). Interestingly, IgE also has been shown to induce production of these Th2-type cytokines in mice, leading to the development of airway hyperresponsiveness and inflammation, which are characteristic of chronic asthmatic responses (Mizutani et al. 2012).

Due to the strong correlation between IgE and allergic asthma, treatment with an anti-immunoglobulin E monoclonal antibody, omalizumab or Xolair, has become an option for those adults and children over the age of 12 with moderate-to-severe

allergic asthma in which inhaled corticosteroids have been ineffective. Omalizumab works by interrupting the asthmatic response at its initial step when IgE binds to the high-affinity mast cell surface FcεI receptor. By reducing free IgE and the binding to its receptor, there is a decrease in allergen processing and presentation to Th2 lymphocytes, along with a decrease in Th2 cell differentiation and thus a lower expression of cytokines.

In addition to Th2 responses playing an important role in allergic asthma, recent evidence has revealed that other mechanisms, such as Th1 and Th17 responses, have been implicated in the immunological and clinical phenotype of allergic asthma (Kerzel et al. 2012a; Langier et al. 2012; Mizutani et al. 2012). Much of the data on the role of Th17 immunity in the allergic response are derived from animal models. However, IL-17 has been shown to be increased in human sputum, blood, and bronchoalveolar lavage fluid (BALF) from adult and pediatric patients with allergic asthma (Kerzel et al. 2012a, b; Mizutani et al. 2012). One recent study evaluated whether Th17 cells (CD3+ CD4+ CD161+ CCR6+ lymphocytes) are increased in children with allergic asthma and whether they are correlated with disease activity. This study found that the proportion of Th17 cells in peripheral blood was significantly increased in children with allergic asthma. In addition, the study revealed a higher proportion of Th17 cells in patients with uncontrolled and partly controlled asthma versus well-controlled asthma (Kerzel et al. 2012a). Another recent study found that the concentration of C3a in BALF is elevated in human patients undergoing a late-phase asthmatic response compared to nonatopic control patients. The researchers then looked at an IgE-sensitized mouse model and established that IgE mediates the increase of IL-17 + CD^{4+} cells in the lungs through C3a production. Because of this finding, they examined the effects of an anti-IL-17 monoclonal antibody. The mAb reduced the late-phase asthmatic response, as they witnessed a reduction in airway resistance, airway hyperresponsiveness, and neutrophilic accumulation (Zhao et al. 2012).

4.1.3 Pollen as a Critical Allergen Facilitator of the Asthmatic Phenotype

The exact mechanisms of allergic asthma have yet to be confirmed and continue to be researched extensively, as stated above. One area of investigation focuses on the interactions of particular allergens with the human respiratory epithelium. It is known that several cysteine and serine protease allergens function as Th2 adjuvants, thus explaining their role in asthma (Wills-Karp et al. 2010). For example, Der p 1, a cysteine protease, and Der p 9, a serine protease, both induce release of cytokines from human bronchial epithelium and respiratory epithelial cells (Röschmann et al. 2009). Proteases from *Aspergillus fumigatus* induce IL-6 and IL-8 production and Pen ch 13, a serine protease from *Penicilllum chrysogenum*, induces prostaglandin-E2, IL-8, and TGF-B1, from human airway epithelial cells (Röschmann et al. 2009).

In addition, removal of proteases from *A. fumigatus*, German cockroach frass, American cockroach Per a 10 antigen, Epi p1 antigen (from the fungus *Epicoccum purpurascens*) or Cur 11 antigen (from the mold *Curvularia lunata*) was reported to decrease airway inflammation and airway hyperresponsiveness in mouse models of allergic asthma (Wills-Karp et al. 2010).

Extracts from different pollen have been shown to induce cytokine release as well. A study was recently conducted examining Ph1 p 1, the major allergen of timothy grass *Phleum pratense*, and its role in IgE reactivity in allergic individuals. The aim of the study was to investigate whether Ph1 p 1 has the ability to activate human respiratory epithelial cells and whether this ability is protease dependent (Röschmann et al. 2009). The authors concluded that pollen grains in general function as allergen carriers and contain bioactive lipids that attract cells in allergic inflammation. Ph1 p 1 induces expression and release of IL-8, IL-6, and TGF-β in alveolar epithelial cells in vitro, without using a direct proteolytic mechanism (Röschmann et al. 2009). Using this pollen allergen in vitro model, we can further elucidate the pathogenesis of allergic asthma. IL-6 is known to be important in recruitment and activation of neutrophils. IL-8 is also known to recruit neutrophils and is a key chemokine in the attraction of monocytes and macrophages to sites of inflammation. Thus, the Ph1 p 1 allergen itself plays a vital role in perpetuating the allergic response in both early- and late-phase asthmatic reactions.

Other studies have demonstrated that in addition to releasing particular allergens, pollen extrudes bioactive lipids, or pollen-associated lipid mediators (PALMs), that then stimulate neutrophils and eosinophils in vitro (Plotz et al. 2004). Taking from this recent demonstration of pollen-derived lipid components being an important factor in the development of IgE-mediated allergic hypersensitivity, another study examined the ability of birch pollen extracts to affect maturation and cytokine release from human dendritic cells, therefore inducing Th2 responses (Traidl-Hoffmann et al. 2005). The study demonstrated that an aqueous pollen extract from birch (*Betula alba* L.), or *Bet*.-APE, modulates the function of human dendritic cells, which induces Th2-dominated adaptive immune responses (Traidl-Hoffmann et al. 2005). A more recent study also examined the role of PALMs, specifically the E1-phytoprostanes (PPE1), and *Bet*.-APE in the enhancement of dendritic cell mediated Th2 polarization of naïve T cells. This study found that *Bet*.-APE strongly and dose dependently induces intracellular formation of cAMP. Incubation of dendritic cells with *Bet*.-APE leads to enhanced secretion of Th2-attracting chemokines such as CCL17 (Gilles et al. 2009). Because of the strong induction of cAMP and the strong Th2-polarizing effect of the birch pollen extract *Bet*.-APE both in vitro and in vivo, this study suggests that there must be additional bioactive substances contributing to this effect, thus indicating the need for future research in this area (Gilles et al. 2009).

For the first time in 2005, researchers investigated the role of NADPH oxidases in pollens and subpollen particles and their effect on allergic airway inflammation. A landmark study was published looking specifically at ragweed pollen extract, which contains NADPH oxidase activity, in the recruitment of inflammatory cells and the development of allergy. Looking at cultured epithelial cells and murine

conjunctiva, the study found that NADPH oxidases from the pollen extracts generate superoxides which in turn increase intracellular reactive oxygen species (ROS). The conclusion was made that ragweed pollen-induced oxidative stress is an independent process of adaptive immunity. They also showed that inactivation of the pollen NADPH oxidase significantly decreased the accumulation of inflammatory cells into the conjunctiva (Bacsi et al. 2005). Since that study, the role of NADPH oxidase in allergic inflammation has been studied further and a two-signal hypothesis for the induction of allergic inflammation has been proposed, with signal 1 consisting of innate ROS, and signal 2 consisting of the classical pathway of antigen presentation to T cells (Dharajiya et al. 2008). The role of pollen-induced oxidative stress on the airway epithelium in mice has been extended further to include how pollen grain exposure triggers oxidative stress in dendritic cells and mast cells (Chodaczek et al. 2009; Csillag et al. 2010). This adds growing evidence to the fact that oxidative stress from pollen grains contributes to allergic airway inflammation.

4.1.4 Adaptive vs. Innate Immune Responses: Contribution to Heterogeneous Asthma Phenotype

There are complex interactions between both innate and adaptive immune cells that lead to the symptoms of both the early and late-phase asthmatic response. The innate immune cells that may control allergic responses in the lung include phagocytic cells such as alveolar macrophages, dendritic cells, and neutrophils; nonphagocytic leukocytes such as natural killer cells, mast cells, eosinophils, and basophils; and epithelial cells, specifically bronchial epithelial cells (Verstraelen et al. 2008).

Bronchial epithelial cells are involved in allergic airway inflammation by secreting cytokines and augmenting inflammatory cells (Suarez et al. 2008). It has been shown that upon exposure to a particular allergen *Dermatophagoides pteronyssinus*, bronchial epithelial cells will produce the proinflammatory cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-8, and TGF-α, which all contribute to allergic airway inflammation (Lordan et al. 2002). Bronchial epithelial cells are also a source of thymic stromal lymphopoietin (TSLP) which is a cytokine that enhances Th2 responses. It has been shown that TSLP is abundant in the airway epithelium of mice and human subjects with allergic asthma (Suarez et al. 2008). TSLP can activate dendritic cells and mast cells to produce Th2 cytokines (Kool et al.2012; Suarez et al. 2008).

Alveolar macrophages are important in first-line defense, along with bronchial epithelial cells, against respiratory pathogens and allergens. They can attenuate the immune response in the lung, thus suppressing some of the clinical manifestations of allergic asthma (Suarez et al. 2008). Although macrophages can have both

proinflammatory and suppressive functions in the lung, overall, evidence indicates that they tend to inhibit immune activation and inflammatory cell entry into the lungs after the inhalation of respiratory allergens (Verstraelen et al. 2008). Upon inhalation of an allergen, alveolar macrophages secrete substances such as TGF-β and prostaglandin E2, thus suppressing airway inflammation. However, it is shown that once these macrophages are sensitized to allergen, their ability to produce these anti-inflammatory mediators is diminished (Suarez et al. 2008).

Dendritic cells are involved in the pathogenesis of allergic asthma due to their role in the chemotaxis of T cells in ongoing inflammation (Verstraelen et al. 2008). It has been shown that dendritic cells can recruit Th2 cells at sites of inflammation during the late-phase asthmatic reaction. As stated above, dendritic cells can be activated by TSLP, thus enhancing the Th2 response, a step necessary in allergic sensitization. Dendritic cells have also been found to be involved in memory immune responses with regard to allergic disorders (Suarez et al. 2008).

Innate immune molecules like toll-like receptors (TLRs) and NOD (nucleotide oligomerization domain) -like receptors (NLRs) also play an important role in airway epithelial cells generating a response to a complex allergen. Respiratory epithelial cells express endosomal TLRs, which signal a series of adaptor proteins upon ligand attachment (Lambrecht and Hammad 2012). This results in the activation of NF-κB and the production of several proinflammatory cytokines, interferons, chemokines, prostaglandins, and defensins. In addition, activation of NODs, specifically NOD-1 and NOD-2 in respiratory epithelium, results in MAPK and NF-κB-dependent production of inflammatory mediators (Lambrecht and Hammad 2012).

4.2 Gaseous, Volatile, and Particulate Environmental Triggers as Determinants of the Asthmatic Phenotype

4.2.1 Small Gas Molecule Environmental Pollutant Triggers: Phenotype Shapers or Interactors?

Sulfur Dioxide

General Characteristics

Sulfur dioxide (SO_2) exists as a colorless tri-atomic gas, having two oxygen atoms bound to each sulfur atom, through an alternating double bond that is shared by each of the oxygen atoms. It is also known as sulfurous anhydride or sulfur (IV) oxide and considered the active agent in antibacterial sulfating chemicals utilized in wines and other food products of fruit origin. Its pungent odor is characteristically described as that of rotten eggs, with a typical odor detectability threshold that averages approximately 2.5 ppm (Pohanish 2004). While SO_2 is a relatively stable gas

when dry, in the presence of water, it can form sulfur acid (H_2SO_4) as shown in the following stoichiometrically balanced equation:

$$2SO_2 + 2H_2O + O_2 \rightarrow 2H_2SO_4 \quad (4.1)$$

Sulfuric acid is highly corrosive (hence its classical name “oil of vitriol”) but has a number of commercial uses; therefore, its bulk production is necessary for cleaning products, fertilizers, and water treatment applications. However, sulfuric acid is also readily produced by a similar hydration reaction when SO_2 gas comes in contact with rain in the atmosphere, producing the corrosive “acid rain” (Weathers and Likens 2006) that has been described since the Industrial Revolution, preceding, and into, the nineteenth century (EPA 2013). In a similar fashion, inhaled SO_2 gas can form sulfuric acid when coming into contact with the hydrated surfaces of the nasal, oral, and pulmonary airways, which can result in local oxidative stress that may be important in the development or exacerbation of asthma (Lin et al. 2004; Peden 1997; Schwela 2000).

SO_2 as a Hazardous Gas

SO_2 gas is typically released when fossil fuels are either burned or processed. Examples are the burning of coal for energy, and production of gasoline from crude oil. SO_2 is also released when certain metals are extracted from their crude ores such as copper (Lin et al. 2004). Thus, it is easy to imagine that workers in commercial industries either manufacturing sulfuric acid from SO_2, or engaging in processes in which SO_2 is a byproduct, could be acutely exposed to toxic or even lethal levels of SO_2. Accordingly, NIOSH stipulates the level of SO_2 that is considered to be immediately dangerous to life or health (IDLH) as 100 ppm (CDC 1995). However, a significant amount of SO_2 is produced by diesel engines in agricultural equipment and other nonroad vehicles (Decker et al. 2003) and can therefore be a gaseous hazard that is readily present in areas not necessarily associated with commercial manufacturing and processing. Due to its toxicity, regulations and safety standards for SO_2 have been put into place by governmental bodies such as the EPA and NIOSH, which typically stipulate an exposure concentration and time factor combination. For example, in 2010, the EPA replaced the existing primary SO_2 standards with a new 1-h exposure standard, at a maximum level of 75 ppb. NIOSH standards of exposure acceptability vary from 5 ppm for 15 min of SO_2 exposure, to 2 ppm for 10 h of exposure, which are similar to that of OSHA, setting an acute permissible exposure limit (PEL) of 5 ppm over 8 h (NIOSH 2010).

SO_2 as a Factor in Airway Dysfunction

With the presence of diesel engines in commercial vehicles, levels of gaseous SO_2 in polluted urban air can remain high, which can be problematic, given that since 1971, the EPA primary standard has been 0.14 ppm for 24 h of exposure (EPA 2012d), and given that the SO_2 odor detection threshold (2.5–3.0 ppm) is above the

level at which SO_2 is associated with breathing problems in asthmatics. In other words, asthmatics in those areas can be exposed chronically to SO_2, without being aware of their exposure and the source of their airway dysfunction. Subsequently, in 2010, the EPA revoked the 24-h standard, indicating that no level of SO_2 beyond 75 ppb for 1 h was acceptable (EPA 2012d). While acute high level exposures to SO_2 gas can cause pulmonary edema and derangements of gas exchange at the level of the alveoli, acute exposures at levels as low as 0.1–0.5 ppm can induce bronchoconstriction and bronchospasm in asthmatics, whereas these symptoms are only seen at concentrations of 5–20 ppm in nonasthmatics (Lin et al. 2004; Peden 1997; Schwela 2000; "TOXNET: Hazardous Substances Data Bank (HSDB)," 2013). Of further interest, SO_2 appears to intensify allergic responses to inhaled allergens (Cai et al. 2008; D'Amato et al. 2002), suggesting that it can synergize or amplify asthma, in those with significant atopy.

SO_2 as a Phenotype Shaper or Interactor?

An interesting question about SO_2 is whether it should be viewed as a long-term shaper of the asthma phenotype, or simply an acute interactor with other factors in the airway. Given the known toxicity of SO_2 and its ability to readily form sulfuric acid in the presence of moisture, one could reasonably postulate that the oxidative stress response to inhaled SO_2 due to the presence of an acidic irritant stimulus in the airway would be a most potent aggravating factor in promoting asthmatic symptoms. Thus, in considering SO_2 as a shaper of asthma phenotype, the case of chronic insensible exposure to inhaled SO_2, at levels below the odor threshold mentioned above, could be easily considered. For example, a person genetically predisposed to the development of asthma, e.g., one in which a familial diagnosis is present, might have long-term changes in their airway biology, including irreversible airway remodeling, which are only marginally managed by current asthma therapy rather than resolved or cured (Durrani et al. 2011).

At this time, the state of understanding of the progression of airway remodeling and its potential reversibility over time, as well as a lack of longitudinal data about long-term low-level SO_2 exposure, do not currently allow certainty in assigning SO_2 the status of a persistent asthma phenotype shaper. While a number of studies have been done on SO_2-associated hospital admissions and mortality (WHO 2000), and one study indicated minimal effects on lung function at a low exposure level (Lawther et al. 1975), it is not known whether persons chronically exposed to low levels of SO_2 develop asthma at higher rates than those who are not exposed over the same period of time. Furthermore, it is not known whether those diagnosed with asthma associated with living in areas of chronic low-level SO_2 exposure experience relinquishment of symptoms or are cured of asthma, when the chronic exposure is discontinued. Thus, SO_2 may be an acute asthma phenotype shaper, but little is known about its effects on its long-term phenotypic influence on the disease.

As mentioned above, there is a significant amount of evidence that acute SO_2 exposure can play an interactive role in exacerbating asthma, and this role may be attributed to its ability to form sulfuric acid in the airway and possibly become a

trigger of oxidative stress. There may also be either a synergism or amplification of effects with the interaction of Th2 allergic inflammatory pathways in atopic asthmatics that is also associated with production of ROS in the airway, which may potentiate bronchospasm and bronchoconstriction associated with asthma (Cai et al. 2008; D'Amato et al. 2002; Lee and Yang 2012). In this context, it is interesting to consider that asthmatics have a deficiency of production of IL-10 in the airway (Borish et al. 1996), for reasons as yet unexplained, but which could be critical to the exaggerated effects of SO_2 seen in asthmatics, but not seen in nonasthmatics, who are IL-10 sufficient. A question that remains unsolved is whether asthmatics are extraordinarily sensitive to the effects of SO_2 because they cannot mount a significant anti-inflammatory response, with a deficiency in the production of IL-10.

Ozone

General Characteristics

Ozone (O_3), also known as tri-oxygen, like SO_2, is a tri-atomic molecule consisting of three oxygen atoms that share an alternating double bond, or traveling pair of electrons. The name ozone comes from a Greek word meaning "to smell," which describes the characteristic bleach-like odor of O_3, existing as a pale-blue gas, detectable at concentrations as low as 10 ppb, after lightning storms, and with function of some older toner-based printers and photocopiers, lacking ozone filters (EPA 2012a; Morawska et al. 2009). As shown in the stoichiometrically balanced equation, below, O_3 decomposes to simple diatomic oxygen (O_2) within about 30 min, under standard conditions in the atmosphere (Solutions 2013); however, recent measures suggest half-lives as long as 1,500 min under less-disturbed conditions (McClurkin and Maier 2010):

$$2O_3 \rightarrow 3O_2 \tag{4.2}$$

O_3 is naturally present in the stratosphere of the earth's atmosphere, comprising the "ozone layer," at distances that average about 20 miles from the earth's surface, and which blocks a significant amount of the sun's ultraviolet rays, even though the concentration of O_3 in that layer is typically less than 10 ppm (Sci-Link 1995). Typically, naturally occurring O_3 levels at the earth's surface are negligible but can be significantly elevated locally with the burning of fossil fuels such as gasoline and diesel fuel. The action of sunlight on the combination of nitrogen oxides and volatile organic compounds, which come from power plants, factories, and vehicles, can produce O_3, and in large cities such as Los Angeles, O_3 is a significant component of the smog layer (AQMD 2013; Linn et al. 1986). O_3 is a more powerful oxidizing agent than O_2 and can oxidize metals, other gases (e.g., nitric oxide), and olefin-based polymers, and is the basis for its use industrially and commercially as a

disinfectant and antibacterial/antifungal in numerous applications, including water treatment, swimming pool cleaning, fabric treatments, and fresh fruit decontamination. A further interesting use of O_3 is as a disinfectant in hospital surgical rooms, in which it is pumped into a sealed operating room (de Boer et al. 2006), to kill any remaining bacteria after surface cleaning.

Ozone as a Hazardous Gas

Considering its production with the burning of gasoline in motor vehicles, and its widespread commercial and industrial use, the possibilities for inhaled O_3 exposure are potentially significant in certain populous urban locations. In fact, the amount of exposure to O_3 in large cities has been considered to be a strong risk factor, as at least one study has shown a strongly elevated risk of dying of lung disease, in the cities of Los Angeles and Houston, both being in the top four population centers in the USA (Jerrett et al. 2009). Similar to SO_2, the toxicity of O_3 has resulted in regulations and safety standards by government entities to protect the public health. The NIOSH IDLH for O_3 is 5 ppm (one-twentieth of that for SO_2), while the NIOSH REL and EPA PEL is 0.1 ppm (CDC 1994b). In 2008, the primary standard for ground-level O_3 was stipulated by the EPA to be 0.075 ppm over 8 h (AQMD 2013), but there has been recent desire since 2010 to reduce this even further, potentially as low as 0.060 ppm, due to concern for certain sensitive groups such as children and asthmatics, who may be affected at O_3 concentrations as low as 0.085 ppm (EPA 2010a).

Ozone as a Factor in Airway Dysfunction

O_3 is considered to be a potent source oxygen-free radicals, or reactive oxygen species (ROS), which carry extra electrons within their structures that can cause damage to cell membranes, mitochondria, and DNA, leading to cell dysfunction (EPA 2012c). Among the airway cells affected most are airway and alveolar epithelial cells, which bear the brunt of contact with inhaled O_3, due to a lack of absorption of O_3 at higher airway levels, owing to its poor water solubility. As a result, the epithelial cells leak important enzymes and release inflammatory mediators into the airway, which can promote injury and the development of airway inflammation (Devlin et al. 1997). Furthermore, O_3 can stimulate nociceptive nerve fibers, which can lead to stimulation of the cough reflex and bronchospasm (Devlin et al. 1997). O_3 has been reported to increase the airway responsiveness of allergic asthmatics to inhaled dust mite allergen, measured as drops in FEV_1 (Kehrl et al. 1999), indicating its potential to synergize with allergic asthma triggers to reduce lung function in atopic asthmatics. Thus, as a powerful oxidizing agent, inhaled O_3 can result in significant oxidative stress within the airway, promoting bronchoconstriction, bronchospasm and asthma attacks, and even death (EPA 2012b) as shown in the Ozone Effects Pyramid in Fig. 4.1.

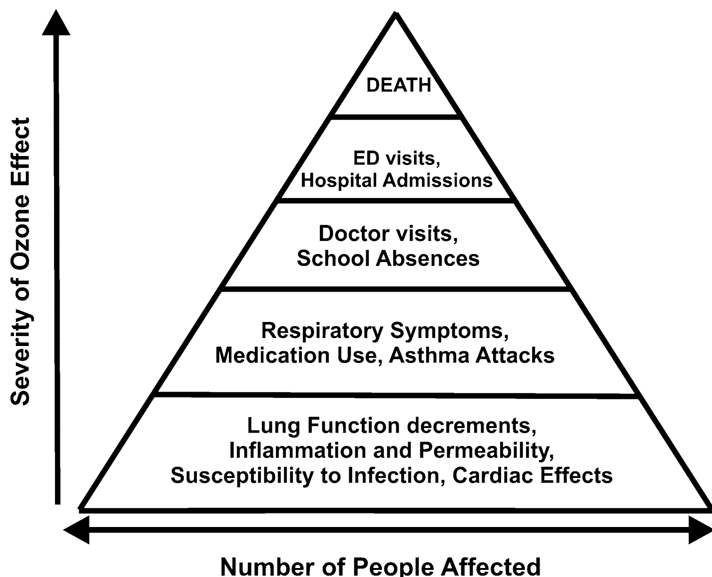

Fig. 4.1 The pyramid of airway effects associated with ozone inhalation. The relationship between the severity of the effect (topmost is most serious) and the proportion of the population experiencing that effect (greater width at base indicates larger segment of people) can be represented as shown. Many individuals experience the least serious, most common effects shown at the larger bottom of the pyramid, whereas fewer individuals experience the more severe effects such as hospitalization or death, as shown at the smaller top of the pyramid. Modified from US EPA (2012c)

Ozone as a Phenotype Shaper or Interactor?

Perhaps more so than SO_2, O_3 may be a stronger factor in determining the phenotype of asthma, due to its significant oxidant-associated toxicity and the promotion of intracellular DNA damage, even at exceedingly small concentrations (Ferng et al. 1997; Steinberg et al. 1990). For example, it is conceivable that if the site of DNA damage were genes controlling the inflammatory response or bronchial hyperresponsiveness, that an individual could become compromised in the ability to resolve inflammation resultant of exposure to other asthma triggers. Interestingly, one study indicated that nitric oxide (NO) could act to repair DNA strand breaks associated with O_3 exposure in human bronchial epithelial cells in culture, which suggests a potential endogenous defense mechanism against the genotoxic effects of O_3 (Cui et al. 2011).

However, it is not currently known to what level of alteration that chronic O_3 inhalation can modify airway cellular function and phenotype. Thus, it is reasonable to expect that chronic O_3 exposure and its attendant oxidative stress would lead to subsequent long-term modifications that are perhaps manifest in irreversible airway remodeling, consequent diminution of lung function, and the development of sustained difficult-to-treat asthma, or potentially, chronic obstructive lung disease (COPD). As in the case of SO_2, it remains open to speculation and further

research, as to whether the inability to mount a significant IL-10-driven airway anti-inflammatory response in asthmatics is a predisposing factor toward allowing chronic O_3 exposure to shape toward a moderate-to-severe asthma phenotype. This consideration may be even more important, when one considers that the deleterious effects of O_3 on lung function become more pronounced with aging (Devlin et al. 1997), which would fit with the idea that prolonged low-level exposure to O_3 could lead to irreversible changes in lung function, particularly in asthmatics. Clearly, given the prominence of O_3 in populous urban settings, and its potent effects in promoting DNA damage, further research is necessary to determine the potential of O_3 to be an asthma phenotype shaper over the lifetime of an individual.

The characteristics of O_3 that make it a powerful oxidant also make it a strong interactive agent that can acutely magnify the effects of other triggers, in people with asthma. The ability of O_3 to acutely amplify allergen-associated decrements in lung function (Kehrl et al. 1999) bears consideration, in this regard. Moreover, the fact that asthma ED and hospital admissions were highly positively correlated to estimated O_3 exposure, only during warmer months (Burnett et al. 1994), when the action of sunlight on nitrogen oxides and volatile organic compounds would be expected to be more effective in producing inhalable O_3, further supports the idea that O_3 can be an important interactive factor in the exacerbation of established asthma. Thus, in the case of O_3, there are reasons to believe that its potency and presence in our lives makes it a factor to be strongly considered as both a long-term asthma phenotype shaper requiring more global efforts toward its reduction, as well as an interactive factor requiring acute therapeutic strategies and attention.

4.2.2 Volatile Environmental Pollutant Triggers: Phenotype Shapers or Interactors?

Acrolein

General Characteristics

Acrolein, also known as propenal, and acrylic aldehyde, is a highly electrophilic unsaturated aldehyde, which, similar to the toxic gases SO_2 and O_3, has a double-bonded oxygen within its molecular structure and is a powerful oxidizer (Garg 2001). As an oxidant, acrolein finds use as a water-treatment algaecide and herbicide in canals, irrigation systems, and other low-flow water settings, as well as in the oil drilling industry (Arntz et al. 2007; Faroon et al. 2008). Acrolein typically exists as colorless or pale-yellow liquid but has a characteristic "acrid" smell; hence, its name, and it is formed with high heating of cooking oils (Bein and Leikauf 2011). Although toxic as a chemical irritant, the World Health Organization has recommended an oral acrolein intake of up to 7.5 mg/day per kg body weight, and typically fried foods result in acrolein intake levels below that threshold (Abraham et al. 2011). Acrolein is a product of many sources of incomplete combustion, including

wood burning, and is also present in significant quantities in cigarette smoke (Stevens and Maier 2008), being inhaled by smokers, but also as sidestream, secondhand, and environmental tobacco smoke, inhaled by nonsmokers (Bein and Leikauf 2011).

Acrolein as a Hazardous Compound

Acrolein is a contact irritant of the eyes, skin, and nasal mucosa, as well as the lower airways (Ghilarducci and Tjeerdema 1995). In humans, acrolein exposure experiments with 0.6–8.0 ppm over time intervals of 1–5 min, produced from slight eye irritation at low concentrations and early times, to profuse lacrimation at higher concentrations and later times (Darley et al. 1960; Henderson and Haggard 1943; NRC 1981; Sim and Pattle 1957). Thus, the NIOSH Recommended Exposure Limit (REL) and OSHA PEL for acrolein are 0.1 ppm over 8 h, and the IDLH is 2 ppm (CDC 1994a). However, the Office of Environmental Health Hazard Assessment (OEHHA) of the State of California EPA has recommended even lower standards as of 2008, with an acute REL of 1.1 ppb, an 8-h REL of 0.3 ppb, and a chronic REL of 0.15 ppb (CDC 1994a). As mentioned above, acrolein is a prominent component of cigarette smoke, but as pointed out by Bein and Leikauf (Bein and Leikauf 2011), it is of considerable interest that acrolein concentrations in sidestream smoke can be as much as 17 times higher than concentrations in mainstream smoke, due to the differences in combustion chemistry as a function of temperature. This apparent concentration of acrolein in sidestream cigarette smoke would indicate that it is a significant hazard for nonsmokers who find themselves in the presence of smokers, provoking insensible and undesirable effects in their lungs that could exacerbate or drive the development of asthma.

Acrolein as a Factor in Airway Dysfunction

It bears notice that acrolein is clearly viewed as a potently hazardous toxic agent, which makes investigation with applied acrolein exposure in humans difficult, in part due to ethical considerations. There is a significant amount of animal study data that is suggestive of the potential effects of acrolein in the human airway, but which is outside the focus of this review. Thus, it has been acknowledged that there are no in vivo experimental human studies demonstrating acrolein effect on airway function and the development or exacerbation of asthma (Bein and Leikauf 2011; OEHHA 2001), which remains problematic for our full understanding of its effects in humans.

While human data about acrolein administration or exposure as a factor in airway dysfunction is scant, there are some indirect studies in humans and human tissues and cells, which indicate its potential for effects. It is of interest, for example, that comparisons of inhaled and exhaled cigarette smoke in humans demonstrate a nearly total removal of acrolein into the airways, particularly into the lower airways (Moldoveanu et al. 2007). As reviewed by Bein and Leikauf (2011), such indirect studies have also shown that acrolein can interact with a variety of cell structures, including the membrane, cell proteins, and nucleic acids, which substantiates

expectations based on its oxidative characteristics. In agreement with these expectations, it has been reported that acrolein at a low concentration of 5 μM induces heme-oxygenase 1 (HO-1; a.k.a. heat-shock protein 32, an indicator of cell stress and inflammation) expression in cultured A549 cells (an alveolar basal epithelial cell line of human origin), but that higher concentrations considered cytotoxic, at 25–50 μM, decreased HO-1 expression (Li et al. 1997). Interestingly, acrolein was also shown to inhibit NF-kappaB activity in cultured human BAL macrophages, suggesting that acrolein may suppress pulmonary host defense and the ability to mount and resolve a proper inflammatory response (Li et al. 1999; Li and Holian 1998). Overall, data such as those above support that higher dose exposures of acrolein may promote human lung injury, while lower dose exposures may represent risk factors for the development of chronic pulmonary diseases associated with airway inflammatory processes such as asthma and COPD (Bein and Leikauf 2011).

Acrolein as a Phenotype Shaper or Interactor?

Based on the lack of data in humans mentioned above, it remains difficult to confidently assign acrolein a role of phenotype shaper in humans with lung diseases such as asthma and COPD. One contributory problem is that no specific long-term studies on acrolein exposure in humans exist from which data are available to better understand its progressive effects. However, given its known presence in cigarette smoke, acrolein would seem to be a strong candidate as a chronic toxicant that could strongly influence the remodeling of the airways in smokers. It also stands to reason that acrolein may be a significant causative agent of lung pathology and dysfunction for people who consistently inhale sidestream smoke in the presence of smokers. Finally, its presence in second-hand and environmental tobacco smoke would also make it a potential candidate for the shaping of phenotype in those instances as well.

Given the fact that acrolein is a powerful oxidizer and mucosal irritant, it is apparent that it could easily be considered to be an interactor, or perhaps an agent that amplifies, or possibly even triggers, acute upper and lower airway responses in asthmatics. Again, the fact that atopic asthmatics can have an immune overresponse to certain allergic triggers such as pollen and dust, and a lack of inflammatory response resolution due to a lack of IL-10 production in the airway, one could imagine that the irritant and oxidative properties of acrolein would amplify those effects, if acrolein exposure occurred in a sensitized and untreated, or undertreated asthmatic. Recent evidence collected in human cell cultures, in vitro, suggests that acrolein may act through noncanonical signaling pathways to exert its effects in the lung (Verstraelen et al. 2009), suggesting that further work is warranted in determining the nature of those pathways. Acrolein has also been reported to increase ROS generation and degranulation in mast cells (Hochman et al. 2012), an important cell determining airway responses to allergic triggers (Krishnaswamy et al. 2001), which was moderated by administration of antioxidants (Hochman et al. 2012), suggesting yet another target of acrolein effects in the lung, and a possible therapeutic treatment for its effects. These interactions are likely complex, but all would seem to be predisposed toward positive synergism in the exacerbation of asthma.

Butadiene

General Characteristics

Butadiene, typically referring to the form of 1,3-butadiene, also known as divinyl, is a conjugated diene which exists as a 4-carbon chain, with two double bonds originating at the 1- and 3-carbons of the chain (i.e., between each of the distal carbons and the next most proximal carbon, moving toward the center of the molecule), and having a total of six hydrogen atoms bound to the carbons; hence, its formula as C_4H_6. As a gas, it has a faint odor similar to gasoline that is discernible by humans at approximately 1–2 ppm (Amoore and Hautala 1983), just above the OSHA PEL (ATSDR 1992), making it relatively easy to discern potential acute exposure without sophisticated measuring equipment. Butadiene is highly volatile and has a low water solubility, and when exposed to sunlight, butadiene gas degrades with a half-life of under 2 h (ACC 1992). Historically, synthesis of butadiene polymers in massive quantities began just prior to World War II, in which they were used in the manufacture of tires for war vehicles and other rubber-based products (Herbert and Bisio 1985). This has continued through today, e.g., polybutadiene, a polymerized form, and styrene butadiene, a combination of styrene and butadiene, account for a major amount of the material in car tires currently manufactured, worldwide. Exhaust from gasoline-powered motor vehicles is a major source of butadiene in the external environment, whereas cigarette smoke is considered to be a major source of butadiene in the internal environment (ATSDR 1992). Also, with the burning of car tires, there are release of significant amounts of butadienes into the atmosphere (EPA 1997), which could pose problems for people living near scrap tire piles throughout the USA, and particularly those that run along the USA/Mexico border (EPA 2010b), in the case that uncontrollable fires were to erupt at those locations.

Butadiene as a Hazardous Compound

Butadiene typically exists as a colorless gas, which has importance for its potential as an inhaled toxicant because it cannot be seen, but it also has the dangerous characteristic of being highly flammable (ATSDR 1992) and is therefore a compound of concern regarding general safety. It is known to be self-reactive (CDC 1992) and form polymers over time when stored in cylinders, which can subsequently rupture the cylinder and result in the release of butadiene gas into the environment and which can be problematic for the induction of acute inhaled toxic effects. Butadiene is listed by the EPA as a carcinogen and is also suspected to be a human teratogen, based on numerous studies, mainly in animals (EPA 2009). Similar to acrolein, definitive experimental and mechanistic data regarding the effects of butadiene in humans is relatively scant, due to its known acute toxicities. For example, acute butadiene exposure is known to cause mucous membrane irritation, typically at levels >10,000 ppm (CDC 1992; CGA 1999), but there are no experimental studies of chronic exposure of toxic levels in humans.

Butadiene as a Factor in Airway Dysfunction

While butadiene gas is considered by some to have low inhalation toxicity (CGA 1999), its main route of exposure is through inhalation and its main effect when

inhaled is to produce sinus and mucus membrane irritation of the upper respiratory tract (Clayton and Clayton 1985). Furthermore, treatment recommendations for acute butadiene exposure in children who develop stridor call for administration of beta agonist aerosol to dilate the airways and relieve difficulty breathing (ATSDR 1992). As a mucous membrane irritant, it would stand to reason that chronic low levels of inhaled butadiene could produce persistent effects such as irritation of sinuses and sore throat, but might be mistaken for common cold or allergy symptoms. However, little is known about airway effects with low level, chronic exposures in humans, particularly in those with chronic airways disease.

Butadiene as a Phenotype Shaper or Interactor?

Under such conditions that an asthmatic might inhale butadiene, it would be expected that butadiene exposure would amplify true allergic symptoms, such that atopic asthmatics could be prone to enhanced sensitivities to common allergen triggers, which could precipitate asthma exacerbations or attacks. The exposure of children to inhaled butadiene, particularly those with asthma, is of some greater concern, as it is thought that they may receive larger doses than adults for a given environmental concentration, due to children having higher surface area:body weight ratios, and greater minute volume:body weight ratios as compared to adults (ATSDR 1992). This idea has been supported by a singular epidemiologic study by Delfino et al. (2003), in which air samples positive for butadiene were associated with adverse effects on asthma in children.

Overall, the evidence for butadiene gas suggests that it is mainly an asthma interactor, serving to exacerbate asthma symptoms, mainly through irritation of sinus membranes and airways. There is not yet enough long-term evidence to determine whether butadiene gas induces an asthma phenotype, as few studies exist in this area. Additionally, unlike the case for SO_2, as presented above, wherein effects on the airways can occur at levels beneath the odor detectability threshold, the acute toxicities for butadiene are mainly considered to be transient and avoidable, because the odor threshold is at, or beneath, the level at which toxic and irritant effects have been shown to occur.

4.3 Particulate Environmental Pollutant Triggers: Phenotype Shapers or Interactors?

4.3.1 Tire Breakdown Particles

General Characteristics

It has been estimated that over 80 % of respirable particulate matter (typically PM_{10}; particles with a mean aerodynamic diameter of 10 μM or less) in urban areas comes from activities associated with motor vehicle transportation (Gualtieri et al. 2005a). Tire breakdown particles, or tire wear debris, have been shown to accumulate in significant amounts near highways and urban high-traffic areas where motor vehicles travel, and it has been pointed out that the smallest-sized particles can be transported

over relatively large distances of 18–30 m from the roadway (Wik and Dave 2009). Reports have indicated that a typical tire loses approximately 30 % of its rubber tread over its lifetime, and that up to 5 % of the resultant tire particulates are airborne, achieving particularly high concentrations in tunnels (Dannis 1974; Pierson and Brachaczek 1974; Saito 1989). The agents within tire breakdown particles that may have significant respiratory health effects include butadienes and other VOCs, latex, as well as some heavy metals, such as zinc (Adachi and Tainosho 2004).

Tire Breakdown Particles as a Hazardous Compound

The hazardous aspects of tire breakdown particles mainly relate to the known toxicities of their components. Thus, the health effects of butadiene, as outlined above, would be among those to be considered for tire breakdown particles, as well as those of other volatile organic compounds (VOCs) contained within them such as benzothiazole and *n*-hexadecane (Brown 2007; Ginsberg et al. 2011). While typically considered toxic in their volatilized gaseous forms, emerging evidence suggests that the presence of VOCs in the form of inhaled particulates may be of some greater concern than previously thought (Brown 2007). For example, experiments in vitro with application of organic extracts of tire debris have been shown to increase cell mortality, DNA damage, and production of ROS in A549 human alveolar epithelial cells (Gualtieri et al. 2005b, 2008). Furthermore, animal studies have suggested the potential that zinc contained within tire wear products can exert cytotoxic effects (Gerlofs-Nijland et al. 2007).

Tire Breakdown Particles as a Factor in Airway Dysfunction

As mentioned above, butadiene would be one potentially toxic chemical agent within tire breakdown particles that might produce airway dysfunction through its irritant effects on sinus membranes and lower airways, which may promote the formation of mucus and subsequent bronchospasm, and difficulties breathing. However, in recent years, latex has been considered to be a potentially important agent within tire breakdown particles that may induce an allergic response within the airways that could promote airway dysfunction. While relatively inert in terms of chemical toxicity, latex is known to promote allergic reactions on the skin of individuals sensitive to it (Hamann 1993), and the latex protein has been shown to share immunologic cross-reactivity with mountain cedar and cypress pollen (Caimmi et al. 2012; Miguel et al. 1996). It has been further postulated that latex may also be an important protein allergen that can induce allergic responses within the airway (Eghari-Sabet and Slater 1993), and that there may be similarities between skin and airway sensitivities. Since the typical particulate size distribution of tire debris has been shown to range from 2.2 to 35.2 μm, with an average of 6–7 μm (Williams et al. 1995), tire dust in the smaller size range would be easily inhalable by humans and could reach relatively far into the airways, especially if inspired by mouth. This has practical ramifications in heavily urbanized areas such as the case of Los Angeles some years ago, in which over 5 tons of rubber particulate <10 μm were typically released into the air on a daily basis (Cass et al. 1982).

Tire Breakdown Particles as a Phenotype Shaper or Interactor?

Given the demonstration of significant amounts of tire debris particulates in the air, there has been concern that such inhaled particulates could be of relevance to asthma (Glovsky et al. 1997), particularly for individuals living near high-traffic areas (Dockery et al. 1993), i.e., that car tire particulates may contribute to the development of the asthma phenotype, potentially due to its role in the increasing numbers of asthma morbidity, mortality, and diagnoses over the past quarter century. In this case, tire breakdown particles could be potentially considered as asthma phenotype shapers from the perspective of long-term exposure to the compounds that make up those particles. More recent epidemiological studies have suggested that asthma can be worsened by increases in levels of PM_{10} (Donaldson et al. 2000), but also that anti-oxidants such as vitamin C and E may mitigate these effects (Canova et al. 2012) in the short term.

However, with the evidence regarding latex acting similar to an allergic trigger, one could also make the argument that small latex particles may act to exacerbate allergic asthma, and thus tire breakdown particles could be considered as interactors with the established disease of atopic origin. Interestingly, this would also be expected to hold true for the use of tire "crumbs" on synthetic turf surfaces used for sports, as those larger particles will eventually breakdown with use and wear, resulting in finer dust-like particulates that can be inhaled by athletes and associated athletic personnel (Brown 2007). Furthermore, this acute mechanism would also apply to the use of tire crumbs now commonly used in children's playgrounds (Brown 2007). In all, the use of these products should be monitored to determine whether these potential health risks develop, as synthetic turf and playground pads have become the norm in recent years.

4.3.2 Diesel Exhaust Particles

General Characteristics

While tire breakdown particles constitute a significant portion of PM_{10} particulate load in the environment, diesel exhaust particles (DEP) constitute a significant portion of the $PM_{2.5}$ particulate load in the environment (particles with a mean aerodynamic diameter of 2.5 μM or less) (Wichmann 2007). These are considered to be fine particles, and as such, may pose an even greater risk to health, in part due to the ease, associated with their small size, with which they can be deposited in the smaller airways, and in part due to other toxicants that can be bound to them. However, it is also worth noting that a significant portion of DEP also comprises ultrafine particles, which are in the $PM_{0.1}$ range (particles with a mean aerodynamic diameter of 0.1 μM or less) (Saiyasitpanich et al. 2006), which are beyond visual resolution of the naked eye and can be deposited all the way down to the alveoli.

Diesel Exhaust Particles as a Hazardous Compound

Similar to tire breakdown particles, the hazardous aspects of DEP mainly relate to the known toxicities of their components, and other toxicants complexed onto them. These components include: acrolein, butadiene, sulfur dioxide, and heavy metals, among others; essentially all of the environmental toxicants described above, as promoting and/or exacerbating the asthma phenotype. Based on a number of epidemiologic studies, DEP are conventionally considered to promote lung cancer (Wichmann 2007), although this concept has been disputed (Hesterberg et al. 2006) and may be lessened in the years to come, with the adoption of new-technology diesel exhaust (NTDE) engines, which utilize low-sulfur fuel and exhaust after-treatment such as particulate filters (Gerlofs-Nijland et al. 2007; Hesterberg et al. 2006). However, there are many older technology diesel engines still in use in the USA and elsewhere; therefore, from present, it will possibly take another decade or two before this transition becomes more complete with vehicle fleet turnover (Blanchard et al. 2013) and will likely lag in time in developing countries.

Diesel Exhaust Particles as a Factor in Airway Dysfunction

Initial experimental studies in humans under controlled conditions demonstrated that 1 h of inhalation of diesel exhaust increased trafficking leukocytes in BAL fluid, indicating an airways inflammatory response (Rudell et al. 1990). As pointed out by Salvi and Holgate (1999), early changes that herald the beginnings of the inflammatory process, as assessed in human-relevant animal models such as guinea pigs, have been shown to occur with diesel exhaust producing eosinophilic infiltration into the nasal airway epithelium and subepithelium (Kobayashi 2000). Those findings are consistent with more recent in vitro studies in human bronchial epithelial (BEAS-2B) and human small airway epithelial (SAECs) cells, which have indicated that DEP can components can lead to release of inflammatory cell markers and cell death (Schwarze et al. 2013). In fact, ultrafine particles have been reported to induce proinflammatory mechanisms and oxidative stress in in vivo animal models, but it is worth noting that a number of those data were obtained form study of surrogate particles (e.g., carbon black) rather than ultrafine DEP themselves (Donaldson and Stone 2003) and that the exact causal relationships between DEP and the development of diseases such as lung cancer and asthma have not yet been established (Hesterberg et al. 2006). The relatively large surface area of DEP is considered to be a major factor (Donaldson and Stone 2003; Salvi and Holgate 1999; Wichmann 2007), but the precise relationship remains unclear and requires further research.

Diesel Exhaust Particles as a Phenotype Shaper or Interactor?

Due to their irregular shape and relatively large surface area, DEP can pick up pollen and other allergenic agents, e.g., grass pollen allergen Lol p 1 (Knox et al. 1997), potentially making it a double-hit, or possibly even a triple-hit threat, where the

asthma phenotype is concerned. Intranasal coadministration of DEP with allergen in atopic rhinitic humans resulted in a 50-fold increase in allergen-specific IgE (Diaz-Sanchez et al. 1997), supporting this concept. There is evidence in animal models that are likewise supportive of this concept (Lee et al. 2013), and it has been pointed out that a number of studies suggest that DEP are associated with induction of the early and late phases of the inflammatory response in asthma (Pandya et al. 2002). Thus, diesel exhaust mitigations such as particulate filters could conceivably lessen the impact of allergic factors on inhaled DEP. Even so, it seems apparent that DEP can be considered to be asthma phenotype interactors from the perspective of the combined nature of particulate/surface area effects, complexed volatile toxicants, and attached allergens, that make up the totality of the DEP that might be typically inhaled by a person in the environment. In this way, DEP could be considered the perfect storm of deep lung administration of environmental factors that can interact and exacerbate existing asthma.

However, some results of mouse studies (Hougaard et al. 2008) suggest that it is conceivable that with prolonged, and possibly early lifetime, or perhaps in-utero exposure, that DEP may promote early disposition to inflammatory effects at the molecular and cellular level, which can be considered precursors to later adverse respiratory health outcomes (Ristovski et al. 2012). For example, epigenetic modifications can occur with repeated exposures to environmental agents, both allergenic and toxicant, over time, that may be important in determining the risk and health outcomes of individuals (Ho et al. 2012) and subsequently have a strong impact on their disease phenotype. In this way, DEP could also possibly be considered as asthma phenotype shapers, if exposure is chronic and life-long.

4.4 Summary

In summary, both allergic and nonallergic/toxicant factors can interact to acutely exacerbate the asthma phenotype; some may also promote its development over prolonged periods of untreated exposure. These interactions can include double- and triple-hit combinations such as allergen + toxicant + particulate effects, which may at least be additive or could possibly be synergistic, producing profound exacerbations. Figure 4.2 illustrates this potential, within a continuum of asthma development, induction, and exacerbation.

However, of more recent consideration, is the possibility that allergen exposures and toxicants (i.e., irritants and promoters in Fig. 4.2) may have direct effects on the genome, which may shape the asthma phenotype and increase susceptibility to the subsequent induction and development of asthma, along the continuum of mild to severe. Furthermore, as mentioned above, there may also be cross-reactivities that may be genetically promoted, which might need to be accounted for, in the determination of treatment options for asthma, in the future. Further research is necessary to better delineate and understand the effects of combined environmental allergens and toxicants on the determination of the asthma phenotype.

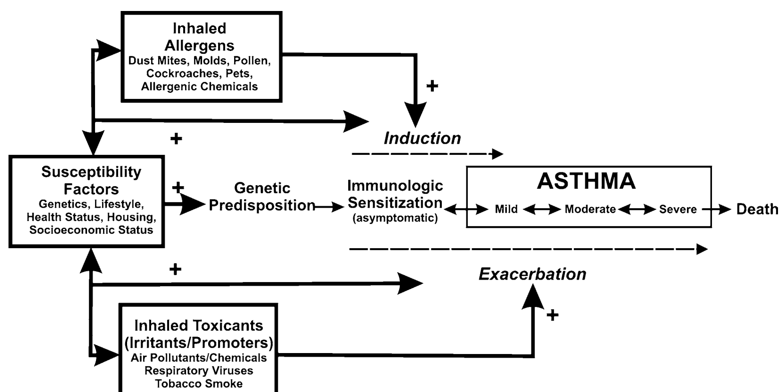

Fig. 4.2 Interactive inhaled factors (allergens and toxicants) involved in induction and exacerbation of asthma. In genetically susceptible people, with exposure to allergens or toxicants, induction and/or exacerbation of asthma can occur (upper and lower dashed arrows), highlighting the importance of the interaction of stimulating factors with the genome. Inhaled irritants and promoters can facilitate induction of asthma through both lung injury and increased uptake of allergens, or by modulating immune responses in genetically susceptible individuals, (as indicated by double-headed thick arrows and plus signs), making them asthma phenotype inducers. More typically, allergens and toxicants are also considered to exacerbate existing asthma, also making them asthma phenotype interactors (also shown by double-headed thick arrows and plus signs). The severity of asthma disease (shown in box on right) can be variable and reversible (as shown by double-headed arrows), depending on environmental factors and treatment, as well as susceptibility to allergens and toxicants. In extreme cases, exacerbation of asthma by environmental factors can lead to death, as shown on far right. Adapted from US EPA, Cui et al. (2011)

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Chapter 5
Current Clinical Diagnostic Tests for Asthma

Shiwan Shah and Gulshan Sharma

Abstract Asthma involves variable airflow obstruction in both large and small airways. The physiological consequences of obstruction include increased airway resistance and decreased expiratory flow rates, which lead to air trapping and dynamic hyperinflation. This chapter reviews current methods for pulmonary function testing to detect these physiological changes for both diagnosis and monitoring.

Keywords Spirometry • Peak expiratory flow rate • Airway hyperresponsiveness • Bronchodilator reversibility • Bronchoprovocation • Methacholine

5.1 Introduction

Asthma is characterized by airway hyperresponsiveness leading to variable airflow obstruction which may occur as a result of both intrinsic (genetic) and extrinsic (environmental) factors. The variable and reversible nature of the obstruction distinguishes asthma from other obstructive lung diseases. Asthma involves both large and small airways, and the physiological consequences of obstruction include increased airway resistance and decreased expiratory flow rates, which lead to air trapping and dynamic hyperinflation. Pulmonary function testing can detect these physiological changes for both diagnosis and monitoring. This is important, as studies have shown that physicians are often inaccurate in diagnosing asthma and predicting reversibility of obstruction based on history and physical examination alone (Russell et al. 1986; Scott et al. 2012).

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5.2 Spirometry

Spirometry is recommended in all adults and children at least 5 years of age in which asthma is a consideration (NAEPP 2007). Spirometry involves the patient inhaling to total lung capacity (TLC) and then blowing out as much as possible to residual volume (RV). The resulting forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV1) are measured. In order to get accurate results, long-acting bronchodilators should be stopped for 12 h and short-acting beta-agonists should be stopped for 6 h prior to performance of spirometry.

Asthma is characterized by an obstructive ventilatory defect, with a reduction in FEV1 out of proportion to FVC, and a low FEV1/FVC ratio. Visually, this is detected as an expiratory scooping on the flow volume loop. Classically, obstruction is defined as an FEV1/FVC ratio less than 70 %. However, recent data suggest that using the lower limit of normal (LLN) is more accurate. LLN is determined by the fifth percentile predicted based on the patient's age, race, gender, height, and other factors. LLN is often greater than 70 % in the younger population, while it is less than 70 % in the older population. While evidence to favor use of either the fixed ratio or LLN for asthma is lacking, it does appear that use of a fixed ratio of 70 % increases the likelihood of underdiagnosing obstruction in the younger patient population, which contains most asthmatic patients (Hansen et al. 2007).

In addition, asthma is characterized by a low forced expiratory flow of 25–75 % (FEF25–75 %), defined as the slope of the spirometry curve between the 25th and 75th percentile. A decreased FEF25–75 % is indicative of small airway obstruction. While an FEF25–75 % less than 60 % of predicted is considered abnormal, values are more variable than FEV1, and the utility of an isolated decrease in FEF25–75 % in the setting of otherwise normal spirometry is otherwise unclear. Nevertheless, evidence suggests that a reduced FEF25–75 % correlates with bronchial hyperresponsiveness on bronchoprovocation testing (Alberts et al. 1994; Drewek et al. 2009).

Spirometry is often repeated after administering a bronchodilator to confirm reversible/variable airflow obstruction. Reversibility is characterized by an increase in either the FEV1 or FVC by more than 12 % and 200 ml. Reversibility supports the diagnosis of asthma, although some patients with chronic obstructive pulmonary disease (COPD) have reversible airflow obstruction. Additionally, in rare cases of severe asthma, reversibility may be minimal until the patient's inflammation is controlled with corticosteroids (NAEPP 2007).

Two important points need to be made. Normal spirometry does not rule out a diagnosis, as spirometry may be normal between asthma flares, especially in those with milder disease. Second, with more severe asthma, one can develop a pseudorestrictive pattern. In this condition, worsening air trapping leads to an increase in the RV and a decline in the FVC with a consequent increase in the FEV1/FVC ratio. Using the ratio of inspiratory capacity (IC) to expiratory reserve volume (ERV) can help distinguish pseudorestriction from true parenchymal

restriction. IC is the maximum volume that can be inspired from resting expiratory levels (tidal volume plus inspiratory reserve volume). The ERV is the maximum volume that can be expired from the end tidal expiration (from functional residual capacity, FRC). IC:ERV is normally 2–3 to 1. With asthma, the increased RV results in a decrease in the ERV, leading to a ratio that may be greater than 6:1. In parenchymal restriction, however, the ratio is often less than 2:1. In addition, pseudorestriction should improve or reverse with bronchodilators.

5.3 Lung Volumes

Asthma is characterized by elevated RV, FRC, and TLC. The most commonly used methods of measuring lung volumes include body plethysmography, nitrogen washout, and inert gas dilution. All three methods measure FRC and, when added to inspiratory capacity determined from spirometry, give TLC. Body plethysmography calculates lung volume based on Boyle's principle: at a constant temperature, the volume of a gas is inversely proportional to the pressure. The patient sits in an enclosed box and then starts to pant against a closed mouthpiece. Changes in the pressure within the box are proportional to the volume of compressible gas within the lung. Because the shutter on the mouthpiece is closed at FRC, this is the volume measured. Both inert gas dilution and nitrogen washout depend on the principle that if an initial volume of gas with a known concentration of either nitrogen or inert gas is inhaled, the concentration in the exhaled gas can allow calculation of lung volume. Starting the process when the patient is at FRC allows calculation of this volume. The downside of these techniques is that only communicating gas is measured. Noncommunicating air spaces, such as distal to areas with high resistances, will not be included. Thus, in obstructive lung disease, gas dilution techniques underestimate TLC, and body plethysmography is preferred (Ferris 1978).

5.4 Diffusion

Diffusion capacity for carbon monoxide (DLCO) is measured by having the patient inhaling air with a certain concentration of carbon monoxide (CO) from RV to TLC. After a 10 s breath hold, the patient exhales, and the amount of CO remaining can be used to determine how much CO was transferred to the blood during that period. DLCO is normal or even elevated in asthma. This is likely due to the increased negative pressures needed for breathing in the setting of obstruction, leading to an increase in pulmonary blood volume. Furthermore, this pulmonary volume will be diverted more toward the upper lung zones, which have more reserve to accommodate the increased blood flow, and are better ventilated (Keens et al. 1979; Collard et al. 1994).

5.5 Peak Expiratory Flow Rate Measurement

The measurement of the peak expiratory flow rate (PEFR) is a simple method to evaluate air flow obstruction. PEFR can be measured in the physician's office or at home. Strict attention should be paid to proper technique, as improper technique can lead to nonvalid results. Patients should stand upright, to take in as deep a breath as possible, and then place the peak flow meter in the mouth, making sure that the tongue does not obstruct the device. The patient should close his/her mouth tightly against the device and then blow out as hard and fast as possible. The patient should then hold the breath normally for a little while and then repeat the process two more times. The highest of the three values should be used. Patients are encouraged to measure PEFR early in the morning as well as in the evening. PEFR is normally lower in the morning and higher in the evening, a difference which is exaggerated in asthma. A diurnal variation greater than 20 % is diagnostic of asthma, and higher variability correlates with poorer asthma control. Nevertheless, PEFR should not be used to diagnose asthma. There is a wide variability in PEFR reference values, and the values will vary with the brand of equipment used. In addition, less severe obstruction tends to be underestimated, while more severe obstruction tends to be overestimated. Nevertheless, if an asthmatic patient establishes a personal best when he/she is clinically well, monitoring PEFR in reference to this baseline can be useful. If the peak flow rates are 80–100 % of the personal best, the patient is in the green zone, and this correlates with good asthma control. Values of 50–80 % represent the yellow zone, and medical care should be sought, and asthma control likely needs to be improved. Values less than 50 % of the patient's personal best represent the red zone and require immediate physician attention. Of note, PEFR monitoring has not been shown to be superior to monitoring of clinical symptoms.[10-12] Nevertheless, guidelines suggest considering PEFR monitoring for patients with moderate to severe asthma, those with a history of severe exacerbations, those who poorly perceive airflow obstruction, and those who prefer this method of monitoring (NAEPP 2007).

5.6 Bronchoprovocative Testing

Demonstration of airway hyperresponsiveness by bronchoprovocative testing is useful in evaluating patients who have asthma symptoms but normal spirometry. A significant proportion of these patients will not have asthma, and given the high negative predictive value, a negative bronchoprovocative test essentially rules out asthma (McGrath and Fahy 2011). On the other hand, a positive test is consistent with asthma. Patients with other conditions, including allergic rhinitis, cystic fibrosis, and COPD, may have positive tests as well. In these cases, the clinical history should be used in conjunction with the results of the bronchoprovocative test.

As the test involves induction of bronchospasms, patients may deteriorate clinically during the test. Patients with lower reserve, specifically those with an FEV1

less than 50 % of predicted volume or those with less than 1 l of reserve, should not undergo testing. An FEV1 between 60 and 70 % is a relative contraindication. Other relative contraindications include acute coronary syndrome or stroke within the past 3 months; severe, uncontrolled hypertension; and significant hypoxemia. Generally, it is necessary to withhold albuterol for at least 8 h prior to the test, ipratropium for at least 24 h, and long-acting beta-agonists for at least 24 h. Caffeine-containing products, including coffee, tea, cola, and chocolate, may affect the test and should not be consumed on the day of the study (Henderson et al. 1993).

There are two types of provocative tests: direct and indirect. Direct testing involves administering medications (usually either methacholine or histamine) that act on smooth muscle receptors to constrict the airway. Histamine is not FDA approved; thus, methacholine is most commonly used in the United States. Methacholine is administered via nebulization and 2 min tidal breathing or with a five breath dosimeter, in doubling concentrations until the FEV1 drops more than 20 % or the highest concentration is reached. The level of methacholine required is noted as PC20. A level less than 16 is characterized as mild airway hyperreactivity, less than 4 as moderate hyperreactivity, and less than 1 as severe hyperreactivity. Values greater than 16 indicate no significant hyperreactivity. The exact cutoff for diagnosis of asthma depends on pretest probability, although in the setting of moderate pretest probability, a level less than 8 is predictive of asthma.

Indirect stimuli result in the release of inflammatory mediators which mediate subsequent bronchoconstriction. These include cold air, exercise, eucapnic hyperventilation, hypertonic saline, mannitol, and adenosine (Argyros et al. 1996). While direct testing allows for better characterization of dose response, indirect testing is often more clinically meaningful. Specifically, for exercise-induced bronchospasms, eucapnic hyperventilation, in which the patient voluntarily hyperventilates a mixture of 5 % CO_2 and 95 % room air for 6–8 min, is the most sensitive bronchoprovocative test (Holley et al. 2012).

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Chapter 6
Management of Asthma: The Current US and European Guidelines

Ashwini P. Reddy and Meera R. Gupta

Abstract Asthma management guidelines aim to improve the implementation of current knowledge into daily clinical practice by establishing a consensus of scientific practices for the management of asthma. Initial guidelines were based on consensus of expert opinion in order to employ a severity-based classification system as a guide to treatment. However, advances in asthma research led to the development of evidence-based guidelines and a major paradigm shift to control-based asthma management. Control-based management is central to the published guidelines developed by The National Heart, Lung, and Blood Institute (NHLBI), The Global Initiative for Asthma (GINA), and The British Thoracic Society (BTS), each one using the same volume of evidence but emphasizing aspects particular to their specific patient populations and socioeconomic needs. This chapter summarizes the evolution of these guidelines and summarizes the key points and evidence used in the recommendations for the assessment, monitoring, and management of asthma in all ages, with particular emphasis on the NHLBI guidelines.

Keywords Asthma • Asthma: management • NHLBI guidelines • Asthma: drug therapy • Humans • Practice guidelines as topic • GINA guidelines • BTS guidelines • Asthma: pediatric guidelines • Asthma: diagnosis • Asthma: treatment

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6.1 The Need for Guidelines

In the early 1980s, asthma prevalence, morbidity, and mortality were increasing in all age groups worldwide. Findings that with optimal treatment many of these asthma deaths were preventable, led to the development of guidelines for the optimal management of asthma by countries worldwide (Bousquet et al. 2007). To address the growing problem of asthma in the USA, in 1989 the National Heart, Lung, and Blood Institute (NHLBI) initiated the National Asthma Education and Prevention Program (NAEPP), and in 1991, the NAEPP Expert Panel published the first comprehensive "Expert Panel Report: Guidelines for the Diagnosis and Management of Asthma" (EPR 1) based on expert opinions. These guidelines aimed to improve the implementation of current knowledge into daily clinical practice by helping healthcare professionals bridge the gap between current knowledge and treatment (Sheffer and Taggart 1991).

6.2 History of the NHLBI Guidelines

6.2.1 Adults

1991: Expert Panel Report 1

The first EPR guidelines focused on the recent discovery that asthma was an inflammatory disease, and transitioned clinical management to a treatment approach focused on controlling inflammation rather than managing bronchospasm (NHLBI 1991). A concept of asthma management consisting of a multifaceted approach was introduced where recommendations for the treatment of asthma were organized around four components of effective management (1) assessment of asthma severity and regular monitoring of the effectiveness of therapy, (2) control of environment factors and comorbid conditions affecting asthma, (3) comprehensive pharmacologic therapy for long-term management and acute exacerbations, and (4) patient education to foster a partnership of care between the patients, families, and clinicians. Patients were classified by the level of disease severity based on a composite analysis of symptom frequency, activity limitation, need for rescue medications, and pulmonary function test results, and treatment recommendations for the type and amount of medications for were outlined at each level. An improved understanding of the pathophysiology of asthma and the addition of new medications, such as long-acting β-adrenergic agonists (LABAs) and leukotriene modifiers, led to the first of continuing updates in 1997 (Myers 2008).

1997: Expert Panel Report 2

The Expert Panel Report 2 (EPR 2) put forth a number of new ideas. Firstly, the increasing scientific base of published articles on asthma allowed for the evolution of guideline development from opinion based to one based on a systemic review of

scientific evidence. Secondly, due to accumulating scientific evidence leading to the definition of asthma as a chronic inflammatory disorder of the airways and identification of ongoing inflammation as the cause for recurrent episodes of bronchospasm, bronchial hyperresponsiveness, and persistent airflow obstruction from airway remodeling (Laitinen and Laitinen 1994a, b), the EPR 2 sought to emphasize the importance of early recognition and treatment to prevent irreversible airway injury by early intervention with anti-inflammatory therapy (Djukanovic et al. 1992; Jeffery et al. 1992; Laitinen et al. 1992; Levy 1995). As a result, the classification of asthma severity was changed from mild, moderate, and severe to mild intermittent, mild persistent, moderate persistent, and severe persistent in an attempt to more accurately reflect the clinical manifestations of asthma (NHLBI 1997). Furthermore, to emphasize that persistent asthma requires daily long-term therapy (Busse 1993; Duddridge et al. 1993), medications were categorized as being either controller or rescue medications (NHLBI 1997). Although an EPR 2 update on selected topics was published in 2002 (NHLBI 2002), the first major revision of the asthma guidelines occurred in 2007 with the Expert Panel Report 3 (EPR 3).

2007: Expert Panel Report 3

Previous guidelines were constructed on the idea of assessing and grading asthma severity to guide management and identify people at risk for severe exacerbations. However, recognition that severity can vary over time and that the responsiveness to treatment is heterogeneous even among patients with asthma of similar severity, raised concerns about classifying asthma by severity alone (Wolfenden et al. 2003; Graham 2006). Furthermore, the use of severity as a single outcome measure had limited value in predicting the treatment required and the patient's response to that treatment (Bateman et al. 2004). As it became recognized that categorizing asthma involved both severity of the disease and its responsiveness to treatment, guideline committees began to propose that asthma severity no longer be used as the basis for treatment decisions and instead focused on assessing and using asthma control (NHLBI 2007).

The EPR 3 proposes that concepts of asthma severity and control are linked by common therapeutic goals that are identical for all levels of baseline asthma severity and the specific measures used to assess these domains (frequency of symptoms, need for rescue medications, limitations to normal activities, pulmonary function tests, and frequency of exacerbations). Both concepts are brought into the guidelines of care by initiating pharmacologic therapy based on asthma severity and adjusting therapy based on the level of asthma control (Colice et al. 1999; Strunk et al. 2002; Bacharier et al. 2004). To emphasize the need to consider asthma's effects on quality of life and functional capacity and the risks for future adverse events, severity and control are defined in two domains: impairment and risk. Impairment is an assessment of the frequency and intensity of symptoms and functional limitations, whereas risk is an estimate of the likelihood of either asthma exacerbations or of progressive loss of pulmonary function over time (NHLBI 2007). Although linked, these distinct domains represent different manifestations of asthma that may respond to differently to treatment (Colice et al. 1999; Fuhlbrigge et al. 2002; Bacharier et al. 2004; Schatz et al. 2005).

6.2.2 Children

Pediatric-specific recommendations for asthma management were first introduced in the 1997 EPR 2 guidelines. The availability of an increasing number of studies on wheezing in children led to the formulation of separate recommendations for asthma management in children 5 years of age and under (NHLBI 1997). Similar to adults, children were classified into four groups based on disease severity: mild intermittent, mild persistent, moderate persistent, and severe persistent, with additional recommendations to initiate daily therapy in infants and children consistently requiring symptomatic treatment more than two times per week and in those with episodes of severe exacerbations occurring <6 weeks apart. The lack of evidence on the safety of ICS use in this age group led to recommendations for the preferred use of nedocromil or cromolyn as first-line treatment for mild persistent asthma, and low-dose ICS as alternative therapy (Silverman et al. 1972; Geller-Bernstein and Sneh 1980; Glass et al. 1981; Bertelsen et al. 1986).

2002: Update to the Expert Panel Report 2

The availability of nebulized ICS and montelukast for children as young as 2 years of age and new studies on the effectiveness and safety of ICS in children (CAMP 2000) led to an update of the pediatric guidelines in 2002 (NHLBI 2002). Initiation of treatment with long-term controller therapy was extended to infants and children who had more than three episodes of wheezing in the past year and a high risk of developing persistent asthma as indicated by a history of atopy or a parental history of asthma (Martinez 1995; Martinez et al. 1995; Castro-Rodriguez et al. 2000). Results from the Childhood Asthma Management Program (CAMP) trial demonstrating no differences between nedocromil and placebo in lung function or symptom outcome led to its removal from treatment recommendations (CAMP 2000). Low-dose ICS became the preferred therapy for mild persistent asthma, with cromolyn (Petty et al. 1989; Konig 1997) or leukotriene receptor antagonists (LTRA) as alternative therapy (Israel et al. 1996; DuBuske et al. 1997; Altman et al. 1998; Kemp et al. 1998; Knorr et al. 1998, 2001; Nathan et al. 1998; Tashkin et al. 1999; Bleecker et al. 2000; Pearlman et al. 2000; Busse et al. 2001). Comparative studies in older children and adults consistently favoring combination therapy over increasing doses of ICS (Greening et al. 1994; Woolcock et al. 1996) led to the preferred approach of adding LABAs to lower doses of inhaled corticosteroids for moderate persistent asthma in children 5 years of age and older. However, due to the lack of data on LABAs in children under 4 years of age (Verberne et al. 1997), monotherapy with medium-dose ICS was recommended as the preferred treatment option (Anhoj et al. 2002), with the addition of LTRA or theophylline to low-dose ICS as a nonpreferred alternative.

2007: Expert Panel Report 3

The EPR 3 divides treatment recommendations into three age groups: 0–4 years of age, 5–11 years of age, and ≥12 years of age. These groupings were chosen based on age-related issues of drug delivery and medication approval, relevance of the

different measures of impairment, potential short- and long-term impact of medications, and the variable levels of scientific evidence available for each age group with limited data on the safety and efficacy of treatments for young children (Baker et al. 1999; Kemp et al. 1999). Additionally, it was recognized that the course of disease changes over time. In children 5 years of age and younger, two general patterns in the progression of asthma symptoms appear: remission of symptoms in the preschool years and persistence throughout childhood (Martinez et al. 1995). Although no absolute markers exist to predict the prognosis of each individual child, longitudinal data from the Tucson Children's Respiratory Study was used to generate an asthma predictive index to identify risk factors for the development of persistent asthma (Castro-Rodriguez et al. 2000; Guilbert et al. 2006); children under 3 years of age with 4 or more episodes of wheezing in the past year that lasted more than 1 day and affected sleep are likely to have persistent asthma at 5 years of age if they also have a positive predictive index, either one of the following: parental history of asthma, atopic dermatitis or aeroallergen sensitization, or two of the following: food allergy, >4 % peripheral eosinophillia or wheezing apart from colds.

6.3 Summary of Recommendations from the 2007 NHLBI Guidelines

6.3.1 Assessing and Monitoring Asthma Control

Initial Assessment of Severity

The EPR 3 links the functions of assessment and monitoring to the concepts of severity, control, and responsiveness to treatment. Although severity of disease is best assessed in patients before long-term controller medications are initiated, severity can also be inferred from the least amount of treatment required to maintain control in the domains of current impairment and future risk. Clinical studies confirm that parameters used for the impairment domain reflect increasing gradients of severity in adults (Schatz et al. 2003, 2005; Antonicelli et al. 2004; Diette et al. 2004). However, regardless of their asthma severity as classified on the basis of symptoms, the majority of children 5–18 years of age have normal FEV_1 values, and FEV_1/FVC appears to be a more sensitive measure of severity (Bacharier et al. 2004; Spahn et al. 2004; Paull et al. 2005). In the risk domain, the frequency of exacerbations requiring intervention with oral systemic steroids has been correlated in observational studies with the designation of persistent asthma; in general, the more frequent and intense the exacerbations, the greater the degree of underlying disease severity (Fuhlbrigge et al. 2001, 2006). Thus, based on specific measures (symptoms, use of rescue medications, frequency of exacerbations, and pulmonary function tests), asthma severity is categorized as either intermittent or persistent, with further classification of persistent asthma as either mild, moderate, or severe. To further emphasize the risk domain, an additional classification for the intensity of exacerbations was added and the designation of mild intermittent asthma was

Table 6.1 NHLBI 2007 guidelines for classifying asthma severity and initiation of treatment by age

Components of severity		Intermittent			Persistent								
					Mild			Moderate			Severe		
Age in years		0–4	5–11	>12	0–4	5–11	>12	0–4	5–11	>12	0–4	5–11	>12
Impairment	Symptoms	≤ 2 days/week			≤ 2 days/week but not daily			Daily			Throughout the day		
	Nocturnal symptoms	0	≤ 2x/month		1-2x/month	3-4x/month		3-4x/ month	≥ 1x/week		≥ 2x/week	Often 7x/week	
	SABA use	≤ 2 days/week			≥ 2 days/week			Daily			Several times/day		
	Interferes with normal activity	None			Minor			Some			Extremely		
	PFT FEV$_1$	n/a	> 80%		n/a	80%		n/a	60–80%		n/a	< 60%	
	PFT FEV$_1$/FVC		> 85%	Normal ratio		> 80%	Normal ratio		75-80%	Reduced by > 5%		< 75%	Reduced by > 5%
Risk	Exacerbations requiring systemic corticosteroids	0–1x/year			≥2x/6 months **OR** > 4x/year + risk factors	> 2x/year →							
Recommended step for initiating treatment		Step 1			Step 2			Step 3			Step 3		Step 4 or Step 5

modified to intermittent asthma to emphasize that patients at any level of severity can have severe exacerbations (NHLBI 2007). Table 6.1 summarizes the classification of asthma for each age group.

Assessment of Control

After treatment is established, periodic monitoring and assessment is used to determine whether the goals of asthma therapy are being achieved, asthma is controlled, and if adjustments in therapy are needed (NHLBI 2007). Similar to the assessment of asthma severity, asthma control is also defined in the domains of impairment and risk in the different age groups, refer Table 6.2. The use of validated questionnaires [Asthma Control Test (Nathan et al. 2004), Childhood Asthma Control Test (Liu et al. 2007), Asthma Control Questionnaire (Juniper et al. 1999), and Asthma Therapy Assessment Questionnaire (Vollmer et al. 1999)] in addition to pulmonary function testing was included to better quantify asthma control (Katz et al. 2002). Once asthma control is obtained, reassessment of asthma severity is recommended, with reclassification by the lowest level of treatment required to maintain control (Lemanske et al. 2001; Hawkins et al. 2003). Recommended intervals for monitoring are 2–6 weeks for new or uncontrolled patients, 1–6 months for those who are controlled, or every 3 months if a change in therapy is anticipated (NHLBI 2007).

6.3.2 *Stepwise Approach for Asthma Management*

The EPR 3 recommendations for long-term asthma management integrate the four components of therapy into a stepwise therapeutic approach in which medications are increased as necessary and decreased if possible to achieve and maintain

Table 6.2 NHLBI 2007 guidelines for assessing asthma control and adjusting therapy by age

Components of control			Well controlled			Not well controlled			Poorly controlled		
Age in years			0–4	5–11	>12	0–4	5–11	>12	0–4	5–11	>12
Impairment	Symptoms		≤ 2days/week but not daily			≥ 2 days/week **OR** Multiple times per day on ≤ 2 days/week			Throughout the day		
	Nocturnal symptoms		≤ 1x/mo	≤ 1x/mo	< 2x/mo	> 1x/mo	≥ 2x/mo	1-3x/week	> 1x/week	≥ 2x /week	4x/week
	SABA use		≤ 2days/week			≥ 2days/week			Several times/day		
	Limitations in activity		None			Some			Extremely		
	Questionnaire	ATAQ	0			1–2			3–4		
		ACQ	≤ 0.75			1.5			N/A		
		ACT/CACT	> 20			16–19			≤15		
	PFTs	FEV_1	n/a	80%		n/a	60–80%		n/a	< 60%	
		FEV_1/FVC	n/a	> 80%	n/a	n/a	75–80%	n/a	n/a	< 75%	n/a
Risk	Exacerbations requiring systemic steroids		0–1x/year			2–3x/yr	> 2x/year		> 3x/year	> 2x/year	
	Reduction in lung growth		n/a	Long-term Follow-up		n/a	Long-term Follow-up		n/a	Long-term Follow-up	
	Side effects of treatment		Side effects can vary. Consider in assessment of risk.								
Recommended action for treatment			Maintain step down if control at least 3 months			Step up 1 step			Systemic corticosteroids Step up 1–2 steps		

long-term control of asthma. The type, amount, and scheduling of mediation is determined by the level of asthma severity (Table 6.1) or control (Table 6.2), and therapy is stepped up as needed for more severe or uncontrolled asthma, and stepped down, when possible (Table 6.3). To simplify previous guidelines where each step had several progressive actions, the EPR 3 expands the stepwise approach to six treatment steps for all age groups. The two new steps sharpen the focus of recommendations at each progressively higher level of treatment (NHLBI 2007).

The general stepwise approach is applicable to all patients who have asthma, with modifications to meet the needs of different patient age groups. Although medications were repositioned within the six steps of care, ICS therapy remained at the heart of the treatment for persistent asthma for all ages to emphasize the inflammatory nature of asthma, and the use of daily therapy only during specific periods of previously documented risk was added to the step 1 recommendations (Rafferty et al. 1985; Haahtela et al. 1991; Jeffery et al. 1992; van Essen-Zandvliet et al. 1992; Dahl et al. 1993; Kamada et al. 1996; Suissa et al. 2000; Pauwels et al. 2003). Due to the FDA black box warning on all medications containing LABA over concerns regarding its safety, step 3 recommendations were modified from the 2002 guidelines, and increasing the dose of ICS is presented as an equally preferred option to adding an LABA to low-dose ICS in all patients ≥5 years of age (Bateman et al. 2004; O'Byrne et al. 2005). Specific recommendations for each age group are presented below.

Treatment Recommendations for Children 0–4 Years of Age

Although administration of ICS early in the disease process does not alter the underlying progression, achieving adequate asthma control does reduce impairment from

Table 6.3 2007 NHLBI Stepwise treatment recommendations by age

Age		Step1	Step 2	Step 3	Step 4	Step 5	Step 6
		Intermittent asthma		**Persistent asthma: daily medications**			
0–4 years of age	Preferred	SABA as needed	Low-dose ICS	Medium-dose ICS	Medium-dose ICS + LABA or montelukast	High-dose ICS + LABA or montelukast	High-dose ICS + LABA or montelukast + Oral steroids
	Alternative		Cromolyn or montelukast				
5–11 years of age	Preferred		Low-dose ICS	Low-dose ICS + LABA, LTRA, theophylline **OR** Medium-dose ICS	Medium-dose ICS + LABA	High-dose ICS + LABA	High-dose ICS + LABA + Oral steroids
	Alternative		Cromolyn, LTRA, nedocromil, or theophylline		Medium-dose ICS + LTRA or theophylline	High-doseICS + LTRA or theophylline	High-dose ICS + LTRA or theophylline + Oral steroids
≥12 years of age-Adults	Preferred		Low-dose ICS	Low-dose ICS + LABA **OR** medium-dose ICS	Medium-dose ICS + LABA	High-dose ICS + LABA **AND** Consider omalizumab	High-dose ICS + LABA + Oral steroids **AND** Consider omalizumab
	Alternative		Cromolyn, LTRA, nedocromil, or theophylline	Low-dose ICS + LABA, LTRA, zileuton, or theophylline	Medium-dose ICS + LTRA,zileuton, or theophylline		
		For all ages at each step: patient education, environmental control, management of comorbidities					
		For 5–11 years of age and ≥12 years of age-adults groups: Consider subcutaneous immunotherapy for patients with persistent allergic asthma					

symptom burden and the risk for severe exacerbations (Guilbert et al. 2006). Based on the long-term clinical efficacy of ICS in controlling asthma (O'Byrne et al. 2005; Guilbert et al. 2006) in this age group, ICS continue to be the preferred treatment for persistent asthma, although for step 2, montelukast in children 2 years of age or older can be considered if inhaled medication delivery is suboptimal due to either technique or adherence. Studies addressing step 3 care in children from 0 to 4 years of age are limited. Although some studies suggest a dose-dependent decrease in exacerbations, symptoms, and short-acting β-agonist (SABA) use with daily ICS

therapy, findings are mixed (Bisgaard 1999; Szefler 2002). Moreover, studies looking at the addition of an LABA to low-dose ICS in children ≥4 years of age demonstrated improved lung function and decreased symptoms (Russell et al. 1995; Zimmerman et al. 2004) but did not show a reduction in asthma exacerbations (Bisgaard 2003a). Due to the lack of studies with LABAs in young children combined with lack of evidence of demonstrating improvement in the risk domain, for step 3 the EPR 3 guidelines recommend increasing the dose of ICS prior to adding on adjunctive therapy. No data were found on add-on therapy in children 0–4 years of age whose asthma was not well controlled on medium-dose ICS; thus, recommendations for step 4 of asthma management are extrapolated from studies in older children and adults. In step 4, the EPR-3 recommends adding a noncorticosteroid medication to medium-dose ICS to avoid the risk of side effects associated with high-dose ICS (Van den Berg et al. 2000; Malone et al. 2005).

Treatment Recommendations for Children 5–11 Years of Age

Long-term studies in children ages 5–12 years of age indicate that daily ICS improves health outcomes for children who have mild or moderate persistent asthma, and that the effectiveness outweighs the potential risk for delayed growth (CAMP 2000). Therefore, similar to the 0–4 years of age category, daily ICS continue to be recommended for the treatment of persistent asthma in step 2. However, in this age group, monotherapy with montelukast has not been found to be as efficacious as ICS on a range of asthma outcomes and is not recommended as an equally preferred alternative in at this treatment step (Garcia Garcia et al. 2005; Ostrom et al. 2005; Sorkness et al. 2007). For step 3, two equally preferred treatment options are available. Data from two trials demonstrated that children 4–11 years of age whose asthma was not completely controlled by low-dose ICS alone achieved improved lung function and symptom control with the addition of an LABA as compared to placebo (Russell et al. 1995; Zimmerman et al. 2004). In another trial, the addition of montelukast to low-dose ICS resulted in a slight increase in lung function and reduction in as-needed SABA use (Simons et al. 2001). Additionally, a systemic review in children 4–16 years of age reported a dose response to ICS for improvement in lung function and symptom control (Masoli et al. 2004). Thus, due to the lack of comparison studies for these various long-term control medications in children <11 years of age, the use of low-dose ICS plus adjunctive therapy with an LABA or LTRA, or increasing to a medium-dose ICS are presented as equally preferred treatment options for step 3. The decision between these options may be made on which domain is affected. Children with low lung function and >2 days per week impairment may be better served by adding an LABA to a low-dose ICS, whereas for the risk domain studies have not demonstrated that adding either LABA or LTRA reduces exacerbations in children (Bisgaard 2003a, b). Based on comparative studies in older children and adults, in step 4 the addition of an LABA is preferred (Greenstone et al. 2005; Masoli et al. 2005), with the use of LTRA or theophylline as a secondary alternative (NHLBI 2007; Peters et al. 2007).

6.3.3 *Treatment for Youths ≥12 Years of Age and Adults*

As many of the treatment recommendations for the 5–11 years of age group were extrapolated from studies in older children and adults, the stepwise recommendations for patients in the >12 years of age to adult group are identical for steps 1 and 2 (Table 6.3). The recommendations for step 3 are derived from studies demonstrating that the addition of an LABA to medications in patients whose asthma is not well controlled on low- to medium-dose ICS improves lung function, decreases symptoms, and reduces exacerbations and the use of SABAs (Bateman et al. 2004; Greenstone et al. 2005; Masoli et al. 2005). However, although less effective than adding an LABA (Ind et al. 2003), escalating the dose of ICS in patients with uncontrolled asthma was able to improve the status of control to well controlled or totally controlled. Furthermore, additional studies show similar rates of exacerbations and nighttime awakenings among patients treated with medium-dose ICS or combination low-dose ICS/salmeterol (O'Byrne et al. 2005). This evidence combined with the increased risk for potentially deleterious side effects with the daily use of LABAs (Mann et al. 2003; Nelson et al. 2006) led to recommendations of two equally acceptable options for step 3 treatments: the addition of an LABA to low-dose ICS or increasing to medium-dose ICS (Table 6.3). As in children, the decision between these options may be made on which domain is affected. For the impairment domain, adding an LABA rather than increasing the dose of ICS has shown to more consistently result in improvements (NHLBI 2002). However, in the risk domain, the balance of potential risks need to be considered; the increased benefit of adding LABA to low-dose ICS with the risk of rare life-threatening or fatal exacerbations from LABA use versus the reduced risk of exacerbations at high-dose ICS with the risk of systemic effects at those doses (Pauwels et al. 1997; Masoli et al. 2005). As an alternative but not preferred treatment option, leukotriene modifiers or theophylline may be added to low-dose ICS, although these have not been found to be as effective in controlling asthma at all outcome measures (Evans et al. 1997; Ukena et al. 1997; Dahlen et al. 1998; Laviolette et al. 1999). The recommendations for steps 4–6 are identical to those in children aged 5–11, with the exceptions of the addition of zileuton as a choice for adjunctive therapy in step 4, and the use of omalizumab for steps 5 and 6 in patients who have sensitivity to perennial allergens (Bousquet et al. 2004; Humbert et al. 2005).

6.4 Other International Guidelines

In addition to the NHLBI guidelines, other international guidelines emphasizing specific patient populations have been published. The Global Initiative for Asthma (GINA) guidelines were first published in 1995 in order to have asthma guidelines that emphasized issues facing developing nations (Bateman et al. 2008). At approximately the same time, the British Thoracic Society (BTS) published their guidelines

in the British medical journal with diagnosis and treatment plans directed towards primary care physicians in their country (Morgan and Higgins 2003). Both the GINA and BTS guidelines have had several revisions since their inception with major changes in classification and treatment.

6.4.1 The BTS Guidelines

The BTS guidelines were initially published in 1990, before the US guidelines were developed. Due to a need for a methodic evidence-based guideline for asthma management, in 1999 the BTS and the Scottish Intercollegiate Guidelines Network (SIGN) partnered together to jointly create the next set of comprehensive asthma guidelines for the UK using explicitly evidence-based methodology. Although guidelines are updated yearly, the last major revision was published in 2008 (BTS/SIGN 2008b). Similar to the NHBLI and GINA guidelines, the goal of these guidelines is to provide recommendations based on current evidence for best practice in the management of asthma and is aimed for healthcare professionals, as well as, others outside the healthcare system actively involved in the care of asthmatic patients.

The BTS/SIGN guidelines are centered on three main concepts (1) the initial diagnosis and monitoring of asthma, (2) pharmacologic and nonpharmacologic management, and (3) the organization and delivery of care, and patient education and self-management. Like the NHLBI guidelines, the BTS/SIGN guidelines provide information on specific medications and recommended doses. The recommendations for the management of patients are divided by age into three groups: <5 years of age, 5–12 years of age, and those greater than 12 years of age, although the guidelines note that many of the recommendations in the 5–12 years of age and greater than 12 years of age groups are the same.

Initial Assessment and Monitoring

The focus of the initial assessment of patients with asthma is making an accurate clinical diagnosis. As there are no standardized diagnostic tests to diagnose asthma, the BTS/SIGN guidelines encourage clinicians to determine the “probability” of someone having asthma when they present with symptoms. The approach to diagnosis is based on the primary care model that uses an integrated approach centered on the patients presenting symptoms and acquisition of additional details (personal or family history of atopic disease or asthma and diagnostic testing) to achieve an accurate diagnosis, and based on the initial clinical assessment, patients are classified as having a high, low, or intermediate probability of having a diagnosis of asthma (BTS/SIGN).

For patients in all age groups with a high probability of asthma, a therapeutic trial with daily anti-inflammatory medications is recommended, whereas in those

with a low probability of asthma, investigations and treatments for other conditions are recommended. In children and adults with an intermediate probability of asthma, watchful waiting with follow-up is advised, with an option to initiate an empiric trial of treatment depending on the severity of symptoms and results of diagnostic tests. Similar to the NHBLI guidelines after the initial assessment, monitoring of asthma symptoms using various tools (validated asthma questionnaires, pulmonary function tests, and peak expiratory flow volumes) to assess and measure asthma control plays a key role in the recommendations for management. The BTS/SIGN guidelines emphasize monitoring to facilitate the diagnostic process by determining the response to treatment and providing clinicians with information to support treatment and referral decisions.

Pharmacologic Management

Guidance on the pharmacologic management of chronic asthma occupies a central position in the 2008 BTS/SIGN guidelines and emphasizes the need to strive for high levels of asthma control with no breakthrough symptoms or exacerbations and minimal side effects. In addition, identifying patient-set targets for control that balances the patients' needs and personal goals for their asthma management with the idea of perfect control to reduce poor adherence to daily medications and poor outcomes is highlighted. Treatment is organized into a stepwise approach, with the aim of treatment to maintain and achieve control by stepping up or down as appropriate. The level of treatment is dictated by assessment of control rather than by severity.

Adults

In adults, treatment is divided into five steps (Table 6.4). In step 1 (mild intermittent asthma) and all subsequent steps, as needed SABAs are required. For persistent symptoms, step 2 (regular preventer therapy) recommendations are to initiate daily therapy with low- to moderate-dose ICS (Adams et al. 2001). For patients uncontrolled at step 2, the step 3 (initial add-on therapy) recommendations are divided into two parts. As patients using various strengths of combination fluticasone/salmeterol inhaler have been found to achieve guideline-defined control more rapidly and at a lower total dose of ICS than with fluticasone alone, the first choice is the addition of an LABA to low- or moderate-dose ICS (Ringbaek et al. 1996; Crompton et al. 1999; Wallaert et al. 1999). However, in patients with a poor response to LABA, a second option of increasing the dose of ICS along with the LABA, or adding an alternative therapy such as LTRA or theophylline in lieu of an LABA is offered (Evans et al. 1997; Ukena et al. 1997; Ducharme 2003). Additionally, at step 3 and above, the use of combination budesonide/formoterol as a rescue medication instead of an SABA (known as the SMART regimen) has been found to be an effective and cost-saving treatment option (Rabe et al. 2006). At step 4 (persistent poor control), the recommendations are for high-dose ICS and the addition of a fourth drug, such as an LTRA, theophylline, or β_2-agonist tablet. In the fifth and final step

(continuous or frequent use of oral steroids), the addition of daily systemic steroids along with high-dose ICS and referral to a specialist is recommended.

Children 5–12 Years of Age

In children 5–12 years of age, treatment is once again divided into 5 steps (Table 6.4), with recommendations similar to those for adults. Although the routine use of ICS in the treatment of viral induced wheezing is not supported by the BTS/SIGN guidelines, symptoms ≥3 times per week, the use of SABA ≥3 times per week, nocturnal symptoms once a week, or a history of exacerbation requiring oral steroids in the preceding 2 years are indications of poor asthma control and the need for daily ICS therapy (Sporik et al. 1991; Martinez et al. 1995; Dodge et al. 1996). The first major difference occurs at step 3 where 400 mcg per day is defined as the upper limit for moderate-dose ICS along with an LABA and/or adjunctive therapy (BTS/SIGN 2008a). Additionally, due to lack of evidence in patients under 18 years of age, the SMART regimen is not recommended. The step 4 recommendations include increasing the dose of ICS to 800 mcg per day in addition to step 3 treatments, and in step 5, the addition of daily systemic steroids along with referral to a specialist is recommended.

Children <5 Years of Age

For children <5 years of age, recommendations are divided into only 4 steps (Table 6.4). In step 2, treatment with daily dose of ICS at 200–400 mcg per day or LTRA are offered as potentially equal options (Ducharme 2003; Kelly et al. 2008). For initial add-on therapy in step 3, LTRA should be added on in children on maximal doses of ICS and vice versa (Spector et al. 1994; Altman et al. 1998; Reiss et al. 1998). In this age group, LABAs and ICS at doses >400 mcg per day are not recommended at any level of treatment. Finally, for children with persistent poor control (step 4) or in children under 2 years of age, referral to a specialist is recommended.

6.4.2 The GINA Guidelines

The GINA was established in 1993 as a collaborative effort between the NHBLI and the World Health Organization (WHO) with the purpose of developing asthma diagnosis and management guidelines that took into consideration the differences in socioeconomic status of different countries and the availability of healthcare resources. There were two phases to GINA; the first phase encompassing the actual report which included sections on epidemiology, pathogenesis and preventions, complementary medicines, and health economics, and the second phase focused on creating educational materials for widespread dissemination to public health officials, healthcare professionals, and patients. Since their inception, the GINA guidelines have undergone four major revisions. The third revision represents the

Table 6.4 2008 BTS/SIGN treatment recommendations by age

	Step1 **Mild intermittent asthma**	**Step 2** **Regular preventer therapy**	**Step 3** **Initial add-on therapy**	**Step 4** **Persistent poor control**	**Step 5** **Continuous or frequent use of oral steroids**
>5 years of age		Daily ICS (200-400 mcg/day) **OR** LTRA	Daily ICS (200-400 mcg/day) **+** LTRA	Referral to specialist	
5–12 years of age	SABA as needed	Daily ICS (200–400 mcg/day)	1. ICS + LABA 2. Assess control –Benefit from LABA but not controlled: ↑ICS to 400 mcg/day +LABA –No benefit from LABA: ↑ICS to 400 mcg/day + LTRA **OR** theophylline	↑ICS to 800 mcg/day **+** Step 3 therapies	Daily oral steroids at lowest dose providing control **+** Maintain high-dose ICS **+** Referral to specialist
≥12 years of age		Daily ICS (200–800 mcg/day)	1. ICS + LABA 2. Assess control –Benefit from LABA but not controlled: ↑ICS to 800 mcg/day +LABA –No benefit from LABA: ↑ICS to 800 mcg/day + LTRA **OR** Theophylline	Consider trials of: • ↑ICS to 2000 mcg/day • Addition of fourth drug (LTRA, theophylline, β-agonist)	Daily oral steroids at lowest dose providing control **+** Maintain high-dose ICS **+** Alternative treatments **+** Referral to specialist

transition of guidelines from opinion based to evidence based, and the fourth and most recent GINA guidelines represents the paradigm shift in the way asthma is classified.

Initial Assessment and Monitoring

Similar to the EPR 3, the 2007 GINA guidelines center the long-term management of asthma on four components of effective care (GINA 2011). However, in the GINA guidelines, classification of asthma is based solely on the level of control (Bateman et al. 2008). Previous guidelines emphasized severity as a major indicator of asthma disease, but the misperception that asthma severity correlated with

control, and results of the GOAL study (Bateman et al. 2004) demonstrating that control could be achieved at all levels of asthma severity led to a paradigm shift for asthma care at the international level. Classification uses several composite measures in both domains of risk and impairment, including history of symptoms, exacerbations, and pulmonary function testing, to categorize asthma status as being controlled, partially controlled, or uncontrolled. To be considered well controlled, all of the following criteria must be met: no daytime symptoms, no limitation of normal activities, no nocturnal symptoms, no need for rescue treatment, and normal pulmonary function tests. If any one of these criteria is abnormal, the patient is classified as partially controlled and if three or more criteria are abnormal, the patient is classified as uncontrolled. The patient's current level of asthma control and current treatment determine the selection of pharmacologic treatment.

Pharmacologic Management

Treatment options are organized into five steps reflecting the increasing intensity of treatment required to achieve control, with a step up in therapy for patients who are not controlled and consideration to step-down therapy for those who have been well controlled for at least 3 months (Table 6.4). To maintain adaptability in different socioeconomic regions, treatment recommendations are general with a preferred option and other alternatives identified in each step. Treatment options are divided into two age groups: those ≤5 years of age and >5 years of age to adults.

Step 1 treatment with intermittent use of SABA is reserved for patients with intermittent symptoms. For frequent symptoms or periodic impairment, step 2 or higher level of treatment is recommended. Treatment steps 2–5 combine as-needed SABA with regular controller treatments. At step 2, a low-dose ICS is recommended for all ages, with alternative controller medications including LTRA for patients who are unable or unwilling to use ICS for any reason. Other non-ICS options are not recommended for routine or initial step 2 care due to their comparatively low efficacy. At step 3, the recommended option for adolescents and adults is to combine low-dose ICS with an LABA, whereas increasing the dose of ICS or combining low-dose ICS with leukotriene modifiers are the alternatives. However, for children 5 years of age or younger, increasing the dose of ICS is presented as an equally preferred alternative, as there is no clear evidence in this age group for the use of low-dose ICS with leukotriene modifiers. At step 4 of treatment, two or more controller medications along with a rescue medication are recommended, and for step 5, the addition of oral glucocorticosteroids or anti-IgE therapy in selected patients is added to the therapy.

6.4.3 Comparison of Guidelines

In all of these guidelines, the overriding goal is to establish a consensus of scientific practices for the management of asthma centered on common themes: to assess

asthma symptoms and control, the importance of both nonpharmacologic and pharmacologic treatments to maintain control and manage exacerbations, and to develop a partnership between patients and healthcare providers through patient education and use of self-management plans. Similarly, the evolution of guidelines from opinion based to evidence based and the shift from the classification of asthma by disease severity to symptom control are mirrored by the NHLBI, BT/SIGN, and GINA. As national guidelines, both the NHLBI and the BTS/SIGN guidelines provide specific recommendations for diagnostic modalities, and medications with dosage recommendations commiserate with the availability of resources in those countries. In contrast, the GINA guidelines were established to create a more internationally focused set of guidelines taking into consideration the disparities in the socioeconomic status and access to healthcare resources that exist across the world. As such, the GINA guidelines provide more general treatment strategies for management and diagnosis, sections on acceptable alternatives utilizing affordable medications, and added recommendations for more comprehensive asthma education on a global level. The 2008 GINA treatment recommendations are given in Table 6.5.

The major difference between the guidelines is in the initial assessment of patients with asthma. Whereas in the NHLBI guidelines, an initial assessment of severity to initiate treatment is used, the GINA guidelines solely use markers defining the level of control to both initiate and manage asthma treatment. In contrast, the evaluation of asthma in the BTS/SIGN guidelines focuses on making an accurate diagnosis of asthma which likely reflects the primary care-based medical system in the UK. In the long-term management of asthma, all three guidelines outline a stepwise approach to asthma management utilizing the assessment of control to determine the appropriate level of pharmacologic management. As the GINA guidelines arose from a collaboration with the NHLBI, it is not surprising that the many of the concepts regarding the use of the impairment and risk domains in the assessment of control and how control is defined are similar, as well the number of defined treatment steps. In contrast, whereas the BTS/SIGN guidelines also base management on control, the division into specific domains is not explicitly defined. Additionally, definitions of control are more stringently defined by BTS/SIGN, with no tolerance for exacerbations or breakthrough symptoms, and five steps in asthma management of asthma instead of six. Overall, these differences likely reflect the variations in how healthcare is managed in these nations rather than divergent ideologies or interpretations of the literature.

6.5 The Current Status of Asthma Care and Future Directions

Despite the increasing prevalence in asthma, the last decade has seen reductions in death rates and hospitalizations due to asthma (Spahn and Szefler 1996; Szefler 2011a, b). Improved asthma management and new medications have reduced the number of patients receiving systemic steroids, and the number of patients with

Table 6.5 2008 GINA treatment recommendations

	Treatment steps				
	Step 1	Step 2	Step 3	Step 4	Step 5
	Asthma education				
	Environmental control and control of co-morbidities				
	As-needed SABA				
Controller options	SABA	**Select one**	**Elect one**	**Add one or more**	**Add one or both**
		Low-dose ICS	Low-dose ICS + LABA	Medium- **OR** High-dose ICS + LABA	Oral steroids
		Leukotriene modifier	Medium- **OR** High-dose ICS	Leukotriene modifier	Anti-IgE treatment
			Low-dose ICS + Leukotriene modifier	Sustained-release theophylline	
			Low-dose ICS + Sustained-release theophylline		

adverse effects due to these drugs. However, racial and gender disparities, and the continued variable response to treatment amongst patients has spurred the growing concept of personalized medicine, which could significantly advance current asthma management. Identification of biomarkers and epigenetic markers could prompt a more effective treatment strategy to prevent exacerbations, halt disease progression, and define asthma phenotypes and specific phenotype related interventions. To date, several biomarkers such as exhaled nitric oxide levels and sputum eosinophil levels have been studied as prototypic markers for disease activity and targets for therapeutic intervention, and exploration of genetic markers continues in relation to clinical application for asthma management. Better understanding of asthma physiology at the individual level and new discoveries on ways to better manage asthma could lead to another revision in asthma guidelines both in the USA and globally.

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Chapter 7
Community-Based Interventions in Asthma

Sharon Petronella Croisant and Lauren Scott

Abstract Community and public health interventions provide potentially powerful means of decreasing morbidity, hospitalizations, emergency room visits, and mortality from asthma. This chapter thus provides an overview of community-based interventions, which have been demonstrated to be effective—and/or ineffective—in reducing the burden of disease, including development of asthma coalitions, interventions for both provider and patient education, environmental controls to reduce exposure to asthma triggers, and institutional policy and systems change. Perhaps most important is the demonstrated effect of integrated, comprehensive approaches to asthma management and control. A multidisciplinary approach spanning T1 through T4 translational research, coupled with public health activities is promising and has already demonstrated success in reducing the burden of disease.

Keywords Community asthma interventions • Asthma coalitions • Provider and patient education • Environmental control of asthma triggers • Integrated approaches to asthma control

7.1 T1 to T4 Research: From Bench to Curbside

An integrated approach to asthma management and control is increasingly important, given the increase in prevalence of asthma nationally and globally over the past decades. Basic science provides us with a better understanding of mechanisms, which in turn is leading to development of new and innovative pharmaceutical

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interventions to enable better control and management of the disease. Research reveals, however, that community and public health interventions also provide potentially powerful means of decreasing morbidity, hospitalizations, emergency room visits, and mortality. It is important to note that many large organizations are primarily focused on achieving improved outcomes for asthma. Among these are the American Lung Association; the American Thoracic Society; the American College of Allergy, Asthma, and Immunology; the Asthma and Allergy Foundation of America, as well as charitable organizations including the Robert Wood Johnson and Kaiser Family foundations. However, these groups are likely to approach interventions from different perspectives, with disease-based organizations more likely to address provider and patient education and community-based coalitions more likely to focus on environmental, public health, and policy interventions (Keirns 2009). This chapter thus provides an overview of both types of community-based interventions, which have been demonstrated to be effective—and/or limited in effectiveness—in documenting reduced burden of disease, including the development of multidisciplinary coalitions, interventions for both provider and patient education, environmental controls to reduce exposure to asthma triggers, and institutional policy and systems change. Perhaps most important is understanding and appreciating the collective impact of integrated, comprehensive approaches to asthma management and control.

7.2 Public Health Interventions for Asthma

7.2.1 Role of Community Coalitions

One of the earlier and arguably among the strongest community-based initiatives in combating asthma was a national program funded by the Robert Wood Johnson Foundation, Allies Against Asthma Initiative (Allies). The Allies program, directed by the University of Michigan School of Public Health from 2000 to 2006, supported the development of seven community-based coalitions in an effort to improve health care and coordination of efforts while also developing programs and resources that were site specific but could be adopted or adapted as best practices by other communities. An important contribution was the use of standardized tools to evaluate the coalitions as they evolved and their functionality (Clark et al. 2006).

The overarching goals of the coalitions were to improve access to health care as well as the quality of care received, consistent with The National Asthma Education and Prevention Program (NAEPP) guidelines, as well as to provide patient, family and provider education and support. They also sought to address environmental issues associated with asthma and to support development of evidence-based policies and systems change. These combined efforts were designed to reduce hospitalizations and emergent care visits for treatment of asthma and consequent missed school days as well as to improve quality of life in children with asthma and to

develop strategies for managing asthma through sustainable community initiatives. The seven coalitions funded included both established and new initiatives including both professional and grassroots partners:

- Alianza Contra el Asthma Pediátrica en Puerto Rico (ALIANZA)
- Consortium for Infant & Child Health (CINCH)
- Fight Asthma Milwaukee Allies (FAM Allies)
- The King County Asthma Forum (KCAF)
- Long Beach Alliance for Children with Asthma (LBACA)
- National Capital Asthma Coalition (NCAC)
- Philadelphia Allies Against Asthma (PAAA)

The Controlling Asthma in American Cities Project (CAACP), funded by the National Asthma Program of the National Center for Environmental Health within the Centers for Disease Control and Prevention, was initiated in 2001. A complex and ambitious undertaking, also involving coalitions, the CAAAP was largely based upon the experience of the Allies program. The CAAP initiatives required integrating and coordinating multiple interventions at multiple levels. Based upon program theory, the CAAAP sought to establish community asthma coalitions that would include both professionals and community members, nurtured by external funding and access to expertise and resources including paid staff and technical assistance. Following a period of capacity-building, coalitions were expected to complete community needs assessments from multiple sources with a goal of developing community-specific asthma action plans involving coordinated efforts among community, change leaders, policy makers, and healthcare payers. Outcomes of interventions were to be rigorously monitored and evaluated for both process and efficacy, with plan revisions made accordingly. Each coalition was to target children with asthma and their families as well as healthcare providers, community-based organizations, healthcare systems and payers, and policy makers in order to effect environmental and psychosocial improvements (Herman 2011).

Due to the broad-based and ambitious nature of the program, there were both successes and failures in achieving goals. Multiple sites reported difficulties in reaching children with uncontrolled asthma as well as engaging healthcare providers in interventions to improve the quality of care for children with asthma. Nonetheless, they also reported improvements in asthma outcomes related to innovative interventions. Davis et al. (2011) documented improvements in asthma medication prescription and use in targeted Chicago neighborhoods by analyzing a large pharmacy database. Findley et al. (2011) reported dramatic improvements in asthma management for preschool children through a multifaceted educational program targeting early childhood centers, parents, and children as well as healthcare providers. The Oakland, CA coalition also reported significant achievements in asthma control in adolescents in a 4-week educational curriculum designed to improve asthma self-management (Davis et al. 2008). While many of the coalitions used or adapted existing educational programs developed by NIH or professional organizations such as the American Lung Association, several created innovative new programs that are now available for use. Among these are the *Asthma Basics for*

Children handbook for parents and early childhood teachers (available from the Asthma and Allergy Foundation of America) and Oakland's *Kickin' Asthma* educational curriculum, which was designated by ALA as a best practice program (Herman et al. 2011)

While these examples are representative of effective community coalitions, it should be noted that many other community-based, multisite efforts have been implemented. Among these are seven Centers for Children's Environmental Health and Disease Prevention Research, jointly funded by the NIEHS and EPA, which have carried out diverse studies of children with asthma and environmental exposures and generated a large body of scientific literature that have, in turn, informed the development of multiple intervention efforts which have commonalities across sites, while taking into consideration local and site-specific circumstances (Eggleston et al. 2005).

A different type of networked initiative emerged from the Merck Childhood Asthma Network (MCAN), which was funded by the Merck Company Foundation and developed to provide evidence-based interventions in three, widely ranging settings, including schools, clinics, and communities in five program sites. This ambitious program employed multidisciplinary teams utilizing a translational approach to the interventions. While MCAN shared many similarities with other such coordinated efforts, an important distinction was its emphasis on rigorous evaluation not only of outcomes but also of the processes and infrastructure required for and barriers imposed by implementing clinical or research intervention protocols in the real world setting. Perhaps more importantly, lessons learned are being used to guide funding initiatives for future intervention research (Viswanathan et al. 2011; Ohadike et al. 2011).

7.2.2 Patient and Provider Education

Numerous patient and provider education curricula have been developed that are consistent with NAEPP guidelines. Among these is the Physician Asthma Care Education (PACE) program, which is an interactive educational seminar that employs a multimedia approach. PACE has been highly effective in increasing physicians' efficacy in dealing with patients with asthma and in reducing morbidity from the disease in terms of symptom-free days and reduced emergent visits for treatment (PACE 2012). The National Asthma Control Initiative, initiated by the NHLBI in conjunction with CDC, offers numerous patient and provider education resources, as do the AAFA and the ALA, including *Open Airways for Schools* (ALA 2012), as well as *You Can Control Asthma*, and *Wee Wheezers* (AAFA 2012).

Other innovative provider education programs have demonstrated success. Morrow et al. developed an innovative, educational program that combines clinical vignettes with interactive discussions, allowing providers to synthesize the educational components with their own experience and knowledge, i.e., utilizing a problem-based learning approach, yielding significant changes in healthcare

providers' decisions about care of patients with asthma (Morrow et al. 2007). Kattan et al. (2006) demonstrated the efficacy of a program geared toward education of both providers and parents of childhood asthma patients to prompt appropriate prescription and use of asthma medications, reduced asthma symptoms, and healthcare utilization. This novel program involved bimonthly telephone calls to caretakers of enrolled children to collect information about the child's symptoms and asthma control. Following the call, a computer-generated letter was sent to providers summarizing the child's symptoms as well as healthcare usage based upon the discussion with the caretaker, with recommendations for provider action to improve patient outcomes. A second telephone-based intervention involved coaching of parents of children with asthma to provide education and support, resulting in significantly improved quality of life for enrolled parents (Garbutt et al. 2010). Another "hybrid" intervention focused on both provider and patient education also achieved remarkable results. Lob et al. (2011) illustrated significant improvement in patient outcomes via a program, which provided healthcare practitioners with training on the NAEPP diagnostic and treatment guidelines while concomitantly increasing clinic-based patient education through a team-based effort involving the practitioner as the "clinician asthma champion," an asthma coordinator, and non-clinical staff. This continuous quality improvement intervention not only resulted in improved patient outcomes but also documented improved care processes in the clinics and quality of life for study participants.

Multifaceted, clinic-based programs geared toward increasing patient self-efficacy through counseling, ongoing education, and development of personalized asthma action plans have also been effective in adults with moderate-to-severe asthma (Cote et al. 2000) as well as in high-risk, primarily African American patients of low socioeconomic status with poorly controlled disease (Martin et al. 2009). While a pilot effort, there is some indication that efforts to improve parental health literacy and provide education in the emergency department setting are also effective, even among parents with low health literacy (Macy et al. 2011). Other successful initiatives include school-based education (Mansour et al. 2008).

7.3 Asthma, the Environment, and Respiratory Health

The disparate foci of communities and researchers often lend itself to a Community-Based Participatory Research (CBPR) approach. Environmental interventions at community-levels have become increasingly common, and to varying degrees, highly successful. Community Action Against Asthma (CAAA) utilized a CBPR approach in a series of community-based asthma environmental interventions. Originally established in 1998, the initial project had two primary objectives, to carry out home-based intervention studies to reduce asthma triggers, and to conduct an epidemiologic assessment of exposure to ambient air and concomitant health effects. The group utilized Community Health Workers to carry out the household interventions, achieving increases in some measures of lung function and reducing

morbidity for children with asthma in the form of reduced emergent care visits, reduction of asthma triggers in the home, and increasing appropriate use of asthma medications. An important part of these community-based interventions, however, was building and evaluating community capacity to effect change (Edgren et al. 2005; Parker et al. 2003, 2008, 2010).

A predecessor to the work of CAAA was the "Healthy Homes" approach to identify and control environmental indoor asthma triggers that was developed in the late 1990s and approved for use by the NIEHS, the Department of Housing and Urban Development, and the EPA, which heavily funded such efforts. Krieger et al. utilized such an approach to conduct a randomized control trial using community health workers to provide a series of in-home environmental assessments over a 1-year period, during which they also provided support and resources for asthma education (Krieger et al. 2005). As with the CAAA initiative, investigators documented reduced asthma morbidity and symptom days as well as reductions in emergent visits for asthma treatments. Also, like the CAAA program, they revealed self-reported improvements in caregiver quality of life. Similar results were observed for a study which focused on environmental improvements through better insulating homes and improving heat sources. This study was significant in that the authors cited such interventions as more effective than individual behavioral intervention alone (Howden-Chapman et al. 2011).

Our own work has focused on both indoor and outdoor air quality, the study of which has been prompted by community inquiries regarding respiratory health and safety. In 2002, in response to concerns of the local Galveston/Houston community over poor air quality, researchers from the University of Texas Medical Branch (UTMB) initiated the Gulf Coast Study of Urban Air Pollution and Respiratory Function (GC-SURF) as a pilot study with the Galveston County Beach Patrol, hypothesizing that exposure to pollutants would adversely affect the lung function of healthy, athletic young adults working as beach lifeguards (Petronella et al. 2004; Thaller et al. 2008). Deleterious effects of particulate matter and ozone appeared to be transient, but occurring at pollutant levels far below national standards, suggesting that even low levels of exposure may have small but significant effects on healthy individuals. This study was an outgrowth of previous studies in which environmental research initiatives were developed in response to community concerns that further developed into educational and policy interventions to benefit community health, as well as engendering additional, more mechanistic research (Thaller et al. 2005).

Significantly for the Galveston community, and for the three million annual visitors to its beaches, these efforts have evolved into a model that uses the results of these scientific studies to inform public health education and policies. Since 2006, Galveston beaches have included in the public notification flag system an orange "environmental alert" flag, deployed on the city's 26 lifeguard towers and seven freestanding system stations, as well as at strategic locations along the seven-mile Galveston seawall and the entrances to the community "pocket" parks. At each of these locations, information is posted detailing the specific environmental condition of concern, along with guidelines for practical steps to ensure health. In this study,

the community served as partners in the research and used findings to implement intervention/education measures to protect public health.

A second community-based project was developed based upon the GC-SURF study, this time focusing on development of a notification system for parents of children with asthma to apprise them when air quality at local levels had been determined to be unhealthy. The overall goals of the Breathe Easy: Air Quality Index Notification System (BEAQINS) project are to reduce children's health risks from exposures to ozone and other outdoor air pollutants and to evaluate the use of an educational and early warning system in impacting participants' knowledge and subsequent behavior to effect risk reductions. By implementing a daily indicator of air quality at area schools along with appropriate educational programming, we hope to reduce student exposures to unhealthy air, to inform regional school districts and the Texas Interscholastic League of the need to limit prolonged outdoor exertion and the benefits of providing alternative indoor activities, and to increase public awareness of the Air Quality Index (AQI) and its prescribed behaviors appropriate to vulnerable populations. The flags, colored green, yellow, orange and red, correspond to the colors of the AQI and advise parents, students, and school staff of the air quality forecast for the day. The flag's color thus acts as a parent and school advisory and could help to determine choices for outdoor activities. In the event that air quality decreases during the day, the Texas Commission on Environmental Quality's automatic email warning system will advise schools, allowing them to post a warning flag during the day.

7.4 Systems Changes

While admittedly outcomes of the Allies Against Asthma programs varied across sites, the community-based coalition approach to asthma management was largely documented to be successful, especially related to changes in policy (89 documented across site) and systems changes in five key areas: clinical practice, coordination and/or standardization of care, improvement of environmental conditions, family management of asthma, and other improvements that ranged from inter and intrainstitutional changes to statewide legislation related to exposure to environmental tobacco smoke in public places, improvement of air quality in schools, and assurance of children's rights to carry and administer asthma medications in schools (Clark et al. 2010). Coalitions were encouraged to develop community-specific, innovative programs. One such effort in Philadelphia, "the Child Asthma Link Line" demonstrated that children enrolled in a telephone-based care coordination and system integration program were less likely to require hospitalization or ED visits for their asthma and more likely to see their outpatient provider for regular management of asthma symptoms (Coughey et al. 2010). Allies coalitions also documented improvement in children's asthma symptoms as well as parents' emotional control in management of their children's disease. Significantly, they found that the coalitions which affected the greatest number of policy and system changes had

more core and ongoing partners as compared to those with a greater number of peripheral or transient partners (Clark et al. 2010).

A comprehensive review of asthma policies developed to improve asthma outcomes at the community level was carried out by Lyon-Callo et al. (2007) to assess degree of policy implementation and effectiveness. The authors found many examples of successful policies, including, albeit not limited to, reductions in exposures to triggers in the workplace and schools, improvement in both indoor and outdoor air quality, and increasing funding for asthma coalitions to launch comprehensive approaches to asthma education, treatment, and management. Nonetheless, they cite numerous deficiencies including a sparcity of peer-reviewed interventions, few policies focused on educating school staff regarding childhood asthma, and large disparities across local, state, regional, and national initiatives. They suggest that to effectively assess systems changes and efficacy of asthma policies, it is imperative to establish comprehensive monitoring databases that include information on socioeconomic status, race, and ethnicity. However, to do so, federal dollars and guidance will be necessary to ensure uniform implementation and assessment (Lyon-Callo et al. 2007).

7.5 Challenges in Real World Assessment of Program Impacts

Prior to definitively stating that community-based asthma interventions are uniformly successful, it is essential to consider and comprehend the difficulties in establishing and documenting change in a real world setting. Spiegel et al. (2006), for example, describe moderate successes achieved by the Inner City Asthma Intervention, yet revealing that actual implementation was affected by variations across sites and a lack of a clear evaluation design and data collection strategy that was capable of rigorous statistical analysis to document outcomes (2004). An umbrella review of public health interventions for asthma (Labre et al. 2012) found a decided lack of conclusive evidence of efficacy in most, although not all, asthma programs. However, as the authors point out, the ability to determine effectiveness of programs is complicated by the lack of consistent study designs, the interventions carried out, the inclusion criteria for subjects, the outcomes measured, and the means by which outcomes were measured. Of thousands of peer-reviewed studies published, they could only manage to find 42 which lent themselves to comparison. Similar results were obtained by Crocker et al. (2011) as they attempted to assess the effectiveness of multifaceted environmental interventions in the home with a focus on reducing asthma morbidity. While the literature search found over 10,000 citations, only 23 studies met criteria for conclusion in the analysis. Both sets of authors suggest, and rightly so, that if we are to clearly document our successes and failures (and thus establish best practices) we must begin by standardizing definitions and endpoints and integrating practices across programs conducted by federal, academic, and nongovernmental agencies to ensure the possibility of determining best practices in public health asthma intervention.

7.6 Summary

Comprehensive, community-based efforts have been demonstrated to be effective in improving outcomes for patients with asthma and their families. As observed in the preceding pages, coordinated initiatives encompassing environmental controls to minimize exposure to allergens and irritants, appropriate pharmacologic therapy, patient and healthcare provider education, and a systems change approach can collectively reduce morbidity, mortality, and financial costs associated with asthma. That said, rigorous attempts must be made to integrate programs, adopt best practices, and carefully track outcomes if we are to realize their full potential.

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Chapter 8
Heterogeneity of Response to Therapy

William J. Calhoun

Abstract As noted in prior chapters, US national and international guidelines provide a consistent approach to initial management of asthma, based on easily observed physical and physiologic findings, and a detailed history. From those data, a rational initial therapeutic regimen can be prescribed. In many cases, such therapy results in near complete control of asthma symptoms, restoration of normal lung physiology, and elimination of exacerbations. In fact, such improvement is frequent enough that therapeutic responsiveness to asthma treatments was thought to be nearly universal. However, it is not uncommon for patients to return with incomplete, or even trivial improvement in these clinical metrics of control (Langmack and Martin Curr Opin Pulm Med 16:13–18, 2010). In this chapter, we review the current literature on the variability of response to commonly used therapeutic agents in asthma.

Keywords Asthma • Therapeutic response • Molecular phenotype • Predictive phenotype • Heterogeneity • Inhaled steroid • Montelukast • Periostin • IL-13

8.1 Introduction

As noted in prior chapters, US national and international guidelines provide a consistent approach to initial management of asthma, based on easily observed physical and physiologic findings, and a detailed history. From those data, a rational initial therapeutic regimen can be prescribed. In many cases, such therapy results in near

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complete control of asthma symptoms, restoration of normal lung physiology, and elimination of exacerbations. In fact, such improvement is frequent enough that therapeutic responsiveness to asthma treatments was thought to be nearly universal. However, it is not uncommon for patients to return with incomplete or even trivial improvement in these clinical metrics of control (Langmack and Martin 2010). In this chapter, we review the current literature on the variability of response to commonly used therapeutic agents in asthma.

8.2 Variation in Response to Corticosteroids

An early evaluation of variability in response to inhaled corticosteroids (ICS) was published by Malmstrom et al. (1999). This work was one of the first publications to evaluate the distribution of individual responses to therapy (ICS and montelukast) in patients with mild asthma. From this analysis, it was clear that well characterized, and clinically homogeneous populations of asthma showed remarkable heterogeneity of responses (improvement in FEV1), with as much as 40 % of the population failing to demonstrate a clinically significant improvement in lung function.

The US Asthma Clinical Research Network (ACRN) has added much information to this field. An early study by Szefler and colleagues demonstrated using a rigorous design, and well-characterized subjects, that a significant fraction of patients with asthma had no improvement in FEV1 or bronchial hyperresponsiveness following 6 weeks of treatment with several ICS preparations including a 2 mg/day dose of fluticasone propionate (Szefler et al. 2002). These data formalized the understanding of marked therapeutic heterogeneity with ICS in asthma. A subsequent ACRN study demonstrated that reduced FEV1, FEV1:FVC, and a large bronchodilator response to albuterol administration were the best predictors of response to ICS therapy, but the variability in such predictions remains great (Martin et al. 2007).

Tantisira and colleagues identified a genetic variant that was associated with corticosteroid response (Tantisira et al. 2004a). Variation of the corticotropin hormone releasing hormone receptor 1 (CRHR1, now called CRF1) was found to be associated with enhanced improvement in FEV1, in three distinct asthma populations. Moreover, the genetic variation showed "dose response," in that subjects who were homozygous for the polymorphism had even greater benefit from inhaled corticosteroid therapy. The same group, in another publication, demonstrated that variation in the TBX21, the gene encoding the nuclear transcription factor T-bet, was an important determinant of response to inhaled steroid therapy in children (Tantisira et al. 2004b).

Observable characteristics may be associated with steroid responsiveness. Cigarette smoking blunts the therapeutic response to ICS by a variety of mechanisms (Lazarus et al. 2007) but does not affect the bronchodilator response to montelukast. These findings have been amplified by a recent meta-analysis (Zheng et al. 2012). Sputum neutrophilia has been associated in several studies with blunted responsiveness to ICS for a variety of asthma outcomes (Green et al. 2002). Less abnormality of FEV1 and FEV1:FVC ratio may also be associated with less

favorable ICS responses. A variety of other clinical metrics may have limited value (Montuschi and Barnes 2011; Hoffman et al. 2008). However, none of these characteristics, aside from smoking, appears to have sufficiently good predictive performance to be translated into clinical practice. The biochemical mechanisms by which steroid resistance occurs in asthma have been reviewed recently (Matsumura 2010).

8.3 Variation in Response to Leukotriene Pathway Inhibitors

The promoter for the ALOX gene exhibits variation in the number of repeat sequences. Common variants are four or five repeats in the SP1 binding motif. This ALOX polymorphism has been associated in two studies with variation in the response to antileukotriene therapy. As outlined elsewhere in this book, there are other important polymorphisms of the leukotriene pathway that are associated with asthma susceptibility and aspirin exacerbated respiratory disease (Tantisira and Drazen 2009).

This promoter polymorphism affects the level of gene expression, and accordingly the level of 5-lipoxygenase (ALOX5) in cells and patients (Silverman and Drazen 2000). In patients with the polymorphism, reduced therapeutic benefit from a 5-lipoxygenase inhibitor was observed (Drazen et al. 1999), suggesting that a measurable parameter (ALOX5 promoter structure) could be useful as a biomarker of response to therapy. To date, this reasonable postulate has not been brought widely into clinical practice.

In another study of the same promoter variation, Telleria and colleagues demonstrated that response to montelukast, a competitive antagonist of the leukotriene D4 receptor, was increased in patients with at least one copy of the promoter with five repeats. Those patients homozygous for four repeats had a blunted clinical response to montelukast (Telleria et al. 2008). These observations demonstrate that (1) heterogeneity of response to therapy is present and (2) an identifiable genetic variation explains at least part of the variation in treatment response. The interested reader is referred to an older, but still current review (Lima 2007).

In summary, although important variation exists in therapeutic response to antileukotrienes in asthma, and an emerging, pathway based understanding of the mechanisms underlying the variation has emerged, there is not presently consensus on how best to use these insights to improve the care of patients with asthma.

8.4 Variation in Response to Beta-Receptor Agonists

Considerable controversy exists regarding the causes of variability in responsiveness to inhaled beta-receptor agonist therapy. A well-designed, randomized, ipratropium-controlled, genotype-stratified study from the ACRN (BARGE) suggested that continuous use of short-acting albuterol, in the context of homozygosity for a specific polymorphism of the beta2 receptor (Arg for Gly), was associated with

impaired bronchodilator responses and reduction in baseline lung function (Israel et al. 2004). The BARGE study was based on several earlier observations from the ACRN and elsewhere that implicated frequent use of albuterol with deterioration of lung function in patients with the Arg/Arg genotype. One clear implication of the BARGE study was that continuous stimulation of the beta2 receptor with long-acting beta2 agonists, such as salmeterol and formoterol, might also be associated with deteriorating lung function over time. This concern led the ACRN to conduct the LARGE trial, in which a small potentially deleterious effect of salmeterol was demonstrated (Wechsler et al. 2009). However, at least two larger studies (Bleecker et al. 2007, 2010) have been conducted which failed to demonstrate any genotype-specific adverse effects of long-acting beta agonists. Accordingly, the factors that associate with variability in bronchodilator response to beta2 agonists have not been brought widely in to clinical practice.

8.5 Variation in Response to Biologic Agents

Few biologic agents are currently employed for asthma management; of these, omalizumab has had the greatest penetrance. To date, there have been no definitive studies that characterize the nature, extent, and quantity of variation of asthma control metrics in response to omalizumab. Similarly, the experience with inhibitors of the TNF-alpha pathway is insufficient to discern predictors of the variation in responses.

In contrast, one new agent in Phase III studies does appear to have a predictive biomarker. Lebrikizumab, a monoclonal antibody against IL-13, shows significant efficacy in patients with asthma inadequately controlled on ICS alone (Corren et al. 2011). However, in those patients whose serum periostin was high, the improvement in FEV1 after therapy was significantly greater than those patients with a low serum periostin. Periostin is downstream of IL-13, suggesting that periostin levels may be a biomarker for high IL-13, and consequently a biomarker for response to lebrikizumab.

Very recently, a trial of dupilumab for management of moderate-to-severe asthma was reported (Sally Wenzel et al. 2013). Dupilumab is a monoclonal antibody directed against the IL-4 receptor alpha chain and inhibits signaling through that receptor from ligation of either IL-4 or IL-13. In this trial, about 100 subjects with moderate-to-severe asthma and elevated blood eosinophils (>300/μl) were enrolled. In these patients, dupilumab therapy was associated with significant improvement in most important asthma outcomes. Whether simple blood eosinophil counts are robust predictive biomarkers for response to dupilumab was not tested in this trial, but the robust improvement in this selected subset of difficult-to-control asthma suggests some utility in that regard.

As biological molecules undergo development, it is likely that predictive biomarker development will proceed in parallel. The costs of biologic therapy dictate that their use be limited to those patients whose benefit can reliably be predicted.

8.6 Summary

Considerable variability in the response to asthma therapy exists for all of the current classes of asthma therapy (Langmack and Martin 2010). The biologic mechanisms that underlie such variation are well known for some agents and are as yet obscure for others. At present, no reliable predictive proteomic or genomic biomarker has been incorporated into clinical practice for any class of asthma therapy. However, the promise of such predictive biomarkers, particularly for expensive biological molecules used in asthma therapy, remains bright.

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Part II
Genetics and Genomics in Asthma

Chapter 9
Introduction to Genetics and Genomics in Asthma: Genetics of Asthma

Rasika Ann Mathias

Abstract While asthma is a heterogeneous disease, a strong genetic basis has been firmly established. Rather than being a single disease entity, asthma consists of related, overlapping syndromes [Barnes (Proc Am Thor Soc 8:143–148, 2011)] including three general domains: variable airway obstruction, airway hyper-responsiveness, and airway inflammation with a considerable proportion, but not all, of asthma being IgE-mediated further adding to its heterogeneity. This chapter reviews the approaches to the elucidation of genetics of asthma from the early evidence of familial clustering to the current state of knowledge with genome-wide approaches. The conclusion is that research efforts have led to a tremendous repository of genetic determinants of asthma, most of which fall into the above phenotypic domains of the syndrome. We now look to future integrative approaches of genetics, genomics (Chap. 10), and epigenetics (Chap. 11) to better understand the *causal* mechanism through which, these genetic loci act in manifesting asthma.

Keywords Genetics • Linkage analysis • Positional cloning • Genome-wide association study • Linkage disequilibrium • Population stratification • Heritability • Complex traits • Asthma

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9.1 Evidence for a Genetic Basis to Asthma

9.1.1 Familial Aggregation of Asthma

Heritability is the proportion of variation in a quantitative trait or risk of disease for a dichotomous trait that can be attributed to genetic variation. Familial aggregation or clustering of asthma was recognized early in the twentieth century (Wiener, et al. 1938; Sakula 1988). The earliest familial studies performed by Cooke (Cooke and Vander Veer 1916; Spain and Cooke 1924) in 1916 and 1924 established an increased occurrence of asthma in relatives of subjects having the disease as compared to relatives of normal controls. Numerous family studies performed in the 1920s and 1930s (Wiener, et al. 1938; Schwartz 1952) and more recently (Gerrard et al. 1976; Dold, et al. 1992; Aberg 1993) found similar results of familial aggregation of the disease. Twin studies which have a greater advantage over the above-mentioned family-based studies: easier detection of nonadditive genetic effects (dominant or epistatic effects) and matching for environmental effects have consistently shown higher concordance between monozygotic twins in contrast to dizygotic twins. A tremendous range in the heritability of asthma is seen from these studies range from 36 to 95 % (Edfors-Lubs 1971; Duffy et al. 1990; Laitinen, et al. 1998; Skadhauge et al. 1999; Koeppen-Schomerus, et al. 2001; Hallstrand et al. 2005; Nystad et al. 2005; van Beijsterveldt and Boomsma 2007; Fagnani, et al. 2008; Willemsen et al. 2008; Thomsen, et al. 2010), with higher estimates generally observed in studies implementing more objective diagnostic criteria.

This wide spectrum of heritability estimates for asthma is not unexpected as heritability is a feature of the sample at hand particularly with respect to the relative contribution of genetic and environmental variability representing the complex interplay of genes and environment. It is useful to summarize these observations in the context of the relative risk to sibs (λ_s), which for monogenic diseases or largely genetic disorders, tends to be high (e.g. $\lambda_s \sim 500$ for Cystic Fibrosis). In contrast the λ_s is only about 2.0 for asthma (Cookson and Palmer 1998) which along with the complex genetic background implicated above makes the search for genetic loci that cumulatively contribute to this risk incredibly difficult. Despite this, there has been tremendous success in identifying genetic determinants of this disease as illustrated below.

9.1.2 Inheritance Models for Asthma

Further illustration of the complex nature of asthma comes from segregation analysis where evidence in support of a wide range of inheritance models has been noted. Segregation analysis finds its roots in Mendel's Law of Segregation: every individual inherits *factors* from his/her parents, and in the formation of gametes, these factors *segregate* into separate gametes, manifested themselves as specific genotypic, and consequently phenotypic distributions in the offspring generation. The evaluation of

complex models of genetic transmission to explain the observed distribution of asthma in a sample of families (Khoury and Beaty 1993) has provided evidence for codominant models with a correlation between age of onset and number of disease alleles (Wiener et al. 1938), dominant models (Schwartz 1952), and even polygenic and recessive models (Wang et al. 2000; Holberg et al. 1996; Martinez and Holberg 1995). From these studies, it is apparent that asthma should be considered as a paradigmatic complex genetic disease, manifesting through the interaction of multiple susceptibility genes with environmental.

9.2 Linkage Studies for Asthma

The promise of genetic mapping for disease-gene identification is that it requires no prior assumptions on the candidacy of a gene or locus in the biology of asthma—a so-called hypothesis-free approach. The first such application as a genome-wide approach was linkage analysis, a family-based mapping strategy designed to detect susceptibility loci (i.e., disease-susceptibility genetic regions) with large effect sizes that co-segregate with disease in either large pedigrees or nuclear families (Box 9.1). Families are ascertained by design; typically contain multiple affected and unaffected individuals (e.g., multiplex families with affected and unaffected individuals) identified on the basis of an index case (proband) and require genotype and phenotype information on affected and unaffected individuals. The affected-only ascertainment is an alternative whereby allele sharing between affected relative pairs (e.g., affected sibling pairs) is compared against the expected allele sharing given the relative-pair kinship. By relying solely on genetic co-segregation, linkage enables the discovery of novel genes and pathways without preconceived biases regarding the underlying biology. Parametric models that explicitly specify the mode of inheritance (i.e., dominant vs. recessive vs. co-dominant) have proven particularly effective for mapping variants underlying rare Mendelian diseases like cystic fibrosis. However, for complex diseases like asthma, where the correlation between individual mutations and disease risk (that is, genetic penetrance) is relatively low, less powerful nonparametric approaches are commonly used. These latter methods that compare allele sharing given the phenotype of the relative pair against the expected sharing for the relative-pair kinship are limited in power to detect smaller effect sizes (evidenced by the observation that linkage signals discovered thus far typically fail to meet strict genome-wide linkage thresholds of LOD >3.7 and $p<2\times10^{-5}$ (Lander and Kruglyak 1995). Furthermore, identified regions of linkage are typically wide (often more than 10 million bases) and encompass numerous genes that may cumulatively explain the overall linkage signal. Nonetheless, since the first genome-wide linkage screen for asthma susceptibility loci was published in 1996 (Daniels et al. 1996), >20 independent chromosomal regions have been identified though linkage approaches, many of which are widely replicated (chromsomes 2p, 4q, 5q21-33, 6p24-21, 11q13-21, 12q21-24, 13q12-14, 16q21-23, and 19q, Fig. 9.1) (Wills-Karp and Ewart 2004). Large scale meta-analyses of individual linkage scans (Bouzigon

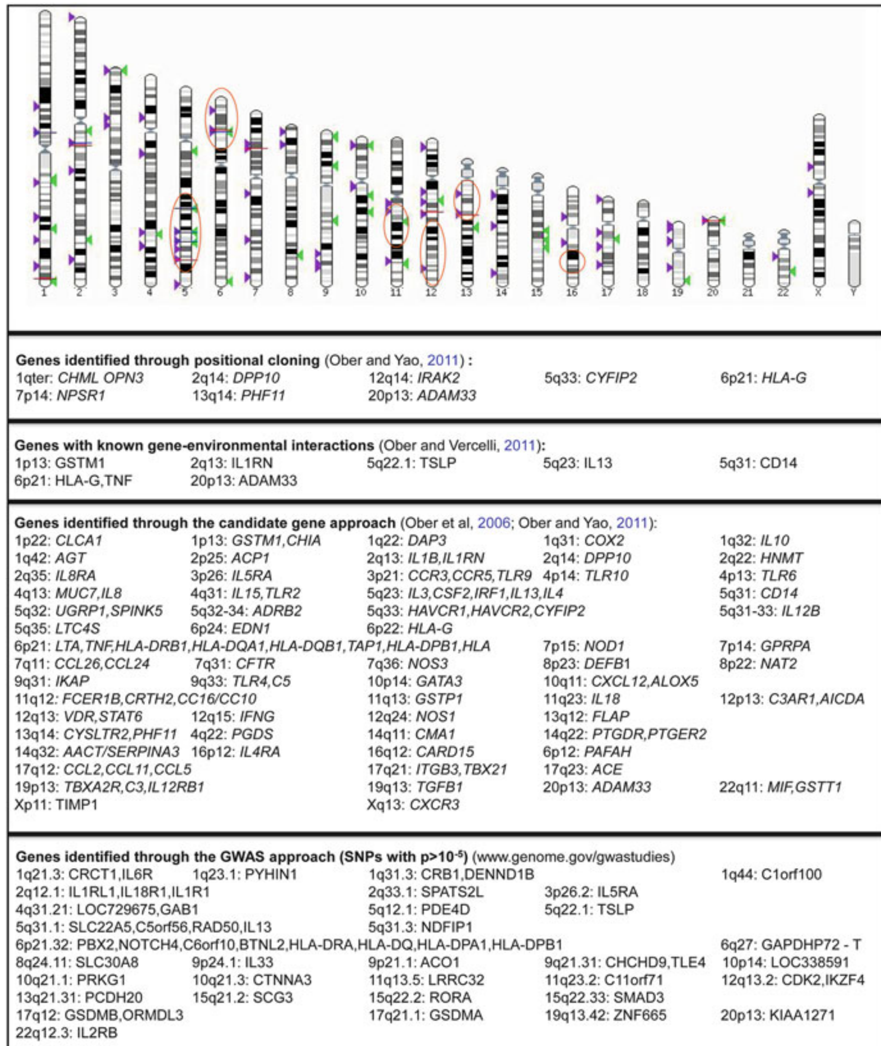


Fig. 9.1 Genetic loci discovered for the phenotype of asthma to date. Genes identified through the candidate gene approach are identified in *purple arrows* to the left of each chromosome. GWAS-identified genes with *p*-values $<10^{-5}$ are identified in *green arrows* to the right of each chromosome genes involved in gene*environment interactions are identified in *horizontal red bars* and genes identified through positional cloning are identified by *horizontal red bars*. Regions of peak linkage evidences are represented by the *orange lasso* on chromosomes 5, 6, 11, 12, 13, and 16

et al. 2010; Denham et al. 2008) have revealed the value of a combined approach leading to the identification of a novel 2p21-p14 locus not noted in any single study. Meta-analyses have also revealed high between-study heterogeneity, which may be reflective of study design and family ascertainment differences, but probably also reflects the intrinsic complexity of the disease. While an analysis of 11 studies of

Caucasian asthma populations (1,267 pedigrees, $n=5{,}832$) did not identify any region showing genome-wide significance with asthma, significant linkage with bronchial hyper-responsiveness (BHR) was observed with 2p12-q22.1, 6p22.3-p21.1 and 11q24.1-qter (Denham et al. 2008). A separate linkage-based meta-analysis of 20 different populations of differing ethnicities (3,024 pedigrees, $n=10{,}027$) found genome-wide evidence for linkage with asthma on 2p21-p14 and 6p21 in the subset of European families.

Once linkage is observed, identification of the causal genes and variants first requires further narrowing of the candidate region through the process of positional cloning. Positional cloning typically consists of association testing of dense panels of single nucleotide polymorphisms (SNPs) across the linked regions to define those variants and their corresponding haplotype blocks that show strong genetic association with disease. *ADAM33* was the first report of a positionally cloned asthma gene (Van Eerdewegh, et al. 2002). A multistep approach of (1) linkage analysis in families yielding evidence for a novel locus on chromosome 20p13; (2) determination of a homologous region on mouse chromosome 2 previously linked to BHR; (3) subsequent case–control association approaches; (4) validation of association in family-based approaches; and (5) demonstration of *ADAM33* expression in lung cell types yielded the strongest evidence for associations to variants within the *ADAM33* gene identifying it as the most likely gene from a set of ~40 within the linkage peak. Additional successes using similar positional cloning approaches for asthma include *DPP10* (Allen et al. 2003) on 2q14, *PHF11* (Zhang et al. 2003) on 13q14, *NPSR1* (Laitinen et al. 2004) on 7p14, *HLA-G* (Nicolae et al. 2005) on 6p21, *CYFIP2* (Noguchi et al. 2005) on 5q33, *IRAK2* (Balaci et al. 2007) on 12q14, and *OPN3/CHML* (White et al. 2008) on 1qter.

While linkage analysis in asthma has suffered from lack of replication between studies, meta-analysis has emphasized select regions that may be robust to study-specific heterogeneity. In general, the identification of a single gene as the source of the highly replicated linkage signals has been limited. However, as summarized by Ober and Hoffjan (2006) and as illustrated in Fig. 9.1, many in the set of *most associated* genes map to regions of *most replicated linkage*. A striking illustration of this is the widely replicated linkage to chromosome 5q31-33 (Ober et al. 1998; Ober and Hoffjan 2006; CGSA 1997; Yokouchi et al. 2000; Yokouchi et al. 2002; Haagerup et al. 2002); at least 14 genes in this region have been shown to be associated with asthma and its related atopy phenotypes including some of the most replicated associations (*IL4*, *IL13*, *CD14*, *ADRB2*, *SPINK5*, *LTC4S*) (Ober and Hoffjan 2006; Ober and Yao 2011). This region also includes a positionally cloned gene (CYFIP2) (Noguchi et al. 2005), genes with documented environment interactions (Baldini et al. 2002; Zambelli-Weiner et al. 2005), and genes that influence drug response (Martinez et al. 1997). Given the complex nature of the genetic architecture of asthma, including the polygenic model established by multiple gene–gene and gene–environment interactions, the cumulative relative risk (λ_s) conferred by each locus is small. Families most probably segregate multiple loci that determine family-specific risks, with strong heterogeneity between families. It is likely that the next frontier of asthma genetics, which includes sequencing of entire genomes and therefore regions of prior linkage, will allow the direct evaluation of this hypothesis.

9.3 Association Studies for Asthma

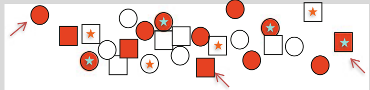
Association testing explicitly tests for the nonrandom correlation between the observed phenotype of asthma and genotyped markers (most often SNPs) in a population and this is typically performed in a case–control design setting wherein allele frequencies at a measured SNP are compared between case and control samples from the population (Box 9.1). It is based on the concept of linkage disequilibrium; the nonrandom association of alleles at two or more loci (Box 9.1). While in linkage two or more loci on a chromosome have reduced recombination between them simply because of their physical proximity to each other, in LD combinations of specific alleles at genetic markers occur more or less frequently in a population than would be expected. If the allele under consideration is at higher frequency in cases in the population, then it is referred to as a *susceptibility* or *risk* allele. One of the major drawbacks of the case–control design is the potential for spurious associations due to population stratification: the presence of a systematic difference in allele and disease frequencies between subpopulations in a population give rise to confounding effects and false associations when cases and controls are not matched on subpopulation membership. Family-based designs such as the case-parent trio design where an affected individual and his/her parents are included rely on the transmission disequilibrium test (TDT) and are free from confounding due to population stratification. In principle the case-parent trio design tests for excess transmission of a specific allele from parents to affected offspring in a comparison of transmitted vs. untransmitted alleles.

9.3.1 The Candidate Gene Approach

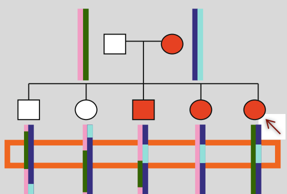
This approach is founded on prior knowledge; genes are selected to be tested as determinants of asthma using principles of association illustrated in Box 9.1 because they are either (1) believed to be biological candidates given their known function; (2) physically located within a region of linkage evidence; or (3) physically located within a region of prior association evidence. The main advantage to this approach is that it is narrow in hypothesis and thereby not limited by the stringent thresholds set in place with significance testing in the more unbiased (by prior knowledge) genome-wide approaches. On the other hand, the approach is limited in that it does not include novel loci that may add to the understanding of biology, each candidate gene study is generally insular only considering a gene in contrast to the pathway from which the biological candidacy is determined and often there is lack of replication between studies because of the lack of consideration of environmental effects further discussed below.

Despite these limitations, the candidate gene approach has had many successes in asthma, and these successes are elegantly summarized by a number of excellent review articles (Ober and Hoffjan 2006; Ober and Yao 2011; Vercelli 2008). Most of the >100 loci found to be harboring genetic determinants of asthma and its associated allergic phenotypes have evidence based in this approach, of which the most

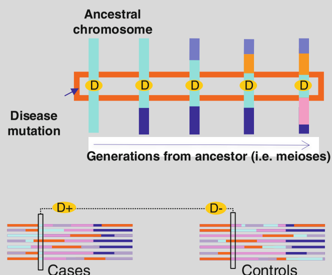
Box 9.1 An overview of the concepts in linkage and association analysis for the discovery of asthma susceptibility loci



Linkage reflects the tendency for chromosomal segments to be inherited intact from parent to offspring. Here, all affected offspring share a chromosome segment (blue) inherited from the mother that is not shared with the unaffected offspring which suggests a susceptibility locus within the shared chromosome segment.



Linkage disequilibrium is the nonrandom association of alleles at two or more loci. Here, association testing would identify a correlation between SNPs in the block of high LD (blue) and case status which would suggest the presence of an unobserved disease variant (D).



(continued)

Box 9.1 (continued)

- An allele-sharing approach that specifically tests for co-segregation of a genetic locus along with phenotype across individuals *within* each family. Then adds information across the families.
- Families are selected on the basis of an index individual (proband).Typically probands are a specific sample of *affected* individuals from the reference population.
- Genetic variant allele that co-segregates with phenotype has to be the same allele within a family, but does not need to be the same allele between families.
- Because closely related individuals share extensive regions of their genome, ~500 polymorphic markers are adequate to identify linkage.
- However, because relatively few recombinant events are seen within a single family, there is simultaneously difficulty in refining the genetic below the order of megabases.

- Traditionally tests for association of a specific allele of a genetic variant with phenotype across *independent, unrelated* individuals.
- Individuals are selected on the basis of affection ( ) in the reference population: cases = affected / controls = unaffected.
- An allele that is higher in frequency in cases is the susceptibility/risk allele.
- Because of LD, SNPs are correlated with each other and therefore “tag SNPs” are usually sufficient to adequately capture all variation in and around the unmeasured disease locus.
- Difficulty in tagging arises when the variant allele is rare or is common but from multiple ancestral chromosomes. If rare, then a SNP is usually not adequately tagged by other SNPs. If common but from multiple ancestral chromosomes then there may be multiple haplotype backgrounds on the variant.

frequently replicated are listed in Fig. 9.1. Asthma susceptibility genes have been argued to fall into four main categories, and candidate genes with identified associations fall into each of these categories as has been reviewed in-depth by Vercelli (2008). Briefly the asthma susceptibility loci fall into the following categories: (1) a class of genes associated with innate immunity and immunoregulation (examples include CD14, Toll-like receptors TLR2, TLR4, TLR6, and TLR10, cytokines such as IL10, TGFβ1, and HLA class II molecules); (2) genes associated with Th2-cell differentiation and effector function (including IL13 the central effector of allergic inflammation where genetic determinants of asthma are perhaps some of the best understood to date in their functional consequence) (Vladich et al. 2005; Cameron et al. 2006); (3) genes expressed in epithelial cells and involved in mucosal immunity (genes in the CC-chemokine cluster for example); and (4) the final class of genes that appear to determine lung function, airway remodeling, and asthma severity (two of the most consistent asthma loci *ADRB2* and *TNF*).

9.3.2 The Genome-Wide Association Approach

The advent of dense oligonucleotide microarrays that enable multiplex genotyping of large numbers of variants at low cost has made feasible the extension of genetic association beyond the study of candidate genes or regional positional cloning to a truly genome-wide survey. A variety of commercially available arrays enable typing of hundreds of thousands to several millions of variants simultaneously (Distefano and Taverna 2011) and rigorous statistical methods, including SNP genotype imputation methodologies have been developed to facilitate comprehensive testing of virtually all common genetic variation, including more than 35 million sequenced variants cataloged in the Thousand Genomes Project (Abecasis et al. 2012). Taking advantage of these advancements, the genome-wide association (GWA) era combines the strength of the unbiased nature of the query for genetic determinants of disease risk (similar in spirit to genome-wide linkage) along with the ability to recover most common human variation using a relatively small set of tagging genetic variants (similar in spirit to candidate gene association) (Risch and Merikangas 1996). The premise of the tagging approach is that given genetic architecture wherein SNPs are often found within blocks of LD where all SNPs within a block are highly correlated to each other, it is not necessary to genotype all variants in a single block to capture association between the disease locus within the block and phenotype; a reduced set of SNPs is sufficient to represent most of the variation contained within a block and can be used as a proxy for all remaining variants within the block.

The foundation for the GWA approach is the "common disease, common variant" hypothesis, wherein common diseases are argued to be attributable (in part) to common genetic variants (Reich and Lander 2001; Collins et al. 1997) and the leverage of the case–control design in place of the traditional family-based approaches necessary for genome-wide linkage. There are two obvious ramifications of this hypothesis: common genetic variants influencing disease are not

expected to have a large effect size (highly deleterious variants are generally recent and therefore uncommon in human populations (Tennessen et al. 2012); and, for common alleles with small effects to explain common disorders multiple loci each with small effect must cumulatively influence disease susceptibility.

The precise number of asthma GWA studies (GWAS) is hard to identify as many of the individual studies are folded into larger meta-analyses; several recent reviews are now available (Ober and Yao 2011; Akhabir and Sandford 2011). Table 9.1 is a comprehensive list of all GWAS publications relevant to asthma and highlights three points: (1) GWAS-identified loci are generally common in frequency; (2) GWAS-identified loci have modest effect sizes; and (3) although most loci are not replicated across the studies, there are several that are novel, highly replicated, and perhaps most importantly, robust to ethnicity.

The first asthma-susceptibility locus to be identified by GWAS is that on chromosome 17q21 (Moffatt et al. 2007). The associated variants reside on a common, cosmopolitan (i.e., observed in populations of diverse ancestry) haplotype that spans more than 100 kb and includes four genes: *ORMDL3*, *GSDMB*, *ZPBP2*, and IKZF3. This association with asthma has been among the most highly reproduced (Sleiman et al. 2008; Tavendale et al. 2008; Bouzigon et al. 2008; Galanter, et al. 2008; Hirota et al. 2008; Bisgaard et al. 2009; Wu et al. 2009; Leung et al. 2009; Halapi et al. 2010; Flory et al. 2009; Madore et al. 2008), observed in both children and adults, and across diverse ethnic groups (Galanter et al. 2008). The haplotype has regulatory potential, as it is associated with the expression of ORMDL3, GSDMB and ZPBP2 and functional fine-mapping studies suggest the causative variant regulates the differential binding of the insulator protein CTCF (Verlaan et al. 2009). However, due to the extensive linkage disequilibrium at this locus and it's impact on the expression on multiple genes, it remains unclear which of these genes is the culprit target. It is interesting that this locus overlaps with meta-analyses linkage regions for atopy and not asthma (Bouzigon et al. 2010; Denham et al. 2008), but it should be pointed out that these genes have never been studied under the candidate gene approach, supporting the importance of the unbiased GWAS approach in identifying novel loci for asthma. The ORMDL3 gene encodes ER-resident transmembrane protein and has high expression in cells involved in the inflammatory response (Moffatt et al. 2007). Alterations of protein folding or Ca(2+) levels within the endoplasmic reticulum (ER) result in the unfolded-protein response (UPR) which is an endogenous inducer of inflammation. ORMDL3 has been shown to alter ER-mediated Ca(2+) homeostasis and thereby facilitate the UPR (Cantero-Recasens et al. 2010). It has been shown that heterologous expression of ORMDL3 protein increased resting cytosolic Ca(2+) levels and reduced ER-mediated Ca(2+) signaling, an effect reverted by coexpression with the sarco-endoplasmic reticulum Ca(2+) pump (SERCA). Increased expression also promoted stronger activation of UPR transducing molecules and target genes. In contrast siRNA-mediated knockdown of ORMDL3 potentiated ER Ca(2+) release and attenuated the UPR adding further support for a likely biological explanation to the associations seen at this locus with asthma risk.

Another consistently replicated GWAS locus is that mapping to a region upstream of *IL33*, the gene encoding interleukin 33 (IL-33) located on chromosome 9q24.

Table 9.1 Summary of GWAS studies on asthma as the primary phenotype summarized from the Catalog of Published Genome-Wide Association Studies highlighting three regions with replication across multiple studies and ethnicities. Studies include those with a panel of >100,000 SNPs and reported *p*-values on the discovery data of $p < 10^{-5}$

Pubmedid	First author	Year	Sample size of discovery cohort(s)	Region	Hg19 chromosomal Position	Reported gene(s)	SNPs	Predicted function of peak SNP	Risk allele frequency	p-Value	OR or beta
Asthma	Moffatt MF	2007	994 cases,1,243 controls	17q12	38069949	ORMDL3	rs7216389	Intron	0.52	9.00E-11	1.45
	Himes BE	2009	422 cases, 1,533 controls	5q12.1	59369794	PDE4D	rs1588265	Intron	0.29	3.00E-08	1.18
	Mathias RA	2010	464 African American cases, 471 African American controls, 1,028 African Caribbean family members	NR		NR	NR		NR	NS	NR
	Sleiman PM	2010	793 European ancestry child cases, 1,988 European ancestry child controls	1q31.3	197325908	DENND1B, CRB1	rs2786098	Intron	0.85	2.00E-13	1.43
	Li X	2010	607 cases, 3,294 white controls	5q31.1	131901225	RAD50	rs2244012	Intron	0.21	3.00E-07	1.64
				15q21.2	51969668	SCG3	rs17525472	Intergenic	NR	2.00E-06	NR
				20p13	3827309	KIAA1271	rs4815617	nearGene-5	NR	8.00E-06	NR
	Himes BE	2010	359 non-Hispanic white cases, 846 white controls	NR		NR	NR		NR	NS	NR
	Moffatt MF	2010	10,365 cases, 16,110 controls	2q12.1	102986222	IL18R1	rs3771166	Intron	0.62	3.00E-09	1.15
				5q31.1	131723288	SLC22A5	rs2073643	Intron	0.45	2.00E-07	1.11
				5q31.1	131995843	IL13	rs1295686	Intron	0.2	1.00E-07	1.15
				6p21.32	32625869	HLA-DQ	rs9273349	Intergenic	0.58	7.00E-14	1.18
				9p24.1	6190076	IL33	rs1342326	Intergenic	0.16	9.00E-10	1.2
				15q22.2	61069988	RORA	rs11071559	Intron	0.86	1.00E-07	1.18
				15q22.33	67446785	SMAD3	rs744910	Intron	0.49	4.00E-09	1.12
				17q12	38062196	GSDMB	rs2305480	Missense	0.55	1.00E-07	1.18
				17q21.1	38121993	GSDMA	rs3894194	Missense	0.45	5.00E-09	1.17
				22q12.3	37534034	IL2RB	rs2284033	Intron	0.56	1.00E-08	1.12
	DeWan AT	2010	66 case children, 42 control children	NR		NR	NR		NR	NS	NR
	Ferreira MA	2010	986 European descent cases, 1,846 European descent controls	17q12	38095174	ORMDL3	rs6503525	Intergenic	0.43	5.00E-07	1.33
	Ege MJ	2011	850 European asthmatic children, 348 European children with atopy, 510 European child controls	NR		NR	NR		NR	NS	NR
	Schauberger EM	2011	112 European ancestry children cases, 165 European ancestry children controls	NR		NR	NR		NR	NS	NR

(continued)

Table 9.1 (continued)

Hirota T	2011	1,532 Japanese ancestry cases, 3,304 Japanese ancestry controls	4q31.21	144003159	LOC729675	rs7686660	Intergenic	0.27	2.00E-12	1.16
			4q31.21	144357737	GAB1	rs3805236	Intron	0.25	7.00E-08	1.12
			5q22.1	110401872	TSLP	rs1837253	Intergenic	0.35	1.00E-16	1.17
			6p21.32	32155581	PBX2	rs204993	Intron	0.58	2.00E-15	1.17
			6p21.32	32184345	NOTCH4	rs404860	Intron	0.5	4.00E-23	1.21
			6p21.32	32338695	C6orf10	rs3129943	Intron	0.62	3.00E-15	1.17
			6p21.32	32358513	BTNL2	rs3117098	ncRNA	0.25	5.00E-12	1.16
			6p21.32	32414273	HLA-DRA	rs3129890	Intergenic	0.61	5.00E-13	1.15
			6p21.32	32658079	HLA-DQB1	rs7775228	Intergenic	0.63	5.00E-15	1.17
			6p21.32	32687973	HLA-DQA2	rs9275698	Intergenic	0.79	5.00E-12	1.18
			6p21.32	32961361	HLA-DOA	rs9500927	Intergenic	0.26	4.00E-09	1.13
			10p14	8972018	LOC338591	rs10508372	Intergenic	0.433	2.00E-15	1.16
			12q13.2	56412487	IKZF4	rs1701704	Intergenic	0.18	2.00E-13	1.19
			12q13.2	56364321	CDK2	rs2069408	Intron	0.23	1.00E-10	1.15
Torgerson DG	2011	2,088 European American cases, 1,612 African American and African Caribbean cases, 1,688 Hispanic ancestry cases	1q21.3	152492559	CRCT1	rs4845783	Intergenic	NR	6.00E-06	NR
			1q23.1	158932555	PYHIN1	rs1101999	Intron	NR	4.00E-09	NR
			2q12.1	102953617	IL1RL1	rs3771180	Intron	NR	2.00E-15	NR
			5q22.1	110401872	TSLP	rs1837253	Intergenic	NR	1.00E-14	NR
			9p24.1	6193455	IL33	rs2381416	Intergenic	NR	2.00E-12	NR
			11q23.2	114231255	C11orf71	rs11214966	Intergenic	NR	6.00E-07	NR
			17q12	38064405	GSDMB	rs11078927	Intron	NR	2.00E-16	NR
Noguchi E	2011	938 Japanese ancestry cases, 2,376 Japanese ancestry controls	6p21.32	33042880	HLA, DPB1	rs987870	Intron;nearGene-5	0.14	2.00E-10	1.4
			8q24.11	118025645	SLC30A8	rs3019885	Intron	0.31	5.00E-13	1.34
Ferreira MA	2011a, b	12,475 European ancestry cases, 19,967 European ancestry controls	1q21.3	154426264	IL6R	rs4129267	Intron	0.37	2.00E-08	1.09
			10q21.1	53493473	PRKG1	rs7922491	Intron	0.11	5.00E-07	1.13
			11q13.5	76270683	LRRC32	rs7130588	Intergenic	0.34	2.00E-08	1.09
			13q21.31	63638329	PCDH20	rs3119939	Intergenic	0.51	8.00E-06	1.08
Du R	2012	395 European ancestry asthmatic children	NR		NR	NR		NR	NS	NR
Anantharaman R	2011	490 Chinese ancestry cases, 490 Chinese ancestry controls	NR		NR	NR		NR	NS	NR
Karunas AS	2011	330 European ancestry cases, 348 European ancestry controls	NR		NR	NR		NR	NS	NR
Tantisira KG	2012	418 European ancestry cases	6q27	166534742	T	rs6456042	Intergenic	NR	6.00E-06	NR
Wan YI	2012	933 European ancestry cases, 3,346 European ancestry controls	2q12.1	102971200	IL18R1,IL1R1	rs9807989	Intergenic	NR	6.00E-08	1.33
			5q31.1	131796922	C5orf56	rs11745587	Intron	NR	2.00E-06	1.26
			5q31.3	141445980	NDFIP1	rs6867913	Intergenic	NR	4.00E-06	1.33
			9p21.1	32433526	ACO1	rs10970976	Intron	NR	4.00E-06	1.28
			17q12	38089344	ORMDL3	rs4794820	Intergenic	NR	1.00E-08	1.33
			19q13.42	53682042	ZNF665	rs16984547	Intron	NR	4.00E-06	1.43
Li X	2012	813 European ancestry cases, 1,011 European ancestry controls	NR		NR	NR		NR	NS	NR
Ramasamy A	2012	1,716 European ancestry cases, 16,888 European ancestry controls	2q12.1	102955082	IL1RL1, IL18R1	rs13408661	Intron	0.84	1.00E-09	1.23
			6p21.32	32379489	BTNL2, HLA-DRA	rs9268516	Intergenic	0.24	1.00E-08	1.15
Lasky-Su J	2012	Up to 1,238 European ancestry cases, up to 2,617 European ancestry controls	6p21.32	32604372	HLA-DQA1	rs9272346	nearGene-5	NR	2.00E-08	NR

Table 9.1 (continued)

Childhood onset asthma	Hancock DB	2009	492 Mexican trios	9q21.31	82039362	TLE4, CHCHD9	rs2378383	Intergenic	0.78	7.00E-07	1.64
	Ricci G	2011	135 European ancestry children with asthma, 134 European ancestry children with rhinoconjunctivitis	NR		NR	NR		NR	NS	NR
	Forno E	2012	573 European ancestry children	1q44	244511176	C1orf100	rs4658627	Intergenic	0.2418	6.00E-06	0.13
				3p26.2	3614887	IL5RA	rs9815663	Intergenic	0.182	2.00E-08	0.17
				11q24.2	127761666	NR	rs7927044	Intergenic	0.0134	7.00E-09	0.16
				13q13.3	36351766	NR	rs7328278	Intron	0.0285	3.00E-06	0.13
				17p12	13559080	NR	rs10521233	Intergenic	0.0735	3.00E-06	0.14
Asthma (toluene diisocyanate-induced)	Kim SH	2009	84 Korean ancestry cases, 263 Korean ancestry controls	9p21.3	20098711	Intergenic	rs16937883	Intergenic	0.02	7.00E-06	5.29
				10q21.3	68088508	CTNNA3	rs10762058	Intron	0.25	6.00E-06	5
				13q12.13	27415673	Intergenic	rs9319321	Intergenic	0.29	3.00E-06	5.2
Asthma (aspirin-intolerant)	Kim JH	2010	80 Korean cases, 100 Korean controls	NR		NR	NR		NR	NS	NR

A GWAS in one Asian and nine European population demonstrated association for asthma of a variant situated ~6 kb upstream of *IL33* (Gudbjartsson et al. 2009). Subsequently, another variant situated ~27 kb upstream of *IL33* was associated with asthma in the European-based GABRIEL Consortium—the largest GWAS meta-analysis to date (>26,000 subjects) (Moffatt et al. 2010). A second meta-analysis of nine North American asthma GWAS—the EVE Consortium—also replicated this association with variants ~22 kb upstream of *IL33* (Torgerson et al. 2011), demonstrating consistency across populations of diverse ethnicity (European American, African American, and Hispanic American). In contrast to the 17q21 locus, *IL33* along with its receptor *IL1RL1/ST2*, also implicated in GWAS (Gudbjartsson et al. 2009; Moffatt et al. 2010; Torgerson et al. 2011) represent extremely strong well-understood biological candidates for asthma (Wjst et al. 2013). Produced by mast cells following IgE-mediated activation (Hsu et al. 2010), IL-33, a member of the interleukin-1 (IL-1) cytokine family is directly involved in eosinophil- and basophil-mediated inflammation and IL-5 production, hallmark features of allergic disease (Schmitz et al. 2005; Cherry et al. 2008; Suzukawa et al. 2008; Smithgall et al. 2008; Pecaric-Petkovic et al. 2009; Smith 2010). Greater *IL33* expression in airway smooth muscle cells (Prefontaine et al. 2009) has been observed in airway epithelium of patients with asthma compared to healthy individuals (Prefontaine et al. 2010). Its receptor, ST2 in its soluble form (sST2) on chromosome 2q12.1, neutralizes IL-33 by acting as a decoy receptor (Sanada et al. 2007) and is another replicated GWAS signal robust to ethnicity. Serum ST2 has been associated with atopic asthma (Oshikawa et al. 2001). It is indeed striking that two genes in the interleuin-1/Toll-like receptor (TIR) superfamily pathway, what has emerged as a central pathway in asthma, have both independently been implicated as asthma determinants in GWAS approaches and points to the merits of the unbiased GWAS approach. It is notable that several additional TLRs have also been implicated as determinants of asthma in the candidate gene approach noted in Fig. 9.1.

The GWA approach is not without limitations as follows. The SNPs identified from the GWAS are not the true causal variant themselves but generally a proxy for some unmeasured disease-causing variant that needs additional follow-up for discovery. Given the vast number of statistical tests performed, stringent thresholds are set in place to control the family-wise error rate; the most commonly used approach being the Bonferroni correction given by $0.05/n$, where n is the number of tests being performed, and 0.05 is the Type I error rate typically selected. This often leads to the failure to address association signal from loci with less striking effects, now being discussed as a part of the missing/residual heritability from GWA studies of complex phenotypes. The comparison across studies can be difficult when differing set of SNPs are analyzed due to differences in GWA genotyping arrays; this, however, can be overcome using in-silico genotyping approaches such as imputations to a standard reference panel of SNPs such as the HapMap or Thousand Genomes panels. The standard for replication in a GWAS is the SNP-to-SNP replication with a consistent direction of risk effect. This last concern is an important one to consider given the tagging strategy upon which the GWA relies; especially in the comparison between ethnic groups where the same causal variant may lie on different haplotype backgrounds and thereby manifest as association to an alternate SNP in the GWA panel of SNPs or the same SNP with a different direction of risk (i.e., the risk allele in one group is the protective allele in another). Finally, GWA analysis generally ignores the presence of multiple loci, gene–gene and gene–environment interactions. Nonetheless, despite these limitations, GWASs have led to numerous successes in asthma and at least three different loci that appear to be robust to ethnicity (17q12, 9q24, 2q12.1 and 6p21.32).

9.4 Gene–Environment Interactions in Asthma

The role of environmental factors as key determinants of allergic inflammation and asthma risk has been well established (Strachan 1989; von Mutius 2004), and the potential for gene–environment interactions in asthma has been well recognized (Kauffmann and Demenais 2012). However, despite the importance of the environment in asthma, and the need to consider gene–environment (G x E) interaction in a systematic fashion, few such studies have been conducted to date. This is largely because of the complexity involved in conducting such studies, including the requisite large sample sizes, the large number of interaction models under consideration, and the difficulties in accurately measuring environmental exposures (Box 9.2A, B, C) (Kraft and Hunter 2005; Khoury et al. 1988). The design of a study that takes into account G x E interactions requires accurate assessment of both phenotype and environment, and depending on the magnitude of the G x E effect, considerably larger samples sizes than those required to detect main effects of just genetic loci. The availability of suitable replication populations is important as well, i.e., additional studies where both gene and environment are measured and the environmental exposures are similar in effect.

Despite these limitations, early (largely candidate-gene) GxE studies have reported numerous examples of gene–environment interaction in asthma (Fig. 9.1, Box 9.2C).

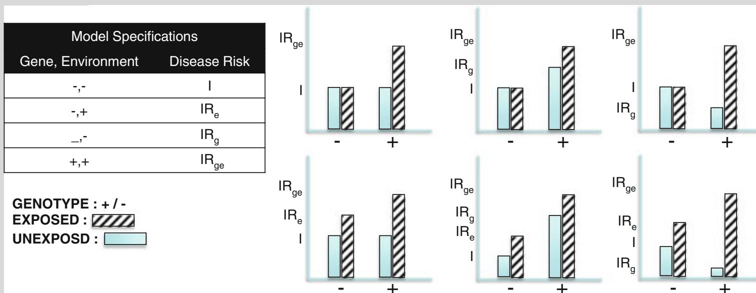
Box 9.2A Ignoring gene–environment interactions can mask genetic effects and thereby lead to the genetic heterogeneity between populations in the evaluation of a single genetic locus

COMBINED		
Gene / Disease	+	-
+	100	100
-	200	200
OR(D|G) = 1.0		

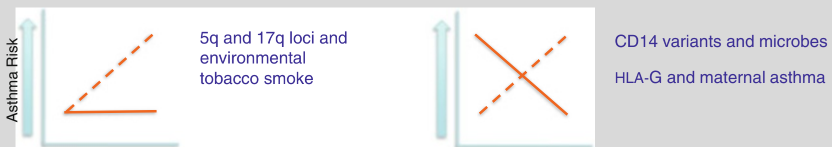
Exposed Group		
Gene / Disease	+	-
+	25	10
-	20	50
OR(D|G) = 6.25		

Unexposed Group		
Gene / Disease	+	-
+	75	90
-	180	150
OR(D|G) = 0.69		

Box 9.2B Complexity in models of gene–environment interaction (adapted from Khoury et al. 1988)



Box 9.2C Established gene–environment interactions in asthma (reviewed in Ober and Yao 2011)



Of these, the most extensively studied are those focused on *CD14*, a component of the toll-like receptor (TLR) signaling complex that facilitates endotoxin responsiveness. A series of association studies on a functional promoter polymorphism (*CD14*-260CT) has revealed protection against asthma (Leynaert et al. 2006), increased asthma risk (Ober et al. 2000), and a plethora of studies with no significant associations (Vercelli 2008). In subjects of African descent, the effect of *CD14*-260CT on asthma is dependent on levels of domestic endotoxin exposure; the TT genotype appears to protect against asthma in low domestic endotoxin exposure, but is a risk factor in high exposure (Zambelli-Weiner et al. 2005). These gene–environmental effects for *CD14*-260CT carry forth to a wide variety of asthma-associated phenotypes as well, including IgE (Eder et al. 2005), atopic dermatitis (Gern et al. 2004), allergic sensitization, eczema, and wheezing (Simpson et al. 2006). In all these examples, the effect of the variant differs based on environmental exposure, and in fact CD14 offers an excellent illustration of how ignoring environment can lead to the appearance of genetic heterogeneity in genetic determinants of asthma risk.

9.5 The Next Frontier of Association Studies: DNA Sequencing

Despite the early successes of GWAS in identifying novel asthma loci, there has been criticism that, for complex diseases broadly, and asthma specifically, the GWAS approach has not provided sufficient insight into the genetic contribution to disease risk. Though more than 30 asthma GWAS have been published, describing 51 genes having p-values $<1.0\times10^{-5}$ specifically for asthma (Hindorff et al. 2013), the cumulative genetic risk explained by the associated variants is relatively low (<15 %), precluding their use as predictive or diagnostic clinical models. This so-called missing heritability problem (Eichler et al. 2010) is a frequent occurrence in GWAS (Frazer et al. 2009), which are based on the premise of common disease/common variant hypothesis (Lander 1996; Cargill et al. 1999; Chakravarti 1999). “Missing heritability” is simply *residual* heritability or the leftover disease risk that is unaccounted for by the GWAS-identified genetic loci. The source of the “missing heritability”(Eichler et al. 2010) could include (1) common variants of smaller effects that fall far below the stringent significance thresholds applied in the GWAS approach; (2) rare variants with large effects or structural variants that are poorly tagged by commercial GWAS genotyping arrays; (3) limited power to detect interactions (Manolio et al. 2009); and (4) unmeasured epigenetic phenomena in the GWAS approach. It has even been argued that what appears to be association signal from a common variant in a GWAS approach could in fact be representative of multiple underlying rare variant association signals (Dickson et al. 2010) due to linkage disequilibrium.

Since the late 1970s Sanger termination sequencing (Sanger et al. 1977) has been the sole method of choice for sequencing studies. Key pieces of technology development in 2005 (Margulies et al. 2005; Shendure et al. 2005) heralded the current era of Next Generation Sequencing (NGS) techniques that have entailed arraying thousands of sequencing templates enabling sequences that can be analyzed in parallel, a

dramatic increase to the 96 sequencing templates on a contemporary Sanger capillary sequencer. Today, NGS systems include SOLiD/Ion Torrent PGM from Life Sciences, Genome Analyzer/HiSeq 2000/MiSeq from Illumina, and GS FLX Titanium/GS Junior from Roche (Metzker 2010) among others and enable the rapid sequencing of either predefined genomic regions (such as all the protein coding regions of the genome, i.e., the exome (Teer and Mullikin 2010; Fu et al. 2013) or entire human genomes (Abecasis et al. 2010). Despite the increasing computational complexities (Hoffmann 2011) inherent to these methods, the dramatic decreases in sequencing costs (Wolinsky 2007) make association studies using this approach highly attractive. Genome-wide sequencing studies in asthma are in their early stages, and it remains unclear what impact they will have on addressing the missing heritability problem.

With respect to asthma, an observation from Fig. 9.1 is the lack of overlap in genes identified through earlier approaches of positional cloning and candidate genes studies and the finding that most of the >100 genes established as asthma loci through these earlier approaches are not rediscovered through GWAS. An elegant argument that many of associated variants in these genes are simply not adequately captured by commercially available GWAS arrays has been demonstrated for asthma (Rogers et al. 2009). In addition to the arguments provided above, this provides compelling reason to extend asthma genetics to the new frontier of sequencing designs which ensures complete coverage of common variants, adequate coverage of rare variants (provided adequate sequencing depth), and importantly, the discovery of novel variants in sequenced cases. Using a theoretical framework that genes with molecular signatures of weak purifying selection are more likely to harbor an excess or rare/low frequency variants, resequencing has revealed that rare variants (in *AGT*, *DPP10*, *IKBKAP*) contribute to asthma susceptibility (Torgerson et al. 2012). Interestingly, the contribution of rare variants to asthma susceptibility was predominantly due to noncoding variants, and these early results of resequencing approaches offer the first promise of the value in a transition from tagging-SNP common-variant GWAS approaches over the past decade to resequencing approaches in the near future. It also provides an argument for consideration of the extensive human variation that exists outside the coding regions of the genome; exciting work by the Encyclopedia of DNA Elements (ENCODE) project (Dunham et al. 2012) has demonstrated that the vast majority of the human genome participates in at least one biochemical RNA and/or chromatin associated event in at least one cell type!

9.6 Heterogeneity in Asthma Genetics

Although a genetic basis for asthma is undeniable and >100 genes have been implicated, the elucidation of causal variants to explain this basis has been fraught with issues of between-study replication that stem from a variety of arguments including (1) heterogeneity in the asthma phenotype wherein "asthma" constitutes multiple overlapping syndromes rather than a single disease entity (Barnes 2011); (2) strong interactions with environment (Vercelli 2008); and (3) the high likelihood of true genetic heterogeneity (different sets of genes determine risk for asthma in different

populations). Large-scale sequencing of the human genome has revealed the dramatic potential for the latter (Abecasis et al. 2010): common human variation (allele frequencies ≥10 %) are almost all found in all of the populations studied, however, 17 % of low-frequency variants in the range 0.5–5 % were observed in a single ancestry group, and 53 % of rare variants at 0.5 % were observed in a single population. Genetic heterogeneity has been noted in asthma linkage signals (CSGA 1997), candidate gene studies (Ober and Hoffjan 2006; Ober and Yao 2011), and GWAS (PYH1N1 is a novel asthma susceptibility locus found only in populations of African descent (Torgerson et al. 2011). Importantly, sequencing approaches reveal rare variant determinants of asthma in four genes (*AGT*, *DPP10*, *IKBKAP*, and *IL12RB1*) among African Americans, but only rare variant determinants of asthma in *IL12RB1* among European Americans, further confirming the potential role of population heterogeneity in genetic determinants of asthma (Torgerson et al. 2012). To date, replication is typically evaluated in the strict sense—a SNP-for-SNP replication with the same direction of effect (Barnes 2011; Vercelli 2008); a transition to NGS approaches opens the window to "burden tests" is to assess association between "clusters" of rare variants within windows (e.g., a gene) and disease status (Li and Leal 2008; Morris and Zeggini 2009; Schaid and Sinnwell 2010; Zhu et al. 2010; Price et al. 2010; Cohen et al. 2004). Briefly, computationally fast tests include (1) cohort allelic sums test (CAST), where the number of individuals with one or more mutations in a window is compared between affected and unaffected individuals (Cohen et al. 2004) and (2) Combined Multivariate and Collapsing (CMC) method (Li and Leal 2008), where all rare variants (e.g., <1 %) are collapsed and treated as a single common variant analyzed along with all common variants in the region using multivariate analysis. More sophisticated approaches include those where variants are weighted according to their frequency (Madsen and Browning 2009) giving more weight to rarer alleles, tests that optimally select an allele frequency cut-off (Price et al. 2010), tests where rare variants in a gene are allowed to have both protective and risk effects (Wu et al. 2010), and tests allowing for misclassification of variant function (Liu and Leal 2010). The general spirit of these approaches is to specifically move beyond any single variant to collapsing information across multiple variants within a window of interest, thereby overcoming some of the limitations of strict replication rules that have plagued asthma genetics thus far.

In conclusion, the road to the discovery of genetic determinants of asthma has had numerous successes as study designs and technology have morphed from small linkage and family-based studies to extensive meta-analyses of GWAS data (Moffatt et al. 2010; Torgerson et al. 2012). One of the biggest successes of the GWAS approach has been the identification of a novel locus on 17q21 that is robust to population ancestry and has been highly replicated since the initial discovery. With the extensive LD and likely coregulation of multiple genes within this association peak, it is yet unclear which gene(s) in this chromosomal region are responsible for the association with asthma (*ORMDL3*, *GSDMB*, *ZPBP2*, or *IKZF3*). Integrative applications of genomic and epigenetic approaches are necessary in further elucidating causal variants behind the genetic association signals described in this chapter, and such applications specific to this 17q21 are further described in Chaps. 10 and 11.

Box 9.3 The genomewide association (GWA) approach

The GWA approach leverages the technological advancements in high-throughput genotyping along with the tagging approach to interrogate the entire genome for common variants that may be determinants of common disease. The completion of the International HapMap project provided the backbone for the design of genotyping arrays containing the smallest set of SNPs that captured the largest amount of common genetic variation given LD block structure and inter-SNP correlation, i.e., a set of tag SNPs to be genotyped as representatives of common human variation. In the design of a GWA study (GWAS), it is important consider to the wide choice of genotyping arrays available and select the array ideal for the population to be studied; for populations with high levels of African ancestry, African Americans, for example, an array that considers the smaller LD blocks that are results of the African representation in the admixed populations are important. GWAS are traditionally done by comparing allele frequencies at genotyped variants between well-phenotyped affected cases and unaffected controls, and clinical characterization is an important consideration for the study design. In asthma, the relevance of age of onset in GWA analyses has been shown to have an impact of strength of association signals observed at the chromosome 17q12 locus, wherein variation in this locus is an important determinant of childhood onset asthma. Careful consideration of population structure differences between the cases and controls is necessary as this can lead to spurious associations. Given the unbiased nature of the ~10^6 SNPs typically on an array (i.e., the vast majority of the SNPs do not in fact correlate with the disease under consideration), the genotype distributions can be used to detect cryptic population structure typically using principal components analysis/multidimensional scaling. An overall difference between cases and controls across the genome measured by the principal components (PCs) is usually indicative of population structure and the PCs can be used to correct for this structure in the single-SNP association tests.

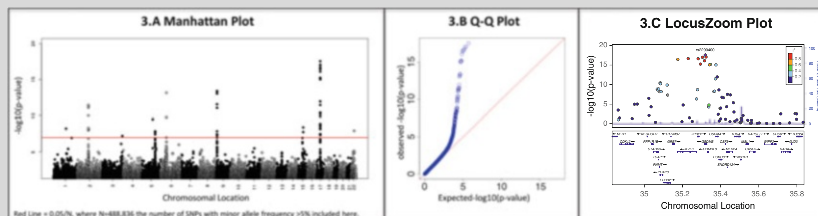
Two commonly used tools in the interpretation of the GWA results are the Manhattan Plot (**3.A**) and the Q–Q Plot (**3.B**). In these illustrations from the publicly available GABRIEL data, one sees the overall GWA p-values plotted as –log10(P-value) against chromosomal position (**3.A**) and quantile–quantile distributions of observed versus expected p-value to show deviation of observed from the expected distribution (**3.B**). In the Manhattan plot, regions of the genome that cross the stringent Bonferroni threshold of significance (red line) required for the multiple testing of SNPs are easily evident. The Q–Q plot supports (i) the lack of population stratification (the vast majority of p-values fall along the red line; they would be expected to be above the red line in the case of population stratification) and (ii) the presence of true association signal

(continued)

Box 9.3 (continued)

denoted by the upper set of strong p-values that are considerably stronger than expected for the range. One final tool is the zoom plot (**3.C**) of the peak association signal showing LD between SNPs in the region to the peak SNP, recombination fractions, and known genes in the region. Often the peak SNPs in a GWAS is a region of LD that includes multiple SNPs; the commonly used LocusZoom plot is a useful tool to identify genes that are potentially implicated by the peak GWAS SNP.

The GWAS has led to numerous discoveries and between 2005 and 2012 there have been over 1,350 publications relying on this approach. However, there are some pitfalls to the approach that must also be highlighted. A narrow and well-defined case/control definition is critical to maximize power, a well-selected GWA array suitable for the population under consideration, a robust control of false positives due to multiple testing, and an efficient way to detect and correct for population stratification are all relevant issues. A more fundamental issue at hand, given the higher degree of unexplained heritability even for some of the most successfully GWA studied phenotypes, is the robustness of the common disease common variant hypothesis. With the advent of high-throughput sequencing that has been dramatically decreasing in cost, it is anticipated that sequencing, targeted and whole genome, will be the next frontier in the tool box of asthma geneticists enabling a query of all variation, common and rare, as determinants of asthma risk.



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Chapter 10
Gene Expression Profiling in Asthma

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Keywords Gene expression • Transcriptomics • Differential expression • Normalization • RNA-Seq • Microarray • Pathway analysis • Systems biology • Ribose nucleic acid

10.1 Introduction

Transcriptomics (gene expression profiling) refers to the quantitative and qualitative characterization of the collection of ribose nucleic acid (RNA) elements expressed in a biological system and represents one of the first truly genome-wide hypothesis-free investigative approaches in molecular biology. The advent of synthetic oligonucleotide microarray technologies has enabled large-scale application of gene expression profiling in the study of human disease, particularly malignant and hematological processes. Due to favorable characteristics of these processes, including their involvement of one cellular compartment (and often a specific, monoclonal cell type), the severity of the underlying cellular perturbation under study (malignant vs. benign cells), and the accessibility to large numbers of available banked samples obtained during clinically indicated medical procedures, the study of transcriptomics in oncology has been quite fruitful, with notable translation of these techniques to novel clinical applications with diagnostic, prognostic, and therapeutic implications. Furthermore, the discovery of large populations of noncoding RNA

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elements, including microRNA and long-intergenic noncoding RNA (LINCC-RNA) has expanded the scope of transciptomic profiling beyond the protein-coding messenger RNAs (mRNA).

In this chapter, we provide a brief survey of prior applications of this approach to the study of asthma, followed by an overview of the primary technical and analytical considerations that should be addressed when conducting such studies. For more detailed review of study protocols and specific analytical platforms, readers are referred to several recent publications (Matson 2009; Yakovlev et al. 2013; Dehmer and Basak 2012; Rodriguez-Ezpelete et al. 2012).

10.2 Applications of Transcriptomics in Asthma Research

Asthma is a complex syndrome arising from the interplay of genetic and environmental perturbations of two multicellular organ systems (the respiratory and immune systems) operating in a developmental context. Thus, in contrast to malignancies that arise from radical molecular alterations in one population of cells, the genomic study of asthma presents a more complicated model with inherent challenges, relating to selection of disease model and tissue sampling, and analytical interpretation. Nonetheless, transcriptomic profiling has been applied widely in the study of asthma and will likely continue to play an important role in biological and translational asthma research. Broad goals of such studies include discovery of novel pathways underlying asthma pathogenesis, reclassification of asthma subtypes on the basis of distinct genetic signatures, and understanding the cellular response of asthma-relevant environmental exposures, pharmaceuticals, and other perturbations, all with the hope of identifying new therapeutic targets.

Tables 10.1 and 10.2 summarize many of the published asthma genomic studies performed in patient populations, in the peripheral immune (Table 10.1) and pulmonary (Table 10.2) compartments. Thus far, virtually all published studies have employed chip-based expression platforms in conjunction with traditional analytical methods of differential gene expression analysis, cluster analysis, or a combination of both. The majority of these studies have focused on contrasts between asthma case and unaffected control status, or across strata of asthma severity. With notable exception, sample size has been small, most studying fewer than 100 patients. Given the complexity of asthma, the heterogeneity in asthma phenotype, and wide spectrum of severity, small-sized studies are of limited value, particularly with regard to broad generalization. Complicated by differences in tissue of study, patient inclusion criteria, expression microarray employed, and analytical methods, the results of these studies are largely nonoverlapping, and few discernable common insights are apparent. Moreover, few of these studies presented rigorous evidence of either intrinsic (technical and computational) or extrinsic (replication) validation, making uncertain the generalizability of their findings. Nonetheless, several of these early efforts have provided important insights worthy of discussion here.

Table 10.1 Asthma gene expression studies (peripheral blood/immune cells)

Study	Experimental description	Sample size	Platform	Implicated genes/pathways
WBCs				
Orsmark-Pietras et al. (2013)	Pediatric and adult populations Primary comparisons: 1. Asthma severity/asthma affection status in children 2. Adult asthmatics- expression in fractionated leukocytes	71 subjects	Affymetrix ST 1.0	Major finding: Bitter taste transduction (TAS2R) differentially expressed in severe asthma, highest expression in blood lymphocytes Other findings: NK cell cytotoxicity, N-glycan synthesis
CD4+/CD8+ T cells				
Tsitsiou et al. (2012)	Gene expression levels compared in severe and mild asthmatic groups vs. controls	24 subjects	Affymetrix U133 Plus 2.0	Activation of CD8+ T cells in severe asthma, with upregulation IL-2 regulatory pathways; less differential expression in CD4+ T cells, with upregulation of vitamin D pathway
CD4+ T cells				
Hunninghake et al. (2011)	Analysis of gene expression and total serum IgE in pediatric asthma population	223 subjects	Illumina Ref8 v2	IL17RB associated with total serum IgE; sexual dimorphism of implicated gene pathways suggest sex-specific regulation of IgE
Kapitein et al. (2008)	Differential gene expression in infant wheeze (transient, persistent) vs. controls	19 subjects	Affymetrix U133A	Wheezing phenotypes show differential expression of apoptosis and T-cell proliferation genes
Neutrophils				
Baines et al. (2010)	Neutrophilic gene expression profiles were studied in none-osinophilic asthma (vs. eosino-philic asthma or control)	28 subjects	Illumina Ref-8 v1.1	Noneosinophilic asthma associated with distinct neutrophil gene expression profile, implicating cell motility and apoptosis regulation

(continued)

Table 10.1 (continued)

Study	Experimental description	Sample size	Platform	Implicated genes/pathways
Basophils				
Youssef et al. (2007)	Expression profiles from "releaser" basophils (produce mediators with FcεRI cross-linking) vs. "nonreleasers" in asthmatics vs. controls	129 subjects	Affymetrix U133A	FcεRI cross-linking induces multiple distinct genes (FcεRI α, β subunit, histamine 4 receptor) in basophil "releasers" vs. "nonreleasers"
PBMCs				
Aoki et al. (2009)	Expression profiles in exacerbation (vs. stable asthma); compared to nonasthmatics with respiratory infection (vs. control)	34 subjects	Illumina Human Ref8	Many of 153 differentially expressed genes in asthma exacerbations also observed in during respiratory infection
Bjornsdottir et al. (2011)	Peripheral expression profiles during asthma exacerbation vs. quiescent asthma	118 subjects	Affymetrix U133A	Distinct exacerbation-associated signatures identified in innate immunity, lymphocyte activation, and downstream adaptive immune pathways
Hakonarson et al. (2005)	Expression patterns in GC (Glucocorticoid)-sensitive vs. GC-resistant asthmatic subjects, at baseline, and after treatment with IL-β, TNF-α	106 subjects	Affymetrix A arrays U95 set	Expression changes in 923 genes after IL-β, TNF-α were reversed in GC responders' cells with GC treatment (15 of these genes predicted GC response in an independent test set)
Subrata et al. (2009)	Expression profiling of PBMCs during asthma exacerbations vs. convalescence; qRT-PCR on specific cell populations	67 subjects	Affymetrix U133 Plus 2.0 Gene Chips	Expression of pathways during exacerbation: arachidonic acid, leukocyte migration, innate, adaptive immunity; gene specific upregulation mainly in monocytes/DCs
Shin et al. (2011)	Differential gene expression in asthmatics vs. controls; of 170 differentially expressed genes, top 8 used in prediction models	52 subjects	Illumina Human Ref8 BeadChip	Best predictive for asthma model contained *MEPE*, *MLSTD1*, and *TRIM37* (98 % sensitivity, 80 % specificity)

Table 10.2 Asthma gene expression studies (lung)

Study	Experimental description	Sample size	Platform	Major genes pathways identified/ findings
Endobronchial biopsies				
Yick et al. (2013)	RNA-Seq for differential gene expression study in asthma	9 subjects	Ovation RNA-Seq/GS FLX+	Differential expression of individual genes (Pendrin, Periostin, and BCL2), ten gene networks in cell morphology, movement, and development
Choy et al. (2011)	Quantitative description of Th2 inflammation in asthmatics; and correlation with inflammatory markers	40 Subjects	Agilent two-color 44K array	Th2 signature correlated with CCL26, IL-13, IL-5, Wnt, TGF-β, PDGF expression, and associated with increased IgE, blood and BAL eosinophils, decreased neutrophilic and Th1 responses
Airway epithelial cells				
Woodruff et al. (2007)	Expression profiling of asthmatics, smoking controls, non-smoking controls for epithelial dysfunction markers, effects of corticosteroids	86 subjects	Affymetrix U133 Plus 2.0	Expression of CLCA1, periostin, and serpinB2, upregulated in asthma vs. smoking controls; corticosteroids associated with decreased expression of CLCA1, periostin, Serpin B2, upregulation of FKBP51 in asthmatics
Woodruff et al. (2009)	Expression of IL-13 inducible genes used for molecular Th2 phenotype classification, validated using cytokine, inflammatory and ICS (inhaled corticosteroid) responses	70 subjects	Affymetrix U133 Plus 2.0	Gene expression analysis classified asthmatics into two subgroups "Th-2 High" and "Th2-low" (indistinguishable from controls) "Th2-high" showed greater peripheral and BAL eosinophilia, ICS response, serum IgE and mucin expression than "Th2-low" subjects

(continued)

Table 10.2 (continued)

Study	Experimental description	Sample size	Platform	Major genes pathways identified/ findings
Bochkov et al. (2010)	Rhinovirus(RV)-induced gene expression compared in asthmatics vs. nonasthmatics	18 subjects	Affymetrix U133 Plus 2.0	After RV-infection, some immune-related genes differentially expressed in asthmatics; most of asthma-related differences in expression seen prior to RV infection
Freishtat et al. (2009)	Human, mouse public datasets used to study overlapping gene expression in asthma, cigarette smoke exposure	4 datasets:2 human, 2 mouse	Affymetrix U133A MOE430A	26 Overlapping genes for expression profiles in asthma and cigarette smoke exposure; 18 genes in lung oxidative stress pathways (Thrombospondin 1, TIMP1 central to gene network)
Kicic et al. (2010)	Study of airway repair mechanisms in asthmatics (compared to atopic subjects and healthy non-atopic controls)	112 subjects	Affymetrix U133A	Slower epithelial cell repair in asthmatics (vs. atopic or healthy controls); differential expression of gene sets for repair/remodeling in asthmatics; fibronectin expression downregulated in asthma
Airway smooth muscle				
Sutcliffe et al. (2012)	Oxidative stress burden evaluated by DNA damage, intracellular ROS; measurements then related to genome-wide expression data in asthmatics and controls	79 subjects	Affymetrix U133A	Asthmatic subjects showed increased oxidative burden in ASM cells and also had higher NOX4 expression vs. controls; blocking NOX4 expression in siRNA knock-down demonstrated abrogated contractility in asthmatic ASM

Alveolar macrophages				
Madore et al. (2010)	Gene expression profiles in allergic asthmatics vs. control subjects	10 subjects	AffymetrixU133A	Of the 50 differentially expressed genes, 19 in stress/immune response pathways, including 9 in the heat shock protein family
Induced sputum				
Baines et al. (2011)	Unsupervised hierarchical clustering of expression data for molecular phenotyping of asthma in adults	59 subjects	Illumina Humanref-8V2	3 Transcriptional asthma phenotypes defined by IL-1,TNF-α, NF-κB pathways; 2 corresponded to neutrophil or eosinophil inflammation Phenotype clusters associated with clinical outcomes (FEV1 and FENO)
Fetal lung tissue				
Melen et al. (2011)	Differential gene expression during fetal lung development	38 subjects	Affymetrix U133 Plus 2.0	No overrepresentation of asthma candidate genes during lung development; asthma GWAS genes differentially expressed during lung development (ROBO1, RORA, HLA-DQB1, IL2RB, and PDE10A)

10.2.1 Immune System Genomic Profiling Studies

The easy access of peripheral blood cells, together with the central role of the immune system, in asthma pathogenesis has motivated multiple genomic studies of cell populations collected by phlebotomy. In theory, studies of specific, homogeneous cell populations (for example, CD4+ lymphocytes, eosinophils, or neutrophils) should provide more readily reproducible biological insights regarding specific pathogenic mechanisms than studies of heterogeneous cell populations (for example, peripheral blood mononuclear cells; PBMCs). However, the latter may be more powerful for genomic classification in clinically motivated studies, for example, when the primary goal is to reclassify patients into molecularly similar subgroups. While this has largely borne true, notable exceptions are evident. For example, Hakonarson and colleagues examined the genomic profiles of 106 glucocorticoid-sensitive and resistant asthmatics using PBMC-derived RNA extracted in a resting state and following in vitro treatment with IL-1 (Hakonarson et al. 2005). A total of 11,812 genes were examined with high-density oligonucleotide microarrays in both resting PBMC (106 patients) and IL-1β/TNF-α stimulated cells treated with or without dexamethasone. More than 5,011 differential expressed genes were detected, of which 923 were reversed by dexamethasone in glucocorticoid responsive patients. A smaller subset of 15 genes classified responders from nonresponders with 84 % accuracy. Technical validation for 11 of these genes was confirmed, with one gene—NFKB—demonstrating predictive accuracy of 81.2 %. Studies in other individual peripheral blood cell types have provided other insights. Tsitsiou and colleagues compared CD4+ and CD8+ T-lymphocyte expression profiles of severe asthmatics vs. controls. They found that compared to CD8+ cells, CD4+ profiles yielded relatively few differentially expressed genes, with the exception of upregulation of the Vitamin D signaling pathway. CD8+ derived profiles showed multiple upregluated genes in severe asthmatics, with enrichment for T-cell activation and inflammation pathways (Tsitsiou et al. 2012). In a transcriptomic study of CD4+ T lymphocytes from mild-to-moderate childhood asthmatics, Hunnighake and colleagues found striking differences between boys and girls in those genes and gene pathways associated with total serum IgE levels (Hunninghake et al. 2011). For example, the gene most strongly correlated with IgE levels—the Interleukin 17 Receptor B (IL17RB)—was only correlated with IgE in boys, with no single gene demonstrating strong correlation in girls. The sets of gene pathways correlated with IgE levels in boys and girls were also nonoverlapping, suggesting distinct molecular mechanisms underlying the noted sexual dimorphism of serum IgE—a critical allergic intermediary phenotype in asthma.

While transcriptional phenotyping efforts aim to describe underlying heterogeneity in asthma cases (which is presumed to be relatively constant), other studies attempt to capture transient changes in gene expression that occur during asthma exacerbations. Asthmatic subjects' gene expression profiles from PBMCs collected during an exacerbation show increased expression in innate immune pathways (TLRs, interferon response genes), adaptive immunity (B-cell and T-cell lymphocyte activation genes), and upregulation of arachidonic acid/prostaglandin pathway

genes as compared to PBMC samples drawn during a quiescent time period (Bjornsdottir et al. 2011; Subrata et al. 2009). PBMC expression profiles during acute asthma exacerbation also show considerable overlap with expression levels from nonasthmatics experiencing upper respiratory infection (Aoki et al. 2009), suggesting that immune pathways activated in response to infection may amplify Th2-mediated responses during asthma exacerbations. An in vitro stimulation experiment of infection-related inflammation (atopic monocytes exposed to IFN-α) demonstrated upregulation of the same atopic pathway genes observed in PBMCs from asthmatic children during exacerbation (Subrata et al. 2009).

10.2.2 The Pulmonary Compartment

Gene expression studies of pulmonary tissues in asthma are equally heterogeneous, ranging from studies of whole lung tissue (from surgical specimens) to studies of bronchial epithelium (derived by endobronchial brushing) to studies of alveolar macrophages from induced sputum. Expectedly, the results from these studies are similarly disparate to those observed in the peripheral compartment. They are also no less revealing. Much attention has focused on a transcriptomic profiling study of bronchial epithelium collected during a clinical trial of inhaled corticosteroid therapies, resulting in the identification of a potential pharmacogenetic biomarker—Periostin (Woodruff et al. 2007). Profiling of airway epithelial brushings obtained from nonsmoking asthmatics ($n=42$) and healthy controls ($n=28$), identified 22 differentially expressed genes, including three genes whose expression reverted to levels similar to those observed in healthy controls following treatment with inhaled corticosteroids: chloride channel, calcium-activated, family member 1 (CLCA1), periostin, and serine peptidase inhibitor, clade B (ovalbumin), and member 2 (serpinB2). In vitro studies confirmed increased expression of these three genes upon cell culture with interleukin-13, a phenomenon also reversed with corticosteroid treatment. A fourth gene, FK506-binding protein 51 (FKBP51), was markedly upregulated in vivo following inhaled corticosteroid treatment, and the expression of all four genes was predictive of clinical corticosteroid response. An independent RNA sequencing study (RNA-Seq) of endobronchial biopsies revealed differential expression of both novel and confirmative asthma-related genes and included upregulation of pendrin, periostin, and downregulation of BCL2 (Yick et al. 2013).

A common observation of many lung compartment studies (of varying cell types) is the induction of oxidative stress response genes in response to relevant exposures, such as mechanical and oxidative stress, among asthmatics compared with healthy subjects. For instance, primary airway smooth muscle cells from asthmatics show a higher burden of oxidative stress (including oxidative stress-induced DNA damage and increased production of reactive oxygen species) and also demonstrate increased expression of NADPH oxidase (NOX) subtype 4, an enzyme which may be involved in airway hypercontractility (Sutcliffe et al. 2012). A systems biology analysis based on the integration of publically available datasets (two mice and two human) revealed 18 oxidative stress genes common to both cigarette-exposed and asthmatic

lung cells, including TIMP1 (tissue inhibitor of metalloproteinase 1) and THBS1 (thrombospondin 1), which were both central to the molecular network constructed using the overlapping transcripts (Freishtat et al. 2009).

10.3 Methods

10.3.1 Tissue Sampling and RNA Isolation

The most important determinants of the quality and reproducibility of genomic data relate to the quality of the input RNA sample, including its purity, integrity, and quantity (Table 10.3); careful consideration of these features is critical during study design and execution. Due the inherent instability of RNA and the ubiquity of RNase enzymes, minimizing RNA degradation is a priority. While many technical issues can be addressed by normalization in the later stages of study (particularly those related to RNA extraction), the choice of tissue type, methods of tissue procurement, and methods of cell isolation must be designed specifically with reference to their impact on RNA quality, as inferiorities introduced in these earliest stages are often not addressable later on. Moreover, the sensitivity of the transcriptome to changes of the cellular environment (including the induction of hypoxia or temperature-related stress responses) mandates that any technical deficiencies that

Table 10.3 Determinants of RNA quality

Feature	Sources of inferiority	Solutions
Purity	Multicellular tissue DNA contamination Organic or inorganic contamination Formaldehyde-fixed paraffin-embedded (FFPE) samples	Tissue microdissection Cell sorting Cell culture Analytical considerations DNase treatment Rigorous protocol adherence/ repeat extraction protocol FFPE-specific extraction procedures and platforms
Integrity	Sampling-related gene expression induction (hypoxia- or temperature-induced stress response, autophagy, and apoptosis) RNA degradation (during extraction) RNA degradation (from multiple freeze–thaw cycles)	Rapid sample preservation, including flash freezing. Modest sample cooling Immediate RNA extraction RNA preservatives Sample storage in multiple aliquots
Quantity	Low cellular yield (small sample size, low percentage of target cell) Low intracellular RNA content (granulocytes) High RNase content (eosinophilic inflammation)	Sample pooling Cell culture and expansion RNA amplification procedures Low yield protocols

arise during sampling and RNA extraction are introduced nondifferentially between study groups (i.e., between cases and controls and treated vs. untreated samples), so as to avoid technical biases that irreversibly confounds data analysis and interpretation. Thus, study design should ensure that all samples are obtained and processed uniformly (Kerr 2003).

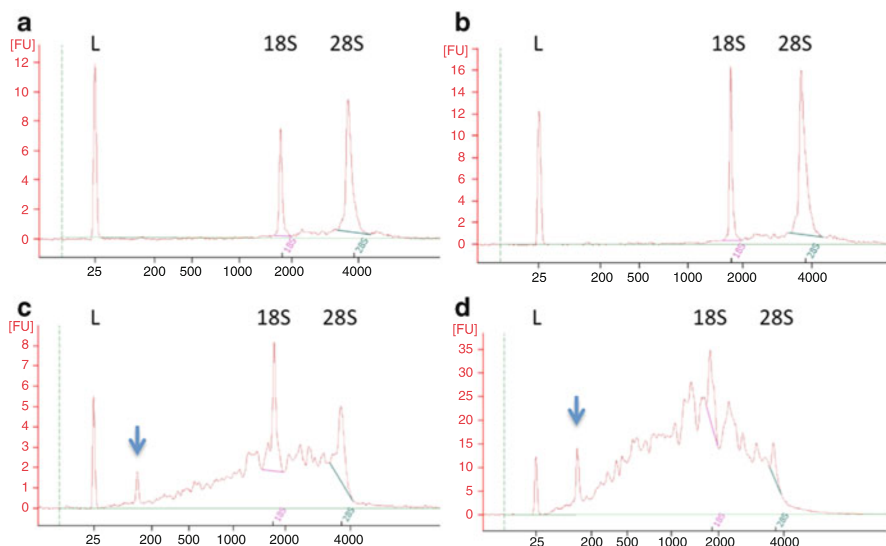
Guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi 1987), a phase separation protocol, is the most commonly used method of RNA extraction. Several extraction kits from Invitrogen (TRIzol), Bioline (Trisure), and Tel-Test (Stat-60) are commercially available that offer high-throughput scalability for large-scale studies and small amounts of starting substrate. Recent modifications include chemistries for isolation of a wider range of RNA moieties, including shorter length microRNAs, without sacrificing yield of larger mRNA populations. Regardless of methods used, the resultant products should undergo rigorous quality assessment. RNA integrity, sizing, and concentration are estimated by either agarose gel (with ethidium bromide) or using microfluidic instrumentation (BioAnalyzer 2100, Agilent Inc.), estimating the ratios of the two ubiquitously expressed 28S and 18S ribosomal protein subunits (see Fig. 10.1). Traditional spectrophotometric analysis (optical density measurement) provides additional information regarding sample purity, with A260:A280 ratios of ~2.0 reflecting an absence of organic contamination. Concentration measurement by fluorescent dye analysis on agarose gel (with ethidium bromide) or by photometer is advised, particularly for low yield samples, though such samples can also be accommodated with the BioAnalyzer using modified (Pico) protocols. Extracted samples should be stored in liquid nitrogen in RNase-free tubing, with division of samples into multiple aliquots to avoid sample degradation from repetitive freeze–thaw cycles.

10.3.2 Sample Profiling (Table 10.4)

Platform Considerations

Oligonucleotide Microarrays

Until very recently, most genome-wide expression profiling was performed using single-channel, hybridization-based oligonucleotide microarray technology (Schena et al. 1995). In this method, RNA samples are converted to fluorescently labeled cDNA by in vitro reverse transcription (IVT) (Rajeevan et al. 2003), generating a pool of targets. This complex pool is subsequently hybridized against a microarray surface densely studded with populations of oligonucleotide DNA sequences, 20–50 bases in length, each of which is complementary to a specific target RNA sequence. These oligonucleotide probes, situated at fixed positions on the microarray slide, bind their complementary targets. Quantitative measures of the fluorescent intensities at each fixed probe site, captured by confocal microscopic



Sample	28S:18S	Concentration	RIN	Interpretation
A	2.1	22 ng/μl	9.9	High quality, modest yield
B	1.8	42 ng/μl	9.9	High quality, high yield
C	0.7	111 ng/μl	6.0	Partial degradation
D	0.3	467 ng/μl	3.1	Extensively degraded

Fig. 10.1 RNA quality assessment: Illustrative examples of BioAnalyzer 2100 RNA assessment analysis from four total RNA samples derived from CD4+ T lymphocytes with plots of fluorescent units (FU) as a function of RNA size (in base pairs). Size standard from ladder (L) correctly situated at 25 bp. Samples A and B represent good quality samples with no degradation and adequate RNA concentrations. Samples C and D are both of poor quality, with evidence of degradation, including accumulation of short RNA products at ~100 bp (*arrows*). Neither sample C nor D is suitable for transcriptome profiling. Note that peaks between 25 and 100 bases are desired in preparations derived from small RNA extraction protocols. Small peaks at 100 bp are often observed following TRIzol or phenol extraction, denoting small ribosome proteins 5S and 5.8S, as well as tRNAs, and do not represent poor quality sample

fluorometry, correspond to the relative abundance of the target RNA in the biological sample. Improvements in chip manufacturing, probe density, and imaging resolution have facilitated development of arrays with more than one million unique features at relatively low cost (<$150 per sample), enabling simultaneous characterization of virtually all known RNA sequences, including numerous splicing isoforms, using relatively small amounts (~100 ng) of starting RNA. Manufacturers have developed a wide range of arrays that assay human and nonhuman model organismal genomes and typically offer a range of chip designs that differ with

Table 10.4 High-throughput gene expression profiling platforms

	Oligonucleotide microarrays		
	Two channel	Single channel	RNA-Seq
Advantages	Low RNA requirement Low array costs More direct comparison of paired samples via competitive hybridization Analytic methods well established	Low RNA requirement Low array costs Wide availability Wide selection of array types/ content Analytic methods well established	Sequence-independent measurement Novel isoform, sequence identification Accommodates both long and short-length RNA species. Detects sequence polymorphism and allelic expression Read-depth measurement of transcript abundance
Disadvantages	More limited availability Lower target content Fixed content Labor intensive (equimolar sample mix) Sequence-dependent hybridization Intensity-based relative measure of transcript abundance	Fixed content Labor intensive (equimolar sample mix) Sequence-dependent hybridization Intensity-based indirect measure of transcript abundance	High RNA requirement High array cost Sensitive, labor-intensive library preparation Intensive bioinformatics support for sequence alignment required Analytic methods less well established Considerable data storage challenges

respect to array content (number of genes, isoforms, and RNA type), as well as the number of samples (arrays) that can be assayed per chip. As a consequence, this technology has been the most widely adopted, resulting in a well-developed understanding among the scientific community of array performance (including each platform's strengths and liabilities) and a comprehensive set of statistical approaches for image processing, sample normalization and quality control, and data analysis (see below). Using standardized approaches, implementing stringent adherence to quality assessment and uniform analysis methodologies, data reproducibility has been demonstrated to be high, both within and across laboratories (Irizarry et al. 2005; Shi et al. 2010).

The earliest oligonucleotide microarray protocols implemented a two-channel competitive hybridization approach, whereby the relative expression of two biological samples are contrasted directly by separate labeling of each with fluorophores of differing fluorescent spectra, hybridizing both to the same array in equimolar concentrations, and measuring their relative intensities by image capture of each fluorescent spectrum (i.e., two channel). There are several limitations of the two-channel approach, most notably the needs for stringent equimolar mixing of two target pools

and for performing technical replicates with dye-swap to avoid dye-dependent bias, as well as a reduced feature (probe) content per array. Though these disadvantages have led to preferential adoption of single-channel protocols for most studies, a natural application of two-channel arrays is in studies of matched, paired samples (for example, within subject comparisons of samples obtained pre- and posttreatment), where the contrast of interest can be assessed directly using individual assays, reducing the potential for cross-array technical bias.

The inherent disadvantage of oligonucleotide microarray expression measurement is the dependency on efficient, unbiased hybridization of target sequence to a predefined, fixed probe set. Due to differences in GC-content and sequence complexity across probe types, hybridization kinetics is not uniform across probe types. Limitations in microarray probe capacity restrict the number of discrete targets that can be assayed (for example, splice isoforms) and also limit the dynamic range of intensity measurement (the upper-limit of which is bound by complete probe saturation). The dependence of efficient hybridization on sequence alignment with features prespecified on the chip precludes novel transcript identification. In addition, DNA polymorphism can differentially impact hybridization between subjects, spuriously generating differences in measured gene expression, even for variants that themselves have no functional impact on RNA transcript abundance (Alberts et al. 2007). Until recently, these limitations were largely not addressable and considered recognized trade-offs for comprehensive, inexpensive genome-wide surveys of expression. However, the development of hybridization-independent, highly parallelized (so-called next-generation) sequencing platforms have largely solved these issues (Schuster 2008).

Next-Generation Sequencing

Over the past half-decade, several platforms have been developed to enable sequencing of oligonucleotide sequence (DNA or RNA) in a highly paralleled fashion, without the need for predefined sequence-dependent hybridization. These include platforms developed by Roche, Illumina, Pacific Biosciences, and Helicos BioSciences, among others. Detailed reviews of these technologies are available elsewhere (Metzker 2010). These methods generate sequence reads of between 30 and 135 bases in length (depending on platform), sampled (fairly) randomly from the target sample. With sufficient sequencing, adequate coverage can be attained to accurately call bases from complete genomes. In addition to quantitative sequence analysis for the detection of genetic variation, sequence data can be analyzed quantitatively, as read-count—the number of times a given base is represented in a random read—correlates with the amount of target sequence starting concentration (Wold and Myers 2008). Unlikely microarrays, where transcript abundance is indirectly quantified by measuring hybridization events, sequencing-based measures represent more direct observations, namely individual transcript reads. As such, next-generation sequencing of RNA (RNA-Seq) represents a powerful tool for comprehensive characterization of transcript abundance at a genome-wide level (Wang et al. 2009), with excellent technical reproducibility (Marioni et al. 2008; Mortazavi

et al. 2008). Freed from the constraints of prespecified probes, RNA-Seq also enables complete enumeration of alternative splicing events and use of alternative transcription start sites, including novel isoform identification. These features make RNA-Seq quite attractive over microarray methods. However, limiting its introduction in most laboratories is the considerable cost, which is currently four to five times that of traditional microarray expression profiling, though it is expected that this will continue to drop to a more competitive price point in the near future. The other critical limitation of RNA-Seq is that regarding sequence preprocessing and downstream analysis, which are considerably more involved, and comparatively less well developed, compared to microarray analysis. Unlike microarrays, where gene identities of each probe are known by their coordinates on the array, the sequences generated from RNA-Seq must first be aligned to a reference genome, then annotated and assigned gene identity. This requires experienced bioinformatics analysis, also adding to the total costs of transcriptome characterization by RNA-Seq. The ability to accurately align sequence is dependent on sequence length (platform dependent), read-depth (which is contingent on the capacity of the platform, the size of the targeted genome, and the number of genomes analyzed per sequence run), and genome complexity. Moreover, the amount of data generated per sequence run (terabytes) is considerably larger than that for microarrays (megabytes), necessitating access to large-scale computing and storage capacity.

RNA-Seq, though powerful, is not without its challenges. Paramount are several recognized sampling biases, and the need for novel analytical strategies to accommodate these issues and perform statistically robust experimental analysis. With regard to sequence bias, the most well understood relate to local sequence complexity, inadequate library preparation, and sampling dependency on gene length. Though a random process, larger genes have greater likelihood of being sequenced than smaller transcripts, introducing gene-specific biases. In addition, the more complex (unique) a particular sequence, the more likely it will be accurately aligned. Similar to microarray-based assessments, target GC content induces systematic differences in read depth (Pickrell et al. 2010). As an additional complication, sequences (genomic regions) are sampled insufficiently if library preparation is inadequate. The process of library preparation demonstrates inter- and intratechnician variability. Thus, like with traditional microarray analysis, it is likely that RNA-Seq is similarly susceptible to technical batch effects, which should be accounted for during study design and analysis (Hansen et al. 2012).

Sample Processing Considerations

Despite substantial advancement in sample processing, labeling chemistries, array synthesis and hybridization protocols, microarray profiling remains highly sensitive to technical artifact, introducing the potential for biased measurement and erroneous data interpretation (Benito et al. 2004; Fare et al. 2003). These biases can be introduced at various stages, during chip manufacturing, sample labeling, hybridization, or image capture. Technician-dependent variance is also frequently observed. Though such issues can be largely overlooked in small studies of a handful of

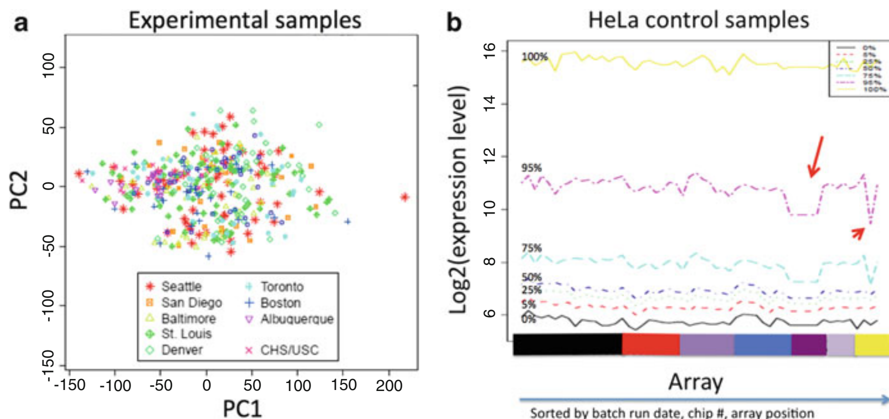


Fig. 10.2 Sample processing strategies: (**a**) Sample randomization for the avoidance of technical bias: dot plot of first two principle components (PC) analysis of whole blood gene expression profiles of 285 samples collected from asthmatic subjects at nine centers across the USA over a 4-year periods. Samples were hybridized and imaged over 6 months on 32 Illumina HumanHT12-v3 BeadChips. The homogenous distribution of samples from disparate sites over the two-dimensional PC space supports an absence of confounding between technical batch and study site, as confirmed by formal statistical testing, illustrating successful sample randomization during processing. (**b**) Longitudinal internal quality control analysis: line plot of global gene expression intensity patterns of a replicate HeLa-cell derived RNA sample hybridized to unique array positions for 32 Illumina HumanHT12-v3 BeadChips over a 6-month period. *Lines* denote 0, 5th, 25th, 50th, 75th, 95th, and 100th percentiles of expression for each sample. Arrays processed together in batches denoted by color coding along abscissa. Note a *purple cluster* of four arrays (*long arrow*) with deviant intensities, suggesting improper processing of one batch of samples that should be considered suspect and candidates for repeat profiling. In contrast, a *yellow cluster* of three arrays processed simultaneously revealed one of three (*short arrow*) with deviant intensities. The 11 experimental samples corresponding to the same chip revealed similar deviations, resulting in removal of these data from consideration and repeat profiling of the samples

samples, processed by one technician over several days, the asthma studies typically conducted in larger patient populations are highly susceptible to these concerns.

While subtle technical variability is both expected and tolerable, it cannot be overstated that, if introduced in a nondifferential way, technical bias is not easily amenable to downstream statistical correction. For example, if samples from asthmatic cases and healthy controls are labeled and hybridized in separate batches, resulting in systematic (technical) differences in global gene expression measurements, no analytical trick can reliably disentangle the true biological expression differences from these so-called technical "batch effects" (Scherer 2009). To avoid this, it is imperative that design strategies aimed at bias prevention and detection be implemented at the outset. Most useful is adherence to a strategy of consistent, repeated sample randomization. At each major processing step (sample extraction, labeling, hybridization, and imaging), samples should be assigned to random batches, irrespective of disease status or other distinguishing clinical characteristics

(treatment group, severity, and gender), so as to minimize the possibility that any relevant covariate is confounded with latent technical artifact. This strategy is particularly useful for studies carried out over many months, where variations in reagent manufacturing, laboratory staffing, or ambient environmental conditions are almost certain (Fig. 10.2a). For such large studies, we also recommend introduction of routine surveillance for reproducibility by randomly selecting samples for repeat testing (to screen for within sample technical variability) and inclusion of standard control samples (for example, a pool of equimolar concentrations of total RNA from all available study sample) that is run with each batch of samples over the course of the study (Fig. 10.2b). Such data can identify outlier batches and potentially be used during preprocessing procedures.

10.3.3 Analytical Considerations

A comprehensive discussion of the many analytical considerations surrounding traniscriptomic analysis is beyond the scope of this chapter, and many detailed, accessible references elegantly explore these issues. Here, we provide a general overview of the basic principles of microarray analysis to orient the inexperienced reader.

Following image processing and data capture, there are four main components of transcriptomic analysis (1) quality control analysis; (2) data preprocessing; (3) feature selection; and (4) experimental analysis. While the last component is of greatest scientific interest, it is entirely dependent on careful execution of the first three.

Quality Control Assessment

Quality control assessment is performed both study wide to screen for systematic bias and for individual samples. Individual arrays with mean intensities >1 SD from the mean should be evaluated as potential outliers and considered for removal. Cluster analysis of genes mapping to the sex chromosomes can be used to identify gender mismatches, suggesting potential sample mixup. Replicate samples should be compared to estimate technical variance.

Preprocessing

Multiple methods have been proposed for sample preprocessing, the most accepted of which include regression based methods, probabilistic models, and multivariate models (including principle components adjustment and surrogate variable analysis modeling). The underlying premise of many regression and probabilistic normalization methods is that, under most circumstances, the majority of genes show either no, or relatively similar, expression across samples. Batch effects can also be modeled using Bayesian (Johnson et al. 2007) or other (Luo et al. 2010) approaches.

Nonparametric procedures, like quantile normalization, scale measures across arrays uniformly, while preserving the rank order of genes (Irizarry et al. 2003). In contrast, multivariate models like PCA and SVA (Leek and Storey 2007) characterize the bulk structure of the data, defining latent variables from the expression data itself that often reflect the effects of either known (for example, differences in genetic ancestry) or unknown technical factors that impact global gene expression patterns. These variables can then be adjusted out during downstream data analysis, provided none are strongly associated with the contrast (i.e., phenotype) of interest. Though described as distinct from the experimental analysis phase, these later normalization methods are often scripted and executed together with statistical inference. Often, these methods can be applied in series, so as to reduce computation time during iterative experimental analyses steps.

Feature Selection

Feature selection refers to the removal from consideration during analysis of subsets of probes with undesirable characteristics, with the goal of reducing the potential for spurious gene detection while preserving experimental sensitivity. Examples of feature selection include filtering of probes that show either no expression, or minimal variance in expression, across the population (there is little utility in formally testing genes whose expression is static), or probes whose sequence aligns to more than one potential gene target. Additional filtering could include those probes that target RNA sequence known to harbor common genetic polymorphism (i.e., SNPs), which would interfere with hybridization and generate spurious association, particularly in integrative genomic studies that consider both expression and genetic variation simultaneously (Murphy et al. 2010). All of these aforementioned filters are nonspecific, in that they are not imposed with reference to the biological question of interest, and thus do not bias statistical inference.

Experimental Analysis

Differential Gene Expression Analysis

The procedures used for experimental data analysis are dictated primarily by the biological question of interest (Table 10.5). Most analyses begin by defining the subset of genes that demonstrate statistically significant fold-differences in expression between cellular states (i.e., asthma vs. no asthma). For studies of dichotomous conditions, standardized *t*-tests, such as those implemented in the RMA procedure (Bolstad et al. 2003), are applied with significance determined using genome-wide thresholds that account for the large number of statistical tests performed. Numerous software packages, including MAS5 (Lim et al. 2007) and limma (Smyth 2005), are available for efficient implementation of these methods. Appropriate statistical models have been developed to accommodate other phenotypes, including continuous or censored phenotypes. The gene lists generated from these analyses is then

Table 10.5 Experimental data analysis procedures

Test procedures	Question of interest	Commonly used methods
Differential expression: *t*-tests, ANOVA, regression	Which genes are differentially expressed in condition X compared to condition Y?	limma, RMA
Gene set enrichment analysis	Are specific pathways/subsets of genes differentially expressed in condition X?	GSEA, GOstats
Network modeling	How are the genes in this condition related to each other?	Coexpression networks, Graphical Gaussian Models
Supervised machine learning	Can I differentiate two or more known cellular states (i.e., cases vs. controls; good vs. poor prognosis) based on gene expression profiling?	Support vector machine, Self-organizing maps
Unsupervised machine learning	Are there subgroups of my disease of which I am not aware?	K-means clustering

examined for biological insight. Though informal gene list interpretation, based on investigators' knowledge, is invariably performed, numerous bioinformatics approaches are available to evaluate the biological significance of observed profiles in a rigorous, statistically motivated framework (Alonzi et al. 2001; Subramanian et al. 2005). These pathway, or gene set enrichment analyses, evaluate whether the observed set of differentially expressed genes are members of specific, predefined gene groups with common biology. Examples of such groupings include membership within specific metabolic pathways, chromosomal locations, similar sequence features, or having similar patterns of expression in response to cellular perturbation. For example, Pietras and colleagues demonstrated enrichment of several previously unrecognized signaling pathways, including the downregulation of N-glycan biosynthesis and the upregulation of the bitter taste transduction signaling pathways in severe asthma (Orsmark-Pietras et al. 2013).

Network Modeling

Though powerful data mining approaches, the ultimate utility of pathway-based analytical approaches are dependent on the quality of the databases queried. Though some gene collections are in near complete form (for example, detailed physical genetic maps), others, including many poorly characterized metabolic pathways, are sparser. Network modeling represents an alternative analytic strategy that attempts to model the biological process under study by developing gene networks using the experimental data itself (Hyduke and Palsson 2010; Vidal et al. 2011). These methods are motivated by the notion that most biological states are determined by the interaction of numerous genes and by the observation that biological systems operate as scale-free networks, displaying a so-called small world property, where any two genes in a network are connected by a small number of links (Barabasi 2009). Using network-modeling approaches, one can define the interrelationship of genes

within the transcriptome and then define the subnetwork of genes that demonstrate greatest change with by experimental state (Chu et al. 2009, 2011; Schafer and Strimmer 2005). Coexpression models define network structure by identifying gene sets with similar expression patterns across disease states, experimental conditions, or temporally. Models that consider additional genomic factors that influence transcriptional regulation, including promoter sequences, chromatin modifications, regulatory genetic polymorphism, and microRNA binding, offer more complete modeling, though are reliant on external data sources. While widely applied in the study of oncology, use of such strategies in asthma to date has been largely restricted to modeling of protein–protein interaction data (Hwang et al. 2008).

Clustering Algorithms and Machine Learning Approaches

Machine learning algorithms represent a broad class of methods that mine multivariate datasets for underlying patterns, with the goal of developing predictive functions (classifiers) that can reliably differentiate samples into specific subgroups (Inza et al. 2010; Larranaga et al. 2006). In gene expression analyses, the premise is that the predictive functions elucidate subgroups that correspond to inherent biological differences between samples. In *supervised machine learning approaches*, the predictive function learns using analyst-predefined labels (for example, case–control status), with the goal of defining gene subsets that can accurately classify samples into their respective subgroups. Similarly, supervised methods can be applied for the classification of expression patterns across samples, to define subsets of genes that follow known patterns of expression (as applied in support vector machine learning). Conversely, in *unsupervised machine learning* approaches, functions are applied with few predetermined notions regarding the underlying data structure, enabling unbiased data mining, with the goal of defining previously unknown sample subgroups with unique biological properties. Numerous clustering algorithms have been developed for these purposes, including both hierarchical and nonhierarchical methods, each with inherent advantages and disadvantages related to underlying assumptions of the data structures, the questions being addressed, and their computational burden. These issues are discussed in detail elsewhere (Kerr et al. 2008). Regardless of method employed, it must be stressed that due to the inherent $p > n$ problem inherent in genomic analysis (where the number of features being tested far exceeds the number of subjects), machine learning approaches are highlight susceptible to model over fitting, resulting in partitioning of data into biologically meaningless, yet statistically robust, subgroups. As such, it is recommended that procedures be implemented at the outset that address this concern, including a priori creation of test and validation datasets and use of both internal and external cross-validation procedures. Only those models that survive stringent validation assessments should be considered viable for further interpretation.

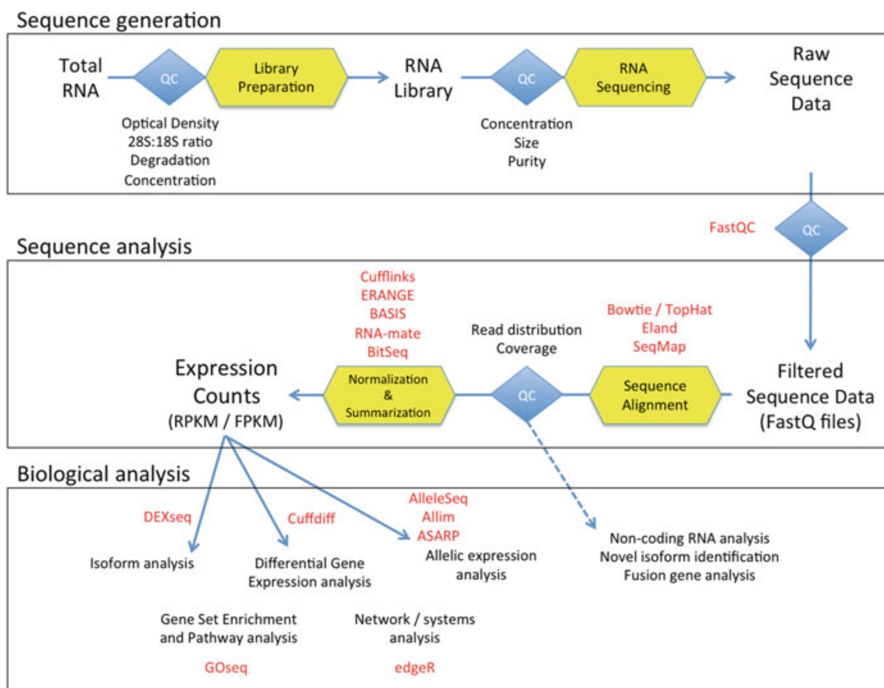


Fig. 10.3 RNA-Seq analysis pipeline: Raw sequence reads are converted to summary measures of transcript abundance through a series of analytic and quality control steps that include filtering of poor quality reads, alignment of reads to reference sequence, gene-based read count normalization, and summarization. Transcript abundance is expressed as either reads or fragments per kilobase exon mapped per megabase sequence (RPKM or FPKM, respectively), metrics normalized by gene length. These counts serve as input for downstream biological inference and interpretation, including traditional differential gene expression analysis, exon-specific (isoform) analysis, or allelic expression analysis, where polymorphic transcript sequence variants are assessed for preferential expression of one allele over the other in heterozygous subjects. Results systems based analyses, including network building, gene set enrichment, or pathways analyses. Exemplars of available RNA-Seq software at each analytic step are indicated in *red*

RNA-Seq Analysis

In broad terms, the analysis of RNA-Seq data is conceptually similar to that of microarray analysis, with a similar framework that includes quality control assessment and preprocessing, with screening for technical covariation, feature selection, and experimental analysis. However, due to the inherent differences in data structures, entirely distinct suites of software are required for RNA-Seq analysis (Fig. 10.3). Data preprocessing includes mapping of reads and alignment to reference genomes, followed by data normalization and summarization, where by aligned reads are translated into more biologically meaningful transcript counts. The number of reads generated for a given transcript is proportional to both the abundance of the transcript and transcript length, as larger transcripts will be

represented by a larger number of random sequence fragments. As such, to avoid systematic biases, normalization techniques must account for differences in gene length. Counts are thus expressed as reads (or fragments) per kilobase exon mapped per megabase sequence. Differential expression testing employs models that consider binomial or Poisson data distributions (in contrast to normal distributions assumed by most microarray-dedicated procedures). Analysis workflows have been packaged for several analytic environments, including the open-source Bioconductor programming environment (Gentleman et al. 2004), Galaxy (Giardine et al. 2005), and MeV (Howe et al. 2011), in addition to several commercial packages.

Postanalysis Considerations

Like all high-throughput, hypothesis-free studies, transcriptomic studies must be considered as hypothesis generating exercises, requiring confirmation, validation, and replication through a variety of means. Individual gene findings deemed of particular relevance should be confirmed by direct technical validation using single-gene based methods, like quantitative reverse transcription PCR. As described above, expression profiles or sample subgrouping derived by machine learning should be validated using both internal and external procedures. External validation of predictive signatures in independently ascertained populations remains the gold standard. However, to date, few studies have successfully met this mark.

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Chapter 11
Asthma Epigenetics

Muhammad T. Salam

Abstract Asthma is the most common chronic disease of childhood, and a growing body of evidence indicates that epigenetic variations may mediate the effects of environmental exposures on the development and natural history of asthma. Epigenetics is the study of mitotically or meiotically heritable changes in gene expression that occur without directly altering the DNA sequence. DNA methylation, histone modifications and miroRNAs are major epigenetic variations in humans that are currently being investigated for asthma etiology and natural history. DNA methylation results from addition of a methyl group to the 5 position of a cytosine ring and occurs almost exclusively on a cytosine in a CpG dinucleotide. Histone modifications involve posttranslational modifications such as acetylation, methylation, phosphorylation and ubiquitination on the tails of core histones. MicroRNAs are short ~22 nucleotide long, non-coding, single-stranded RNAs that binds to complementary sequences in the target mRNAs, usually resulting in gene silencing. While many studies have documented relationships of environmental exposures that have been implicated in asthma etiology with epigenetic alterations, to date, few studies have directly linked epigenetic variations with asthma development. There are several methodological challenges in studying the epigenetics of asthma. In this chapter, the influence of epigenetic variations on asthma pathophysiology, methodological concerns in conducting epigenetic research and future direction of asthma epigenetics research are discussed.

Keywords Epigenetics • DNA methylation • CpG island • Histone acetylation • Histone acetyltransferase (HAT) • Histone deacetylase (HDAC) • Sirtuin1 (SIRT1) • microRNA (miRNA) • Single nucleotide polymorphism (SNP) • Telomerase • CpG pyrosequencing • Chromatin immunoprecipitation-next generation sequencing (ChIP-Seq)

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11.1 Introduction

Epigenetics is the “structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states” (Bird 2007). In simpler terms, it is the study of mitotically or meiotically heritable states of gene expression potential that occur without directly altering the DNA sequence. DNA methylation, histone modifications, and microRNAs are major epigenetic variations in humans that are currently being investigated for asthma etiology and natural history. Furthermore, researchers in the field of environmental epigenetics are interested in evaluating the role of epigenetics as mediating factors that link environmental exposures (e.g., tobacco smoke, air pollution, and dietary factors) to asthma occurrence(Cortessis et al. 2012). In this chapter, our current understanding of the role of epigenetic variation on asthma pathophysiology is described and future direction of asthma epigenetics research is discussed.

11.2 DNA CpG Methylation

DNA methylation refers to the covalent addition of a methyl group to cytosine nucleotides (5-methylcytosine or 5mC) adjacent to guanine residues in the DNA sequence—so-called CpG sites (Fig. 11.1). CpG sites clustered in high frequency near gene promoters are referred to as CpG islands (CGI). In contrast, comparatively low CpG density areas located within 2000bp of traditional CGI are called CpG islands shores (Irizarry et al. 2009). DNA methylation is a nonrandom biological process, mediated by members of the DNA methyltransferases family (DNMTs). While gene promoter DNA hypermethylation is often associated with gene silencing, the effect of hypermethylation of intragenic regions (exons and introns) on gene transcription is complex and less clear.

Although there is a growing interest among researchers in exploring the role of epigenetic variation in asthma etiology (Miller and Ho 2008), only one study has documented the relationship between gene-specific DNA methylation and asthma (Perera et al. 2009). In that study, Perera and colleagues first used umbilical cord blood (UCB) white blood cells and conducted methylation-specific PCR (MS-PCR) to bisulfite-converted DNA. They found that maternal exposure to polyaromatic hydrocarbon (PAH; a constituent found in diet, tobacco smoke and traffic-related pollution) was associated with DNA methylation level in a CpG site in *ACSL3* in UCB white cells. Subsequently, these authors found that DNA methylation level in *ACSL3* was associated with increased asthma risk in children (odds ratio [OR] = 3.9; 95% confidence interval (CI): 1.14–14.3). Using this same study sample, these authors recently reported that subjects with higher benzo[a]pyrene concentration in UCB had higher DNA methylation in IFNγ promoter in UCB white cells and lower expression of IFNγ; however, the authors did not report whether variation in IFNγ promoter was associated with asthma (Tang et al. 2012).

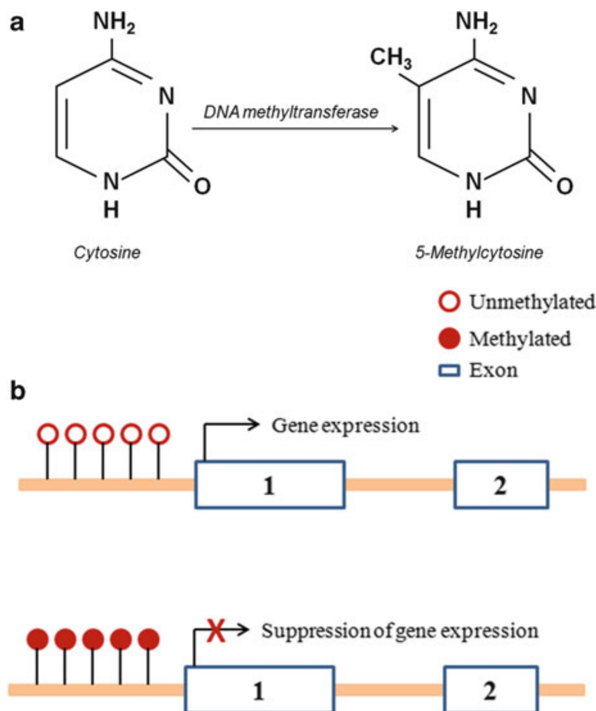

Fig. 11.1 Schematic diagram DNA methylation (**a**) and unmethylated and methylated CpG island in the promoter region of a gene. DNA methyltransferases mediate the conversion of cytosine to 5-methylcytosine by adding addition of a methyl group. Unmethylated CpG (*open red circles*) in the promoter region allows gene to be expressed, whereas methylation of promoter CpGs (*closed red circles*) suppress gene expression

Prenatal exposure to dichlorodiphenyldichloroethylene (DDE) has been associated with increased asthma in children (Sunyer et al. 2005). Morales et al. (2012) examined the role of DNA methylation in mediating this relationship in two cohorts of children. The discovery sample had 122 subjects (17 with persistent wheeze), whereas the replication sample included 236 children (37 with persistent wheeze). Using Illumina GoldenGate Methylation Cancer Panel I array, the authors found 54 CpGs that were differentially methylated by wheeze status; at 4 CpG islands (*ZNF264*, *ALOX12*, *EPO*, and *PDGFB*) the difference was more than 12 % between children with and without persistent wheeze. The methylation levels was lower in children with persistent wheeze at *ZNF264*, *ALOX12*, and higher in *EPO* and *PDGFB* compared to children who never wheezed. *ALOX12* (encoding arachidonate 12-lipoxygenase) belongs to the arachidonic acid metabolic pathway, prompting the investigators to attempt validation and replication of the findings. Technical validation in the discovery sample by Pyrosequencing showed that the Illumina panel overestimated *ALOX15* methylation levels; moreover, the associations with persistent wheeze in the discovery cohort were no longer significant upon repeat testing using the Pyrosequencing-based *ALOX12* methylation level. In the replication sample, however, lower cord-blood DNA methylation levels at two of the four investigated *ALOX12* CpGs was significantly associated with increased risk of persistent wheeze. In the discovery sample, levels of DDE in cord blood (but not

maternal blood samples) were inversely correlated with DNA methylation in one of the *ALOX12* CpGs (labeled as E85). This chapter provides the first epigenetic link between exposure to pesticides and asthma and suggests that *ALOX12* DNA methylation could be an epigenetic biomarker of susceptibility to asthma.

11.3 Histone Modification

DNA is tightly packaged by histone proteins to form a highly organized chromatin structure. The core histones are H2A, H2B, H3, and H4. Two H2A–H2B dimers and one H3–H4 tetramer form the nucleosome (Fig. 11.2a). Histone H1 is not part of the nucleosome but binds the latter to the linker DNA and seals off the entry and exit points of the DNA that wraps the nucleosome. Posttranslational histone modifications, such as acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, and ADP-ribosylation on the tails of core histones, are important epigenetic modifications for gene transcription. Acetylation of histone tails, for example, often results in a relatively loose structure of chromatin, which increases the accessibility of binding sites of transcription factors and thus the activation of gene expression. The status of histone acetylation is reversibly regulated by two distinct enzymes, histone acetyltransferase (HAT) and histone deacetylase (HDAC). Increased histone acetylation by HATs leads to the unwinding of chromatin structure and transcriptional activation, whereas removal of acetyl groups by HDACs causes chromatin condensation and transcriptional silencing (Fig. 11.2b).

Among histone modifications, HDACs are implicated in development and regulation of T lymphocytes, the latter implicated in asthma etiology. In a mouse model, conditional deletion of HDAC1in T-cell lineage resulted in increases eosinophil recruitment into the lung, mucus hypersecretion, parenchymal lung inflammation, and increased airway resistance with parallel increase in Th2 cytokine (IL-4 and IL-13) in HDAC-deficient Th2 cells (Grausenburger et al. 2010), suggesting that HDAC inhibition may result in allergic airway inflammation. Although the role of histone modifications on development of asthma in children has not been published to date, few studies have documented the effect of HDAC inhibitors (trichostatin A and sirtinol) on airway inflammation in humans and in animal models with conflicting results. An in vivo study in a murine model showed that trichostatin A (TSA, a reversible HDAC inhibitor) attenuated allergen-induced airway inflammation and airway hyperresponsiveness (AHR) and reduced IL-4, IL-5, and IgE in bronchoalveolar lavage fluid (BALF) (Choi et al. 2005). Mammalian sirtuin1 (SIRT1) is a class III HDAC that deacetylases several transcription factors (i.e., PPARγ, p53, and NF-κB) and play a role in inflammation and aging (Kim et al. 2010; Yang et al. 2007). In another murine model, ovalbumin (OVA) inhalation resulted in increased SIRT1, hypoxia-inducible factor 1α (HIF-1α), and vascular endothelial growth factor (VEGF) protein levels in lung tissues and increased AHR (Kim et al. 2010). These effects were significantly attenuated by administration of sirtinol (inhibitor of SIRT1).

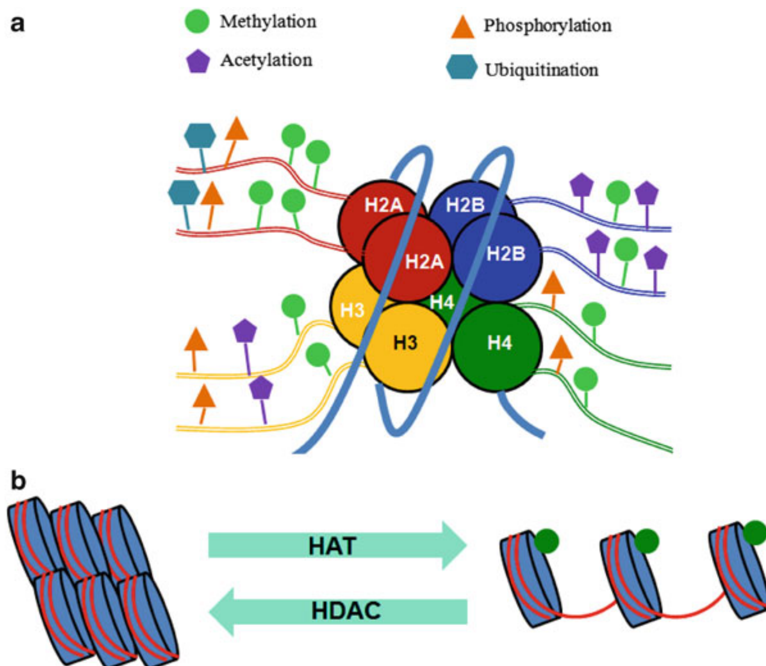

Fig. 11.2 Schematic illustrations of (**a**) a nucleosome with common histone modifications on histone tails and (**b**) function of histone acetyltransferases (HATs) and histone deacetylases (HDACs) o chromatin structure

Contrary to these potential benefits of HDAC inhibitors, other studies showed effects that could be detrimental in subjects with asthma. For example, in a study where human alveolar epithelial cell line (A549 cell) was used, IL-1β induced transcription of transforming growth factor β1 (TGF-β1) and resulted in increased histone H4 and H3 acetylation in distinct promoter regions of TGF-β1 (Lee et al. 2006). Prevention of histone deacetylation by administration of HDAC inhibitor TSA further increased TGF-β1 expression in this study, further supporting the role of histone acetylation in activation of TGF-β1. However, HDAC inhibitor-mediated increase in TGF-β1 concentrations in airway could have detrimental effects in asthma. A large body of evidence indicates that in inflammatory milieu (i.e., in the presence of IL-6 or IL-1β), TGF-β1plays a critical role in the development of pro-inflammatory Th17 cells (Gutcher et al. 2011; Qin et al. 2009). Earlier studies have also shown that the variant -509T allele in a promoter SNP is associated with increase TGF-β1 gene transcription, increased plasma TGF-β1 concentrations, and increased risk of asthma (Salam et al. 2007; Silverman et al. 2004).

Finally, there are conflicting reports on the role of HDACs on steroid-resistant asthma. Earlier studies documented that downregulation in HDAC2 expression is one reason for steroid resistance in asthma (Ito et al. 2006; Li et al. 2010). However,

data from a recent study suggest that downregulation of HDAC1 and HDAC2 expression does not occur in severe asthma and the earlier finding may have resulted due to clathrin cross-reactivity with commercial antibodies (Butler et al. 2012). Based on these contradictory findings, the therapeutic potential of HDAC inhibitors in asthma management remains questionable. Additional studies are needed to fully appreciate the effects of HDAC inhibition in different pathophysiological processes that are involved in asthma.

11.4 MicroRNAs

MicroRNAs (miRNAs) are small (~22 nucleotides), noncoding, single-stranded RNAs that bind to target mRNA through complementary sequences and negatively regulate gene expression at posttranscriptional level. The majority of miRNAs repress gene expression by mRNA destabilization and translational inhibition. The miRNAs are generated from much longer primary miRNA by a multistep process, which is regulated by RNase III endonuclease (Drosha and Dicer). The target sequence of a given miRNA is typically seen in many 3′ UTR sequences, such that one miRNA sequence can regulate the expression of multiple genes in a coordinated fashion (Fig. 11.3).

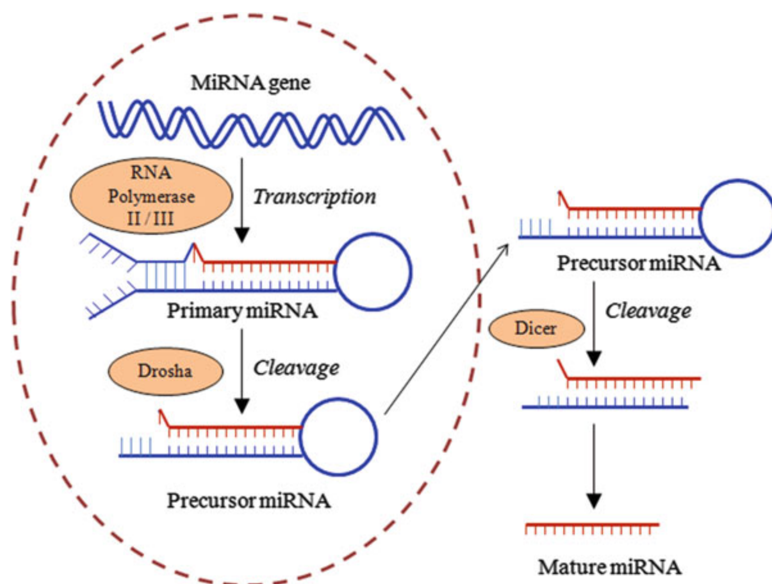

Fig. 11.3 Schematic diagram showing steps of microRNA (miRNA) synthesis

Many miRNAs have been implicated in key pathophysiological asthma processes including immune development and differentiation, AHR, and airway inflammation. While the role of some candidate miRNAs are reviewed here, other candidates implicated in immune development but not yet linked to asthma directly [i.e., miR-9 (Bazzoni et al. 2009), miR-155 (Louafi et al. 2010; Martinez-Nunez et al. 2009; O'Connell et al. 2007, 2010), miR-127 (Xie et al. 2012), miR-147 (Liu et al. 2009), and miR-221 (Mayoral et al. 2011)] are not.

DNA sequence variation in miRNA genes (including the pri- and pre-miRNAs) has been found to influence miRNA function (Duan et al. 2007). Few studies have evaluated the impact of polymorphisms in miRNA target sites on asthma. Following up on the earlier finding that HLA-G as an asthma-susceptibility gene (Nicolae et al. 2005), Tan et al. (2007) found that the +3142C/G SNP (*rs1063320*) in the HLA-G 3′ untranslated region affects binding of miR-148a, miR-148b, and miR-152 to the gene and the SNP interacted with maternal asthma to influence asthma risk in the offspring. The GG genotype was associated with reduced risk of asthma in children born to asthmatic mother, whereas the GG genotype was associated with increased risk of asthma in children born to nonasthmatic mothers. In another study, SNPs in pre-miRNA sequence (miR-146a rs2910164 and miR-149 rs2292832) were associated with a reduced risk of asthma in a Chinese population (Su et al. 2011).

In an experimental study, stretch stimulation of human airway smooth muscle cells (HASMC) induced transcription of miR-26a and resulted in hypertrophic responses in HASMCs (Mohamed et al. 2010). The miR-26a targeted the mRNA 3′ untranslated repeats of glycogen synthase kinase-3β and suppressed GSK-3β protein expression. However, using tissues collected by airway biopsy, no significant difference was detected for miR-26 family and more than 200 other miRNAs (including let-7, miR-125, and miR-30 families) between normal and mild asthmatic subjects (Williams et al. 2009). The investigators also compared miRNAs profiles between airway biopsy samples before and after budesonide treatment; however, no differential expression pattern was revealed.

One of the pathologic hallmarks of asthma is AHR. In animal models of asthma, upregulation of RhoA (a monomeric GTP-binding protein) has been associated with increased contraction of bronchial smooth muscle. Using human bronchial smooth muscle cells (hBSMC), Chiba et al. (2009) found that miR-131a negatively regulated expression of RhoA in BSMs. Downregulation of miR-131a and upregulation of RhoA occurred when hBSMCs were treated with IL-13 and in airway tissues of OVA-challenged mice. Inhibition of miR-131a by treatment with antagomir-133a also resulted in upregulation of RhoA in this study.

Animal models of asthma have implicated several miRNAs. In one study, upregulation of miR-21 was observed in three asthma models (OVA, *A. fumigatus*, and induced IL-13 transgenic mice) compared to control mice (Lu et al. 2009). The authors also found that miR-21 was predominantly expressed in the cytoplasm of cells in monocyte/macrophage lineage with highest expression in dendritic cells in bone marrow. IL-12p35 mRNA was found as the potential target gene of miR-21 and IL-12p35 mRNA was decreased in all three asthma models. Recently,

miR-21 is implicated in allergic rhinitis (AR) in children. In a study where a panel of 157 miRNAs was interrogated using mononuclear leucocytes from human umbilical cord blood, miR-21, miR-96, and miR-126 were significantly downregulated in neonates with high cord blood immunoglobulin E (CBIgE), whereas the expression of miR-21 and miR-126 was significantly lower in children with AR (Chen et al. 2010). The authors found that miR-21 targeted transforming growth factor receptor 2 (TGFBR2) and low miR-21 expression in CB was associated with significantly higher TGFBR2 expression is cord blood leucocytes in children with high CBIgE and those with AR compared to children with low CBIgE and those without AR, respectively. Because rhinitis is a significant risk factor for asthma (Chawes et al. 2010), further studies could examine whether miR-21 expression is associated with asthma risk.

Therapeutic potential of miRNA inhibitors have also been investigated in animal models of asthma. In OVA-sensitized female BALB/c mice, exposure to low levels of OVA resulted in marked increase in miR-126 expression in exposed mice compared to control by 2 weeks; however, the expression was reduced to baseline level by 6 weeks (Collison et al. 2011a, b). Inhibition of miR-126 by administration of an antagomir suppressed eosinophil recruitment into the airways but had no effect on chronic airway inflammation and remodeling. Another study also documented change in miRNA profiles by lipopolysaccharide exposure in male BALB/c without any significant modulating effect of dexamethasone (Moschos et al. 2007). In contrast to these findings, another study documented that exposure to house dust mite (HDM) increased expression of miR-145, miR-21, and let-7b by fivefold in BALB/c mice (Collison et al. 2011a, b). Furthermore, inhibition of miR-145 by using an antagomir reduced eosinophilic inflammation, mucus hypersecretion, Th2 cytokine production, and AHR; however, inhibition of miR-21 or lel-7b had no significant influence on HDM-mediated airway inflammation. The authors concluded that the anti-inflammatory effects of miR-145 were comparable to steroid treatment. The emerging evidence suggests a complex role of miRNAs in mediating the effects of exposure on underlying pathophysiological mechanisms involved in asthma.

11.5 Telomere Length

Telomeres are specialized protein-bound, multiple short DNA repeats that are located at the end of chromosomes (Nandakumar and Cech 2013). They are critically important in chromosomal stability and in the regulation of somatic-cell replication (Allsopp et al. 1992). Telomere shortening may reflect the total number of divisions experienced by a somatic cell and is associated with replicative senescence. Telomere shortening, a marker of biological aging, may represent an additional cellular level biomarker of adversity. While telomerase, a cellular enzyme, extend telomeres in germ cells and stem cells, it is absent in the majority of somatic cells. Therefore, telomere length shortens with each successive cellular division in somatic cells, and increased oxidative stress due to environmental exposures

(e.g., tobacco smoke, and air pollution) can further accelerate telomere shortening (Hou et al. 2012; Hoxha et al. 2009; McGrath et al. 2007; Morla et al. 2006; Valdes et al. 2005; von Zglinicki 2002). In telomerase null mice with short telomeres, exposure to tobacco smoke has been associated with emphysematous changes in the lungs (Alder et al. 2011). In humans, telomere length was found to shorter in subjects with chronic obstructive pulmonary disease (COPD) compared to healthy control in few cross-sectional studies suggesting accelerated aging in these patients (Houben et al. 2009; Savale et al. 2009). However, there has been no published report describing association between telomere length and development or natural history of asthma.

11.6 Assay Methods

In the last few decades, multiple assay techniques have been developed and utilized for characterizing epigenetic variation across including quantitative measures of DNA methylation, microRNA expression, and histone modifications, at a gene or genome-wide level (Bhandare et al. 2010; Laird 2003, 2010; O'Geen et al. 2011; Wei et al. 2012). A review of all such methodologies is beyond the scope of this chapter. Here, we review the most commonly used contemporary methods for studying DNA methylation (i.e., pyrosequencing) and histone modification (i.e., ChIP-seq). The assays for microRNA expression utilize quantitative RT-PCR or commercially available miRNA microarray analysis (Jardim et al. 2012; Levanen et al. 2013; Solberg et al. 2012), protocols similar to the ones used for gene expression, which are reviewed elsewhere in this text.

11.6.1 DNA CpG Pyrosequencing

Pyrosequencing is a real-time DNA sequencing-by-synthesis method in which enzymatic reactions and bioluminescence are used to determine methylation status at one to few CpG sites (Nyren 2007). Initially, bisulfite treatment of the genomic DNA converts unmethylated cytosine to uracil without affecting methylated cytosines. During PCR of the bisulfite-treated DNA, the uracil base is converted to thymine, which results in reading the methylated cytosine sites as virtual C/T polymorphisms. The ratio of cytosine and thymine present at each CpG site is then quantified and reflects the methylation level of that site in genomic DNA.

There could be several major technical issues that might affect measurement of DNA methylation. Insufficient denaturation of DNA may lead to bisulfite conversion failure. In such instances, bisulfite treatment has to be repeated (Tost and Gut 2007). There might be no or very weak PCR products, which may necessitate reoptimizing the assay by reducing extension time or amount of polymerase. If problem persists, then pyrosequencing may not be a suitable method for estimating DNA

methylation. In addition, any problem with annealing and hybridization of the pyrosequencing primer may lead to weak pyrosequencing signal. A redesign of primer and reoptimization of the assay may be required to obtain relatively stronger pyrosequencing signal (Mikeska et al. 2011; Tost and Gut 2007). For some of this problem, this assay may be time consuming and labor intensive.

11.6.2 CpG Genotyping

Although pyrosequencing allows accurate measurement of DNA methylation, one of the major limitations of this method is that only few CpG sites could be analyzed in a single sequencing reaction. Because it is difficult to automate the method to interrogate multiple CpGs at one time, it becomes labor intensive and costly as the number of CpGs investigated in a study increases. Currently, there are many commercial array-based (e.g., Illumina GoldenGate and Infinium assays) and next-generation sequencing-based [e.g., bisulfite sequencing with padlock probes (BSPP) (Deng et al. 2009), reduced representation bisulfite sequencing (RRBS) (Meissner et al. 2005), whole-genome shotgun bisulfite sequencing (WGSBS) (Cokus et al. 2008)] technologies that are available that can measure hundreds of thousands of CpGs in genome-wide scale, albeit the number of CpGs each analysis techniques can measure vary by order of magnitude (Laird 2010). Because these high-throughput technologies have some biases, pyrosequencing of the CpGs that are most significantly associated with an exposure or health outcome of interest remains a standard practice to validate DNA methylation level observed in genome-wide arrays in those CpGs.

11.6.3 ChIP-seq

Chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) allows investigating protein–DNA interactions such as histone modification, transcription factor binding. The principles, guidelines and recommendations for ChIp-seq assay has been recently published based on the work done in the ENCODE and the modENCODE consortia (Landt et al. 2012). The first step of ChIP-seq requires cross-linking proteins to DNA using formaldehyde to stabilize the interaction between protein factors and chromatin (Fig. 11.4). Sonication, cell disruption, or enzymatic digestion (by micrococcal nuclease or MNase) is then used for chromatin fragmentation to obtain 100–300 bp target size for enrichment. The target DNA with its histone mark is then precipitated with specific antibody or epitope tag to purify and enrich the protein (histone mark in this case) (Landt et al. 2012). Once target histone marks are enriched through immunoprecipitation, cross-links are reversed and the DNA sequenced using high-throughput sequencing technologies. Sequence reads are then aligned to reference genome, and “peak calling” within regions of high numbers of aligned reads are performed. There are

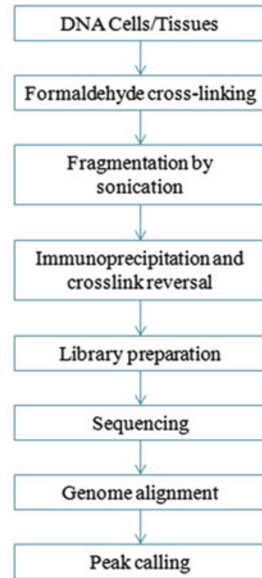

Fig. 11.4 A diagram showing ChIp-seq workflow

many software packages that are available for peak calling, which rely on different statistical models to calculate *p*-values and false discovery rates (Landt et al. 2012; Park 2009). Appropriate thresholds are necessary to detect enriched regions while limiting false discovery rate. Histone modifications may have sharp peaks at regulatory elements and broad peaks at transcribed or repressed regions (Park 2009; Wang et al. 2013).

Although ChIP-seq provides high coverage and high resolution within a reasonable cost per sample, however, there are some of the major constraints of this method. One problem is antibody deficiency that can either cause poor reactivity with the target or cross-reactivity with other DNA-associated proteins (Landt et al. 2012). As much as 25 % of the commercially available histone-modification antibodies were found to have significant problems of antibody specificity (Egelhofer et al. 2011). It is thus recommended to perform primary and secondary tests for antibody characterization and that antibody characterization data should be reported to evaluate the assay method (Landt et al. 2012). For antibody characterization, immunoblot analysis can be used as primary test, while mass spectrometry, peptide binding test, or immunoreactivity analysis in cell lines that contains knockout of specific histone modification could be used as secondary test (Landt et al. 2012).

Potential biases introduced during the sequencing protocol include issues related to DNA amplification (Landt et al. 2012) and sequence alignment. Amplification-related biases can be identified and adjusted for by creating appropriate control libraries using non-ChIP genomic DNA. Mapping of target to genomic region (i.e., sequence alignment) can be challenging, particularly with short tags or for targets in genomic regions with high homology or repetitive sequencing (Park 2009).

As the ChIP-seq technology (including pre- and postprocessing steps) has been evolving very rapidly, the method is poised to be utilized in clinical and epidemiological studies to further our understanding of asthma etiology and natural history.

11.7 Future Directions

Some of the major questions that remain to be addressed in the field of asthma are (1) Do environmental exposures have relatively stable effects on some epigenetic profiles and dynamic effects on other? (2) Do epigenetic changes on individual cell types that have been found to be involved in asthma predicts asthma risk and modulates natural history of asthma? (3) Are there critical developmental windows which modulate exposure-mediated asthma susceptibility? (4) Are there synergistic effects of environmental exposures, and genetic and epigenetic variations on asthma risk and natural history of asthma? (5) Can intervention strategies modulate epigenetic profiles to affect asthma risk and exacerbations in children? and (6) Are there transgenerational effects of exposures that are epigenetically mediated that affect asthma risk? These research questions need to be critically evaluated in future studies to advance our understanding of the role of epigenetics on asthma and to find strategies to reduce the burden of asthma worldwide.

To address these research questions, appropriate study design, data collection, and analytic strategies are needed. First, epigenetic profiles need to be assessed in specific cell types, preferably more than one cell types that are implicated in asthma. With the backdrop of this methodological limitation, studying the role of environment on epigenetic variation and how that relates to future disease risk becomes even more challenging, as a sample collected during susceptible windows (i.e., before disease onset) would be required for etiologic research.

Repeated measure of epigenetic marks from birth to disease onset together with longitudinal evaluation of the effects of environmental exposures on those epigenetic marks could identify whether there are subsets of relatively stable and dynamic epigenetic marks and whether different windows of exposure has differential impact on epigenetic profiles. While the less-modifiable epigenetic marks could serve as biomarker of susceptibility, the modifiable ones could be target for intervention to alter risk profile and/or improve natural history of disease.

There are other methodological concerns that must be addressed. Multiple assay methods are available for measuring DNA methylation that relies on enzymatic digestion, affinity enrichment, and/or bisulfite conversion, discussed in more detail elsewhere (Harris et al. 2010; Laird 2010). Some of the genome-wide approaches for methylation assays are not sensitive to detect DNA methylation with low frequency; however, with next-generation sequencing approaches (albeit more expensive) could be used to detect rare CpGs (Taylor et al. 2007). In terms of assays using common DNA methylation array platforms, genetic variations (SNPs, repeats, deletions, copy number variations, etc.) need to be excluded and background correction and normalization of the data are needed (Adriaens et al. 2012; Johnson et al. 2007; Sabbah et al. 2011).

Once epigenetic biomarkers are identified, technical validation of the most significant loci by another assay (i.e., Pyrosequencing) and replication of the findings in independent samples are needed to assure data quality and to show consistency of findings, respectively. Although the bisulfite-based assay methods are quite accurate, incomplete bisulfite conversion could introduce measurement error in estimation of DNA methylation (Laird 2010). Monitoring of completion of bisulfite conversion is therefore needed (Campan et al. 2009). Linking variation in epigenetic marks to difference in gene expression could provide functional relevance of the epigenetic marks.

Although some animal studies showed transgenerational effects of exposures (diet and obesity) on DNA methylation (Hollingsworth et al. 2008; Waterland et al. 2008), true transgenerational effects of exposure on epigenetic profiles and on asthma have not been reported in humans to date. Although the area is of great interest, some major methodological problems exist. Evaluation of transgenerational effects of exposure on the epigenome requires at least four generations because any exposure to an F_0 female during pregnancy exposes not only the F_1 embryo but also affects germline of the F_2 generation (Perera and Herbstman 2011). In addition, as opposed to controlled animal studies, the exposures in humans could happen postnatally in all subsequent generations and effects of the exposure to paternal sources of chromosomes could further complicate the matter in distinguishing true transgenerational effects from prenatal and postnatal exposure mediated effects on the epigenome.

11.8 Conclusion

Investigation of the etiology and natural history of a complex trait such as asthma in children is difficult. Studying epigenetics as a mediating factor for the associations between exposures and asthma development holds promise to unravel novel biologic pathways. While a good *dose* of optimism will advance the field of asthma epigenetics, too much hype must be avoided as even the robust effect sizes could be small, similar to what has been observed in GWAS. The potentially modifiable nature of the epigenetic profiles may allow us identify intervention approaches to reduce the risk of asthma in children and improve natural history of the disease.

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Part III
Proteomics, Metabolomics, and Systems Biology of Asthma

Chapter 12
Overview

John E. Wiktorowicz

Abstract Although broadly defined in the literature, for the purpose of this section, we define systems biology as the description of the dynamic genomic, proteomic, and metabolomic processes integrated into a functional model of the cell, organelle, or tissue that is capable of accurately tracking the biological system's response to environmental perturbations. The goal of this section is to complete the tripartite description of asthma systems biology, initiated by the previous section (Section II: Genetics and Genomics of Asthma), by reviewing the recent literature—the types and methods of sample collection, processing, analysis, and instrumentation—of metabolomic and proteomic investigations, including functional proteomic studies of the asthma innate immune response and glucocorticoid (GC) receptor signaling with reference to GC resistance in severe asthma.

Keywords Proteomics • Sample prep • Size-exclusion chromatography • Asthma • Airway inflammation • Broncoalveolar lavage • Epithelial lining fluid • Induced sputum • Exhaled breath condensate • Bronchoscopic microsampling

12.1 Introduction

As discussed in this edition and in the supporting literature, asthma is a heterogeneous disease with a complex phenotype that resists clear and absolute classification. To decipher the pattern of symptoms and derive a molecular description of the disease requires multidisciplinary approaches, with an unbiased focus. That is, at a

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basic level, a combination of global analyses spanning genomics, proteomics, and metabolomics may lead to a description of the molecular events that lead to the complex phenotypes collectively defined as “asthma.” The integration of the “omics” observations into a coherent “system” would be described as the “systems biology” of asthma and likely lead to astonishing insights into its etiology.

To more precisely define the scope of our review, in this section we review the current literature and examine the tools used to dissect the proteomics and metabolomics of asthma, with specific application to the innate immune response and steroid resistance and the tools uniquely designed to address the special asthma sample types. Since much has been written regarding these topics, our review covers the most recent literature (since 2009), as well as new technical approaches that may facilitate a better understanding of asthma with respect to these “omics” disciplines.

12.2 Metabolomics of Asthma

Low molecular weight metabolites are the result of biochemical processes dictated by the complement of genes expressed constitutively and as a result of signaling pathways that are activated in response to airway insults. As such they may be indicators of early inflammatory response, detectable from conventional airway sample collection strategies discussed throughout this section. In concert with genomic and proteomic studies, metabolomic investigations may be complementary—completing the tripartite description of asthma as a perturbation of the systems biology of the airway. In addition, metabolomics may serve to guide unique insights into biochemical processes that lead to asthmatic airway inflammation and uniquely suggest therapeutic strategies to lessen the degree of asthmatic morbidity.

The first chapter of this section (Chap. 13), Luxon discusses the clinical rationale for investigating the potential role of low molecular weight metabolites in the diagnosis, treatment, and evaluation of therapeutic efficacy in asthma. In addition, Luxon focuses on a review of the recent literature, a description of sample collection approaches with an eye to their unique application in the study of circulating metabolites as well as volatile compounds that may be exhaled, sample preparation and methods of separation and instrumentation used in their analysis.

12.3 Methods of Sample Preparation for Proteomic Analysis of Airway Samples

As proteins are the drivers of cellular responses to environmental perturbations, they are appropriate targets for the study of the biological processes that lead to asthma morbidity. Moreover, their study may lead to insights into the biology

that complements the other "omics" strategies and may provide new therapeutic targets to diminish or abolish the symptoms that lead to the severe morbidity of the disease.

In Chap. 14, Wiktorowicz and Jamaluddin extend the template provided by the preceding chapter to review the current literature that describes the unbiased proteomic investigations and the approaches used to acquire, analyze, and characterize airway samples from the biofluids of bronchoalveolar lavage, to induced sputum and exhaled breath condensates. Particular focus is given to the proteomics tools and approaches for the sample types that are unique to the airway.

12.4 Measurement of the Innate Immune Response in the Airway

With recognition that asthma is a disease mediated by the innate immune response (IIR) to airway insults, interest in the structure and dynamics of this response has been driven by application of new proteomics technologies and approaches in order to better characterize its molecular components.

In Chap. 15, Brasier and Zhao review the recent literature describing the major molecular players involved in the airway IIR leading to asthma, its coupling to adaptive immunity, the molecular events leading to asthmatic morbidity from allergenic insults and viral exacerbations, and finally review the technology used to quantify and characterize the genes and proteins that constitute or impact the IIR.

12.5 Functional Proteomics for the Detection of Impaired Cellular Response to Glucocorticoids

Glucocorticoids (GC) are a major therapeutic avenue used to limit the inflammatory response in asthma. While most asthmatics respond favorably to this therapy, a small number remain resistant to its benefits. The result is increased exacerbations and higher risk of mortality for these individuals.

In Chap. 16, Pazdrak and Kurosky focus on the role of GC signaling in severe asthma with emphasis on the role of T-lymphocytes, eosinophils, monocytes, neutrophils, mast cells, and smooth muscle cells of the airway. The cell function techniques used to characterize these activities are described in addition to estimation of GC receptor function, and characterization of proteomics of GC resistance—including posttranslational modifications and differential abundance measurements.

Chapter 13
Metabolomics in Asthma

Bruce A. Luxon

Abstract Asthma and airway inflammation are responses to infectious stimuli and the mechanisms of how they are mediated, whether by the innate or adaptive immune response systems, are complex and results in a broad spectrum of possible metabolic products. In principle, a syndrome such as asthma should have a characteristic temporal-spatial metabolic signature indicative of its current state and the constituents that caused it. Generally, the term metabolomics refers to the quantitative analysis of sets of small compounds from biological samples with molecular masses less than 1 kDa so unambiguous identification can be difficult and usually requires sophisticated instrumentation. The practical success of clinical metabolomics will largely hinge on a few key issues such as the ability to capture a readily available biofluid that can be analyzed to identify metabolite biomarkers with the required sensitivity and specificity in a cost-effective manner in a clinical setting. In this chapter, we review the current state of the metabolomics of asthma and airway inflammation with a focus on the different methods and instrumentation being used for the discovery of biomarkers in research and their future translation into the clinic as diagnostic aids for the choice of patient-specific therapies.

13.1 Introduction

As discussed in other chapters (e.g., Brasier and Zhao, Chap. 15), asthma is characterized by episodic airway inflammation coupled with reversible obstruction that is oftentimes linked to specific environmental influences such as

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aero-allergen exposure or respiratory virus infection. Clinicians generally choose therapies for the control of an asthmatic episode based on the patient's symptoms, most of which are primarily due to the airway inflammation. The mechanisms of how asthmatic airway inflammation is mediated, whether by the innate or adaptive immune responses, are complex and involve a number of different cell types and numerous pathways and results in a broad spectrum of metabolic products. The infectious stimuli, in principle, shape the response into a set of symptoms with a characteristic temporal–spatial metabolic profile that could be potentially used as a source of biomarkers or as a spectral "finger print" to identify the stage and trajectory of the episode, which would aid in the choice of therapeutic action.

Thus, being able to determine the degree of inflammation by a readily available and robust laboratory test to identify the presence and relative amounts of biomarkers for inflammation should have a great deal of value in the clinic. Unfortunately, such a system does not currently exist, but there are sophisticated research instrumental analysis methods that have the potential to do so, if they can be modified for use in clinical practice. One candidate Omics technique being intensely studied for this purpose is metabolomics. Metabolomics is interesting to us because metabolism has been scientifically studied for well over two centuries, is well understood, many metabolic pathways have been described in great detail, and a large number of metabolites and their precursors have been chemically identified. Generally, the term metabolomics refers to the quantitative analysis of the full range of metabolites found in a biological specimen. Metabolites are usually (but not always) considered to have molecular mass less than 1 kDa so unambiguous identification can sometimes be challenging. Thus, full detection and identification can be very difficult in practice and many investigations instead have chosen to focus on subsets of metabolites that have well-identified spectral properties that are known to be associated with a specific metabolic state (e.g., a set of symptoms). Others have chosen to use differential multivariate analysis (e.g., by Principle Components Analysis, PCA) to identify spectral patterns that have features characteristic of the specific and instantaneous metabolic state of the donor when the sample was acquired. Though this can be done without identifying each metabolite contributing to the characteristic spectral signature, it has become generally accepted that this should be done in order to validate the biomarker pattern and establish biological context through pathways analysis and other data mining techniques.

Metabolism itself generically covers all the chemical reactions that occur in living organisms and is divided into anabolism, which are the constructive processes where complex molecules are synthesized, and catabolism, those processes that break down large molecules such as proteins into metabolites. Metabolites are thus small molecules, which are the end products of the catabolic digestion of larger precursor molecules. Every protein or other molecule in the body has a metabolic fate such that there is a panel of metabolites that remain after its inevitable degradation, thus in principle, a syndrome such as asthma should have a metabolic signature indicative of its current state and the constituents that caused it.

Metabolomics thus searches for small-molecule metabolite profiles that describe a metabolome, which is the set of all metabolites that characterize the specific state of an organism at a specific point in time. Because different metabolites arise from different processes within specific cells and tissues at a specific time, the metabolome gives us a detailed temporal-spatial description of the current state of the organism, which can then be compared against other states (e.g., the presence or absence of airway inflammation). If we discover a metabolite profile whose signature is diagnostic for a specific physiological state such as early airway inflammation, we call this set of metabolites a panel of biomarkers.

Metabolomics is also attractive because we should be able to rationalize the metabolic signature of an airway inflammatory state according to the regulatory pathways characteristic of that inflammatory state. Metabolic regulation allows organisms to respond to signals and to interact with their environments. Metabolic pathways and reactions are highly regulated to maintain a constant set of cellular conditions in homeostasis. Many diseases, including airway inflammation where a cascade of biochemical events mediates the inflammatory response, are characterized by a disturbance of homeostasis. Inflammatory mediators are generally short lived and are quickly degraded leaving a trail of metabolites, some of which will be potential biomarkers.

The practical success of metabolomics, in asthma specifically and airway inflammation in general, will largely hinge on a few key issues. One is the ability to capture a readily available and reliable biospecimen (e.g., a biofluid) from a patient that can be processed in such a way as to make metabolomic analysis possible. Samples that are currently being used to study the metabolomics of asthma include induced sputum, bronchoalveolar lavage fluid (BAL), blood, urine, and exhaled breath condensate (EBC). Another key issue is the ability to reduce to practice a metabolite instrumental analysis method that can identify metabolite biomarkers with the required sensitivity and specificity in a cost-effective manner in a clinical setting. Several different methods are being actively pursued for this purpose and we discuss them here.

13.2 Current Literature on the Metabolomics of Asthma

At least ten metabolomics review articles have appeared in the literature over the last few years, several focusing on airway diseases, and these have been summarized briefly in Table 13.1. Collectively these contain over 700 (not necessarily unique) references and the number of references in each article is noted in the table for convenience. Adamko et al. (2012) is the most recent as of this writing that focuses specifically on the metabolomics of asthma. Note that the articles that have a download charge were not reviewed for this chapter and are only included in the References and Table 13.1 for completeness. For practical reasons, none of the reviews in the table are discussed in detail here and the reader is encouraged to access them online if interested in the particular subject matter presented therein.

Table 13.1 Recent metabolomics reviews with emphasis and number of references

Year (#Refs)	References	Title	Emphasis
2012 (57)	Adamko DJ, Sykes BD, Rowe BH., Chest. 141(5) (2012 May) 1295–302	The metabolomics of asthma: novel diagnostic potential	Outlines recent work to develop the metabolomic profile of asthma
2012 (124)	Cathcart MP, Love S, Hughes KJ, Vet J. 2012 Mar;191(3):282–91	The application of exhaled breath gas and exhaled breath condensate analysis in the investigation of the lower respiratory tract in veterinary medicine: A review	Discusses EBC analysis in veterinary medicine with potential application in monitoring disease progression and treatment response in animals
2012 ($60)	Snowden S, Dahlén SE, Wheelock CE. Bioanalysis. 2012 Sep;4(18) 2265–90	Application of metabolomics approaches to the study of respiratory diseases	An in-depth discussion of metabolomics for the study of respiratory diseases, including experimental design, choice of clinical material to be collected, and potentially confounding experimental factors as well as particular challenges in the field
2012 (£43)	Zhou B, Xiao JF, Tuli L, Ressom HW. Mol Biosyst. 2012 Feb;8(2):470–81	LC-MS-based metabolomics	Presents a workflow for LC-MS metabolomic analyses for the identification and quantitation of metabolites discussing challenges and solutions in each step of the workflow
2011 (100)	Dunn WB, Broadhurst D, Begley P, Zelena E, Francis-McIntyre S, Anderson N, Brown M, Knowles JD, Halsall A, Haselden JN, Nicholls AW, Wilson ID, Kell DB, Goodacre R; Human Serum Metabolome (HUSERMET) Consortium., Nat Protoc. 2011 Jun 30;6(7):1060–83	Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry	Describes the detailed workflows for long-term and large-scale metabolomic studies involving thousands of human samples with data acquired for multiple analytical batches over many months and years. Protocols for serum- and plasma-based metabolic profiling by GC-MS and UPLC-MS are described including biofluid collection, sample preparation, data acquisition, data preprocessing, and quality assurance
2011 (38)	Sofia M, Maniscalco M, de Laurentiis G, Paris D, Melck D, Motta A., J Biomed Biotechnol. 2011 2011:403260	Exploring airway diseases by NMR-based metabonomics: a review of application to exhaled breath condensate	Reviews the use of EBC as a matrix for NMR metabonomic studies in airway diseases, including separating specific EBC profiles with implications for disease phenotyping and personalized therapy

2010 (£43)	Junot C, Madalinski G, Tabet JC, Ezan E., Analyst. 2010 Sep;135(9) 2203–19	Fourier transform mass spectrometry for metabolome analysis	FT/MS provides accurate mass measurements with ppm to sub-ppm errors and high to ultra-high resolving power. Evaluates the improvements to metabolomics by different types of FT/MS instruments including various analytical requirements, e.g., global metabolite profiling, absolute quantification and structural characterization, and data preprocessing
2010 (79)	Want EJ, Wilson ID, Gika H, Theodoridis G, Plumb RS, Shockcor J, Holmes E, Nicholson JK, Nat Protoc. 2010 Jun;5(6) 1005–18	Global metabolic profiling procedures for urine using UPLC-MS	Describes UPLC-MS for urinary metabolite profiling, including sample preparation, stability/storage and the selection of chromatographic conditions to balance metabolome coverage, chromatographic resolution and throughput. Discusses QC, metabolite identification, and multivariate data analysis
2007 (145)	Dettmer K, Aronov PA, and Hammock BD, Mass Spectrom Rev.; 26(1) (2007) 51–78	Mass spectrometry-based metabolomics	Describes sample preparation, separation, and MS analysis with examples for metabolic fingerprinting, which requires the analyses of all detectable analytes in a sample
2007 (164)	Metz TO, Zhang Q, Page JS, Shen Y, Callister SJ, Jacobs JM, and Smith RD, Biomark Med. 2007 June ; 1(1): 159–185	The future of liquid chromatography-mass spectrometry (LC-MS) in metabolic profiling and metabolomic studies for biomarker discovery	Describes NMR, GC-MS, and LC-MS metabolomics for biomarker discovery with emphasis on LC separations and ESI approaches with their advantages and disadvantages

Amounts in $US or £UK in parenthesis indicate an article which must be purchased and the cost

13.3 Sample Collection and Preparation Methods

Most of the review articles in Table 13.1 have excellent sections on sample collection so this is not discussed here with the exception that due to the increasing interest and potential importance of EBCs to the metabolomics of airway inflammation field, it is worthwhile to discuss it in more detail here.

13.3.1 Exhaled Breath Condensates

Exhaled air contains mixtures of gases, volatile organic compounds (VOCs), nitric oxide (NO), and nonvolatiles such as peptides and cytokines, so differential metabolomic profiling of EBCs is emerging as an increasingly useful method for the determination of airway disease state. The collection of EBC samples is noninvasive, reproducible, and well tolerated by patients. EBC samples are collected by having patients exhale through a mouthpiece where the breath is then cooled resulting in a condensed fluid sample. Further processing may be necessary depending upon the instrumental method of choice. Details of EBC sample collection and preparation, especially for Gas Chromotography-Mass Spectrometry (GC-MS) and Nuclear Magnetic Resonance (NMR), are included in several reviews and articles referenced here and references therein (Sofia et al. 2011; Fens et al. 2009; Loukides et al. 2011; Carraro et al. 2007). Results of EBC analyses are very encouraging and in an early proof of principle study, Carraro et al. (2007) demonstrated that EBC analysis by NMR can provide airway metabolomic fingerprints of low molecular weight (MW) analytes in EBCs that are able to successfully discriminate between asthmatic and healthy children. Recently, the NMR of EBCs has also been shown to discriminate between patients with stable vs. unstable cystic fibrosis (Montuschi et al. 2012). Virtually all these studies have been done at very low statistical power and the use of NMR for the accurate and reproducible determination of the constituent compounds in EBCs is not without some controversy (Izquierdo-García et al. 2011; Motta et al. 2012; Sinha et al. 2011) and this is discussed later. EBCs are clearly very encouraging for use as a biological fluid for metabolomic studies of airway inflammation and asthma. Its use beyond that of a research medium has been hindered by the lack of standardized protocols and reference values but that effort is gathering momentum as interest expands (Loukides et al. 2011).

13.3.2 Sample Preparation

Sample preparation techniques are generally extensible across the range of instrumentation used in metabolomic studies and are chosen as much for the sample type and the type of metabolites to be observed as anything else. A typical biospecimen is a complex mixture of cells, large and small molecules, and salts common to biological fluids, so the successful extraction of metabolites from one is challenging. It

is, however, clearly one of the most crucial steps in the process of conducting a metabolomics experiment. This is particularly true for samples common to asthma studies such as BALs and EBCs which can contain many low abundance metabolites and VOCs. Important metabolite physicochemical characteristics include volatility, hydrophilicity, lipophilicity, polarity, thermal stability, the presence of active hydrogens in functional groups and lability. Thus, choosing the proper sample preparation technique to be used is exceedingly important for achieving high quality and reproducible quantitative results from the instrument. The procedure chosen must be appropriate for both the sample and the instrument and requires a skilled practitioner for the results to be reproducible. This is especially important for low abundance and chemically labile metabolites. Most metabolomics journal articles give reasonably good details of their sample preparation with references and it behooves any investigator to follow the literature in this regard as the field moves forward rapidly. A general review of sample preparation methods and best practices is well beyond the scope of this article and there are several recent and thorough reviews and articles of metabolomics with good discussions of sample preparation (see refs in Table 13.1 and Fens et al. 2009; Loukides et al. 2011; Carraro et al. 2007).

13.3.3 Separation Methods

Liquid chromatography (LC) and gas chromatography (GC) are commonly used methods to serially resolve complex mixtures of compounds commonly found in biological samples into either very small packets of coeluting molecules with similar physicochemical properties, or ideally, as discrete molecules in order to reduce spectral complexity and background noise and to improve detection limits and data quality. It is the mobile phase which is either a liquid or gas and both use capillary columns with varying stationary phases depending on the physicochemical properties of the metabolites of interest. In the case of NMR studies, the chromatographic separation is done offline, but for MS studies it is done in-line with the spectrometer. Besides general high-performance liquid chromatography (HPLC), LC variations increasingly being used in metabolomics studies include reverse phase LC (RPC), which typically uses narrow bore columns with 3–5 μm particle sizes, and ultra performance LC (UPLC) which uses very small, sub-2 μm particle sizes but requires specialized, very high-pressure UPLC equipment (Want et al. 2010; Dettmer et al. 2007). Capillary electrophoresis (CE) is an excellent method for the high-resolution separation of charged metabolites and CEMS should also see more use in metabolomic studies (Dettmer et al. 2007). These methods are almost always performed inline with the mass spectrometer. There are numerous variations in use or emerging in LC and GC practice and one should consult any of the many excellent texts or reviews (Dettmer et al. 2007) on the subject for details, much of which is readily available online.

GC-MS is widely used in metabolomics studies because it is an excellent method for the separation, detection, and identification of small compounds and especially for VOCs. The technique is rapid and the instrumentation has become well suited

for clinical use. GC-MS for urine testing is widely used in forensics, for the detection of illegal or performance enhancing drug use, and increasingly in medicine for the detection of congenital metabolic diseases. This makes it very suitable for airway inflammation studies because small molecules are often found in BAL and urine samples and VOCs are commonly found in EBC samples (Dettmer et al. 2007). Metabolic activity can also be measured using GC-MS by isotopically labeling metabolic compounds then calculating the isotopic ratios (e.g., ^{12}C–^{13}C).

13.4 Instrumental Methods

There are two major instrumental methods being actively pursed as a means of performing metabolite measurements suitable for asthma samples that have the requisite sensitivity and specificity to be useful. These are nuclear magnetic resonance spectrometry (NMR) and mass spectrometry (MS). MS can be further subdivided according to the applied molecular separation technique used as being either LC-MS or GC-MS or Capillary Electrophoresis/Mass Spectrometry (CE-MS) (Dettmer et al. 2007). Each has its advantages and disadvantages and no single one of them is appropriate under all sample types or conditions. Currently, all of them are performed on extremely complex, sophisticated, and expensive research instruments that require highly trained experts to run them and skilled bioinformaticists to analyze the results. A third, emerging nanosensor technique, which has promise to be very cost-effective and suitable for clinical use is the eNose (Adamko et al. 2012; Fens et al. 2009; Dutta et al. 2002; Ideo 2012; The eNose Company 2012).

A commonality across all these methods is that due to the broad range of chemical species represented by even a modest set of naturally occurring metabolites that might be found in an analytical sample, the experimentalist is presented with an equally broad range of chemical and physical properties that can be exceedingly challenging to approach from an analytical chemistry standpoint. The requirement for metabolite identification and quantification across orders of magnitude differences in abundance increases these challenges considerably. Many metabolites are either naturally labile to oxidation, hydrolysis, or other chemical transmutation or become so under the conditions of the instrumental analysis. Thus, samples must be handled appropriately as soon as they are obtained and much care must be taken in the sample preparation phase regardless of the spectroscopic method of choice. This takes both skill and experience and inadvertently mishandling a sample often becomes an insidious source of indeterminate error.

13.4.1 Nuclear Magnetic Resonance Spectrometry

NMR spectrometry is a research method that exploits the behavior of certain atomic nuclei in a strong magnetic field when hit by a high-frequency pulse of radio waves. For the purposes of metabolomics, the primary nucleus of interest is usually the

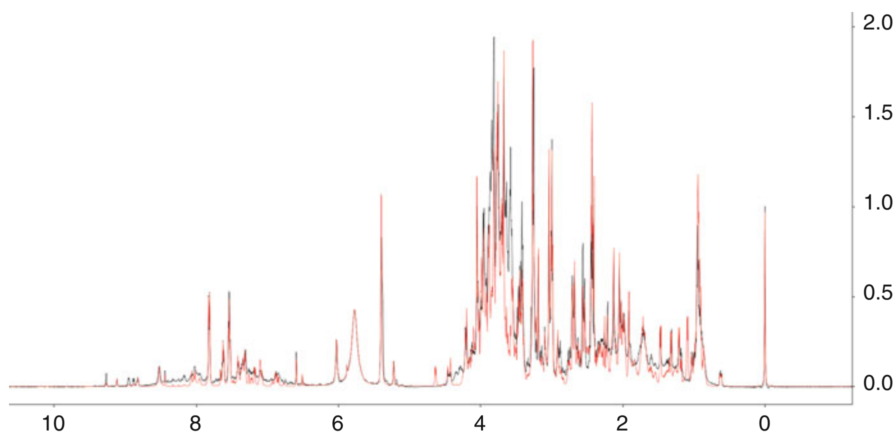

Fig. 13.1 1D proton NMR spectrum. Rat urine *metabolomics* NMR sample showing the original spectrum (*black*) and the computer-generated deconvoluted spectrum (*red*)

hydrogen-1 proton (^{1}H), but carbon-13 (^{13}C) and nitrogen-15 (^{15}N) nuclei are also used. Depending on the chemical structure of a molecule, these nuclei will be arranged by covalent bonds into characteristic spatial patterns that are to varying degrees unique. This spatial organization of the NMR nuclei gives rise to a characteristic spectral pattern, i.e., spectrum for each molecule. NMR spectra for metabolomics studies are generally always taken on samples in solution, which contain from several to many different molecules, each of which has its own characteristic spectral pattern. The resulting NMR spectra of a solution sample will therefore be comprised of the superposition of the unique molecular spectral patterns for each molecule contained in the sample. Thus, each sample spectrum is characteristic of the molecules contained within the sample.

Once you have the specimen's experimentally derived NMR spectrum, the challenge is to deconvolute the composite spectrum into its constituent molecular signature spectra. This is done using knowledge base driven software such as Chenomx NMR Suite (Chenomx Inc. 2012) to identify and quantify the small molecule content of the sample. It does this by searching its database of small molecule metabolite spectra to find one that overlays to a portion of the sample spectrum. It continues to do this until it has accounted for the entire spectrum. The software then superimposes the metabolite spectra from the database onto the experimental spectrum (Fig. 13.1) then produces a list of potential metabolites in the sample solution and their relative concentrations. In practice, this works well if the metabolites within your sample are represented in the spectral knowledge base. You also have the option of running stock samples of specific metabolites yourself and adding these spectra to the knowledge base. Completely unknown metabolites can be challenging, however, and can take some time, skill and effort to resolve. There are also

other software packages available and many of them, including ones for mass spectrometry metabolomics, are described on the Metabolomics Society's Metabolomics Software and Servers webpage (Metabolomics Society 2012).

NMR is being used with increasing success in the determination of asthma and airway inflammation metabolomics. In Adamko et al. (2012), they reviewed several such studies, all preliminary, but highly supportive that the use of NMR in metabolomics studies warrants further investigation. One study they reviewed showed that the NMR analysis of EBCs could discriminate between children with or without asthma (Carraro et al. 2007). Another study used urine samples to discriminate between groups of children with stable asthma vs. those with unstable asthma that were seen in the emergency department (Saude et al. 2011). Future studies will require a significantly larger number of subjects to be statistically conclusive, but these results are very promising.

13.4.2 NMR Studies on Exhaled Breath Condensates

As mentioned earlier, there has been some controversy over the use of NMR for the analysis of EBC samples due to suspected contamination and the inherent insensitivity of NMR. In a 2011 study by Izquierdo-Garcia et al., they concluded that previous studies, which used an EBC sample collection apparatus with reusable condenser parts, showed artificial peaks in the spectrum. It was concluded that the primary source of these peaks was from contamination by residual disinfectant used in cleaning the apparatus. The removal of this contamination appeared to be refractory to standard cleaning protocols. This produced an artificial metabolic fingerprint not related to the endogenous metabolic pathway of the lungs. Furthermore, they posited that because NMR does not have the sensitivity "required to observe the endogenous metabolites presented in the EBC," it should not be used to analyze EBC samples. Based on their study, they then proposed that UPLC-MS and nonreusable collection devices be used as a standard method for metabolomic studies of EBC.

The controversy in their statements arose because in an earlier 2007 study, Carraro et al. were able to clearly discriminate between children with asthma vs. healthy children with a success rate of approximately 86 % using selected signals from EBC NMR spectra using a different reusable sample collection device than the 2011 study (Izquierdo-García et al. 2011).

Furthermore, a 2011 study by Sinha et al., subsequent to the 2011 Izquierdo-Garcia et al. study, used disposable EBC collection tubes to demonstrate reproducible and useful metabolomic fingerprinting using NMR on EBC samples that was able to distinguish clearly between asthmatic and normal patients. They stated in conclusion that their study removed the need for further debate and that the suitability of NMR for the metabolomic analysis of EBC samples was firmly established.

In a very recent study, Motta et al. (2012) assessed the effects of a cleaning procedure, different from that used in the 2011 Izquierdo-Garcia et al. study, on a reusable-part condenser on EBC metabolomics. They determined that when the

same collection device is used repeatedly and using Milton (Milton Pharmaceutical UK Ltd, Gloucester, UK), a pure sodium hypochlorite solution cleaning product, the metabolic profiles of EBC were not altered. Furthermore, to appraise the ability of NMR spectroscopy on EBC samples to discriminate between chronic obstructive pulmonary disease (COPD) patients and healthy subjects, they collected 54 EBC samples from 27 patients with COPD (16 males and 11 females) and 27 HS (healthy subjects; 16 males and 11 females) using an EcoScreen condenser. Using principal components analysis and partial least squares discriminant analysis, NMR-based metabolomics of EBC successfully and decisively discriminated between COPD patients and HS with $r^2 = 99.9\ \%$. They concluded that EBC NMR is suitable and useful for characterizing the metabolic fingerprints of patients with respiratory diseases. They also proposed that the combination of several different techniques, including NMR and MS, might establish "breathomics" as a new noninvasive approach for the assessment of patients with respiratory diseases.

Considering these findings, it seems clear that technique and procedure are exceedingly important in the study of EBCs using NMR or any other method. Thus, NMR has many advantages as a metabolomics method, but there are several drawbacks which require serious consideration as well.

13.4.3 NMR Advantages

NMR samples are relatively fast and easy to run if you have an experienced NMR spectroscopist on your team and access to a modern high-field NMR instrument in the range of 400–800 MHz. Cost of the sample run is relatively low with the single largest material expense being the NMR tube, which is less than $20. It is possible for experienced operators to run 60+ samples in a day.

NMR actually collects the response of each single proton on each molecule in the sample so NMR is inherently highly quantitative. Thus, it is fairly easy to determine relative metabolite concentrations within a sample and absolute concentration quantitation can be improved by spiking samples with a known concentration of a standard compound.

13.4.4 NMR Disadvantages

NMR is an inherently insensitive technique and requires fairly high concentrations of each molecule in the solution to adequately collect a high-quality spectrum across all the metabolites present. In practice, metabolite concentrations in a sample range from fairly high to vanishingly low so NMR will generally find the mid- to high-level metabolites, but the low-level metabolites will be lost in the baseline. If your metabolites of interest are reasonably highly abundant, then you will do well but if they are not you will not see them and need to use an alternate method such as MS.

High-field NMR instruments and highly trained and skilled NMR spectroscopists are not trivial assets and are exceedingly expensive to acquire and sustain so you will have to have access to an established NMR facility. Unless you have both of these your experiment will not be successful. The hourly cost for the use of an NMR instrument varies widely depending on the facility and whether it is a commercial facility or an academic facility. There are commercial services that will run and analyze your samples for you, but these change frequently so they are not referenced here and can be found fairly easily by searching the internet.

13.5 Mass Spectrometry

Mass spectrometry (MS) is an enabling technology for the high-resolution investigation of extremely small samples and for low-abundance molecules. Experimentally, MS measures the mass-to-charge ratio of charged molecular ions or their fragments in order to determine their chemical structures and identities by using a software search engine and database tables containing the spectra of known compounds. In metabolomics, the molecules of interest are usually (but not always) small, typically <1 kDa, and include a wide variety of chemical species.

A mass spectrometer is technically a highly sophisticated, complex, and expensive instrument. It works by ionizing chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios (*m*/*z*) using magnetic and electrical fields in a high vacuum. MS instruments consist of three modules:

1. An ion source to convert gas phase molecules into molecular ions.
2. An analyzer, which uses electromagnetic fields to sort the resulting ions according to their mass and charge.
3. A detector, which provides data for quantitating each ion present.

The resulting signals comprise the mass spectrum, which is typically a graph in which the mass to charge *m*/*z* ratio increases along the abscissa (horizontal axis) and the relative abundance or intensity increases on the ordinate (vertical axis). Oftentimes, the *m*/*z* vs. intensity pattern or "fingerprint" is diagnostic for a specific metabolite but not always and generally needs to be verified, especially for the identification of biomarkers.

13.5.1 MS Advantages

The primary advantage of mass spectrometry in metabolomics is the ability to measure accurately and quantitatively low-abundance metabolites. With recent advances in mass spectrometer technology, this already impressive capability is improving substantially with Fourier transform MS (FT-MS) and FT ion cyclotron resonance MS (FT-ICR-MS), which achieves both high resolution and mass accuracy. 2D

FT-ICR MS is also in development. With these advances, the future of high-resolution MS-based metabolomics is very bright.

13.5.2 MS Disadvantages

The main disadvantages of mass spectrometry are similar to NMR. MS requires very expensive, highly sophisticated instruments that require highly skilled and well-trained operators to run the experiments. MS can be very time-consuming and experiments take substantial preparation. Instrumental results are challenging to analyze and require substantial computational and bioinformatics support. Data management concerns and computational demands can also be significant. Taken altogether, the effort and cost of MS experiments must be carefully considered, but when properly carried out, the results will be worth the time and expense.

13.6 eNose

The electronic nose (eNose) is emerging as having the potential for practical clinical use. It is a portable device that uses an array of composite nanosensors with embedded algorithms to analyze the different fractions of the VOC mixture in exhaled breath to identify a pattern that is diagnostic for the disease phenotype. Both Adamko et al. (2012) and Fens et al. (2009) discuss the use of the eNose in asthma and airway inflammation in more detail. In Fens et al., they used the Cyranose 320 eNose (Dutta et al. 2002; Ideo 2012) (Smiths Detection, Pasadena, CA, USA) to differentiate COPD from asthma subjects and was consistent with their GC-MS findings. An eNose is also being developed by The eNose Company, a small R&D company in the Netherlands (2012). Medically relevant areas of application include the detection of metabolites for medical diagnostics applications such as tuberculosis, asthma, diabetes, and head and neck cancer among many others. If successful in clinical trials, the eNose has the potential to provide an appropriately scaled, simple to use, and highly cost-effective clinical diagnostic aid.

13.7 Conclusions and Projections

Metabolomics studies of asthma and airway inflammation and associated diseases hold tremendous promise. While currently confined to the laboratory, with the substantial amount of talent and resources being increasingly focused on the field, it is virtually inevitable that metabolomics will have significant future impact in the clinic (Adamko et al. 2012; Atzei et al. 2011) as a component of systems and translational medicine. It has the potential to considerably enhance clinical chemistry diagnostics in this area as well as other pathologies.

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Chapter 14
Proteomic Analysis of the Asthmatic Airway

John E. Wiktorowicz and Mohammad Jamaluddin

Abstract Proteomic investigations in general utilize varied technologies for sample preparation, separations, quantification, protein identification, and biological rationalization. Their applications range from pure discovery and mechanistic studies to biomarker discovery/verification/validation. In each specific case, the analytical strategy to be implemented is tailored to the type of sample that serves as the target of the investigations. Proteomic investigations take into consideration sample complexity, the cellular heterogeneity (particularly from tissues), the potential dynamic range of the protein and peptide abundance within the sample, the likelihood of posttranslational modifications (PTM), and other important factors that might influence the final output of the study. We describe the sample types typically used for proteomic investigations into the biology of asthma and review the most recent related publications with special attention to those that deal with the unique airway samples such as bronchoalveolar lavage fluids (BALF), epithelial lining fluid and cells (ELF), induced sputum (IS), and exhaled breath condensate (EBC). Finally, we describe the newest proteomics approaches to sample preparation of the unique airway samples, BALF and IS.

Keywords Proteomics • Sample prep • Size-exclusion chromatography • Asthma • Airway inflammation • Bronchoalveolar lavage • Epithelial lining fluid • Induced sputum • Exhaled breath condensate • Bronchoscopic microsampling

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14.1 Introduction

Proteomic investigations utilize many technologies for sample preparation, separations, quantification, protein identification, and biological rationalization. Their applications range from pure discovery and mechanistic studies to biomarker discovery/verification/validation. In each case, the analytical strategy to be implemented is tailored to the type of sample that serves as the source of the investigations. In general, these may include tissues, cells (primary or continuous), or biofluids. Proteomic investigations take into consideration sample complexity, the cellular heterogeneity (particularly from tissues), the potential dynamic range of the protein and peptide abundance within the sample, the likelihood of posttranslational modifications (PTM), and other important factors that might influence the final output of the study.

Interestingly, proteomic samples types derived from the human airway encompass the entire range of sample types, from natural or derived biofluids such as induced sputum and bronchoalveolar lavage (BALF), respectively; to tissues such as bronchial biopsies; and primary cells such as airway epithelial, alveolar macrophages, and dendritic cells by sampling the epithelial lining fluid (ELF) or recovered from BALF. As a consequence, a review of proteomic technologies applied to airway investigations serves as a comprehensive survey of proteomic technologies in general.

Although the temptation is great to create a complete catalog of all the technologies historically applied to airway investigations, many recent reviews (>2009) address these issues to various degrees (Holguin and Fitzpatrick 2010; Lin et al. 2010; O'Neil et al. 2011a, b; Park and Rhim 2011; Wiktorowicz et al. 2011). We focus on the most recent examples of airway proteomic investigations and refer the reader to other reviews where appropriate. With regard to the most recent publications (>2009) of application of proteomics to asthma, studies are spread evenly between animals models (Haenen et al. 2010; Nagai et al. 2011; Quesada Calvo et al. 2011; Xu et al. 2010; Zhang et al. 2009), human patients (Cederfur et al. 2012; Fitzpatrick et al. 2009a, b; Gharib et al. 2011; O'Neil et al. 2011a, b), and tissue culture (Ali et al. 2011; Altraja et al. 2009, 2010; Cao et al. 2012; Kroeker et al. 2012), primary or continuous.

Airway sampling techniques include those that directly sample the mucosal surface, such as bronchial biopsy or bronchoscopic microsampling (BM) by brush biopsy, or those that indirectly sample the surface, such as bronchoalveolar lavage (BALF), induced sputum (IS), or exhaled breath condensate (EBC).

14.2 Sample Types, Collection, and Studies

14.2.1 Bronchoscopic Microsampling

Bronchial biopsy is an invasive procedure performed to collect a small amount of tissue from a specific region of the lung. This can include excising a tissue sample, or scraping epithelial or other cells that line the airway surfaces by brush biopsy.

In general, a trained pulmonologist performs this procedure using a bronchoscope that is threaded through right or left nostril into the trachea and bronchi of the anesthetized subjects. A small forceps is passed through the channel of the bronchoscope to excise tissues from the bronchi. Generally, multiple samples are collected and pooled for proteomic studies.

Bronchial tissue is an excellent material for unbiased (discovery) proteomic analysis for changes in protein expression in asthmatics. Most of the recent proteomic studies were performed with lung tissues from animal models or from primary tissue cultures, while one notable example utilized human bronchial biopsies. Recently, O'Neil et al. analyzed the global proteome of bronchial biopsies from normal and asthmatic subjects, treated either with an inhaled glucocorticoid steroid or placebo compared pair-wise pre- and posttreatment (O'Neil et al. 2011a, b). Using isobaric tag technology (iTRAQ) and 2D LC-MS/MS to quantify peptides, they identified from ~1,200 to ~1,700 proteins in total. For pre- and postplacebo-treated asthmatics, 115 proteins were analyzed and 3 were identified as statistically significant. In a pair-wise comparison of pre- and poststeroid-treated asthmatics, ~1,200 to ~1,400 proteins were quantified, with 141 analyzed statistically after steroid treatment, with 7 judged significantly changed. The highest scoring pathways by Ingenuity pathway analysis (IPA) for this cohort included prothrombin activation, actin motility, and actin signaling. Although demonstrating eigenvalues below that generally considered compelling (<38 % for components 1 and 2), principal component analysis (PCA) demonstrated tight clustering of healthy controls, with greater scatter of asthmatics, regardless of treatment. This is not only surprising, since asthma is generally recognized as a heterogeneous disease (Moore et al. 2010), but also spotlighting the small sample size of the study (22 patients in total). Nevertheless, this study shows that a small bronchial biopsy sample can be utilized for proteomic analysis and relevant information on the functional biological pathways involved in asthma pathogenesis can be obtained. In addition, the therapeutic effect of glucocorticoid steroids on the proteome level can be analyzed. The major challenge to proteomic analysis of bronchial biopsy samples is the lack of sufficient patient volunteers.

14.2.2 Epithelial Cells and Epithelial Lining Fluid

The airway epithelium constitutes the largest surface area in the human lung and its role in asthma pathogenesis has long been investigated. As epithelial cells and other cells that typically inhabit this environment are sentinels of the airway, it is well recognized that they play an important role in initiating and maintaining asthma pathogenesis by secreting a variety of inflammatory mediators in response to various insults such as allergens and noxious chemicals (Lim et al. 2004). Epithelial lining fluid (ELF), which forms a thin aqueous layer on the bronchial and alveolar epithelial cells, is a suitable material for proteomics as it contains many proteins that are believed to be involved in epithelial functions in normal and diseased state. ELF functions as a protective barrier between epithelium and external environment, and

accumulation of cellular products occurs in ELF during epithelial activation or injury by extracellular or intracellular factors.

ELF is obtained using a bronchoscopic microprobe containing an adsorptive tip that is lowered into the trachea until the lining is contacted (Franciosi et al. 2011). Fluid and cells thus obtained are recovered from the tip without dilution and analyzed. For proteomics studies, ELF has many advantages over BALF and induced sputum (IS). Unlike BALF, it is obtained undiluted and can be collected from the site of disease area without contamination with bacteria and other body fluids. One proof-of-concept study with ELF from rabbits identified 43 proteins, many of which have been previously reported as associated with lung disease (Kipnis et al. 2008). In addition, a recent study indicated that children with persistent bronchial asthma have greater level of nitric oxide oxidation products in ELF (Fitzpatrick et al. 2009a, b). Recently, Altraja et al. have studied the effect of the proinflammatory mediator, leukotriene E_4 (LTE_4), and remodeling factor, TGF-β_1, on the bronchial epithelial cell proteome as these mediators function in an autocrine fashion (Altraja et al. 2009). BEAS-2B culture cells were separated by 2D electrophoresis (2-DE), quantified, and proteins of interest excised and digested, and identified by MALDI-TOF MS. LTE_4 treatment downregulated β_1-tubulin (a marker of ciliated cells), HSP90α, and stress-70 protein, while TGF-β_1 upregulated antioxidant enzyme superoxide dismutase 1, profibrotic enzyme, protein disulfide-isomerase, and heat shock 70-kDa protein 9B, and downregulated lamin A/C, GAPDH, oncogene DJ-1, hnRNP A2/B1, and stress-70 protein.

Of particular interest is HSP90α, which is linked to the glucocorticoid receptor (GR) in a functional complex and is also downregulated by LTE_4 in this study. The authors make the case that HSP90α (α chaperonin) is known to play a special role in regulating the activity of GR, as well as the synthesis of prostaglandin E_2 (PGE_2), and that its downregulation in response to LTE_4 may cause inactivation of GR in bronchial epithelial cells as well as diminishing the bronchoprotective effect of PGE_2. This study clearly indicated that a proteomics approach can identify those proteins that play important roles in epithelial remodeling and illuminate airway pathology.

In a targeted approach to assess the response to increased formation of reactive oxygen species of patients with severe asthma, Fitzpatrick et al. (2009a, b) examined ELF from control and severely asthmatic children measuring reduced and oxidized glutathione (GSH and GSSG, respectively), as well as GSH reductase, GSH-S-transferase, and other indicators of oxidative stress. In comparison to carefully selected controls, children with severe asthma demonstrated lower GSH, increased GSSG, but no change in the glutathione cycle enzymes. In addition, increased malondialdehyde, 8-isoprostane, and hydrogen peroxide were observed in severe asthmatics.

These results were further analyzed using univariate logistic and linear regression analyses to determine the association between altered GSH:GSSG and clinical markers of asthma severity. Hospitalization (<1 year), prednisone use and dose, fraction of exhaled NO (F_{eno}), baseline FEV_1 and FEV_1 bronchodilator reversibility were tested as dependent variables against GSSG as a predictor. Of these dependent variables,

hospitalization ($p=0.001$) and baseline F_{eno} ($p=0.32$) were found to be significantly predicted by GSSG percentage. With these results, the authors conclude that severe asthmatic children exhibit increased biomarkers of oxidative stress (increased GSSG, malondialdehyde, isoprostane, and hydrogen peroxide) in their ELF and that interventions that increase GSH in the ELF might serve as effective treatments.

14.2.3 Bronchoalveolar Lavage Fluids

Historically instillation and recovery of bronchoalveolar lavage fluid (BALF) is the oldest technique commonly used to sample ELF and cells from the airway surfaces, and its analysis in human airway disease has been reviewed extensively since 2009 (Lin et al. 2010; Rottoli et al. 2009; Simpson et al. 2009; Vento et al. 2010). BALF is ideal in many respects for proteomic study, in that it contains different immune and nonimmune cells (alveolar macrophages, lymphocytes, neutrophils, eosinophils, squamous epithelial cells, bronchial epithelial cells, type II pneumocytes, basophils, and mast cells) and different soluble components (proteins, lipids, and nucleic acids).

Some of the soluble components are in common with plasma due in part to leakage from the plasma, and therefore the much discussed use of plasma as a sample source for proteomics studies enumerates several challenges likewise for BALF. Among these are the 10–12 orders of dynamic range in protein and peptide abundance; the interference of high-abundance proteins in the detection and quantification of the often more informative mid- and low-abundance proteins, and the tendency of these high-abundance proteins to absorb the mid- and low-abundance proteins (Gundry et al. 2007, 2009; Seferovic et al. 2008). Finally, since 12 % of serum peptides are naturally occurring (Richter et al. 1999) (i.e., not a result of artifactual proteolysis), the need to discriminate them from artifact requires preseparation fractionation (Brasier et al. 2012a; Wiktorowicz et al. 2011).

Using conventional techniques, different components of BALF can be separated and utilized for a comprehensive asthma proteomic study. In the last two decades, many proteomic analyses of BALF from healthy control and asthma patients have been conducted and databases of BALF proteins constructed (Li et al. 2009; Noel-Georis et al. 2002). However, most of the proteins identified have not attained the status of asthma biomarkers due to lack of verification by alternate methods. As BALF is a mixture of cells, and plasma proteins, lipids, nucleic acids, and salts, its use for proteomic analysis requires careful consideration of sample prep treatments. We have developed an approach that minimizes the confounding tendency of high-abundance proteins to adsorb lower-abundance proteins, allowing plasma protein immunodepletion strategies without loss of informative proteins, and provides reproducibly generated protein and peptide pools from the same biofluid sample, permitting the discrimination between natural and artifactual peptides. This approach has been in extensive use in our NIAID funded Clinical Proteomics Center for Infectious Disease and Biodefense and is described below in detail (Brasier et al. 2012a).

In a more targeted approach, Cederfur et al. have studied the glycoproteomic profile of BALF from asthmatics using galectins (small carbohydrate-binding proteins) and identified about 160 galectin-enriched proteins including several surfactant proteins (SP-A2, PIGR, and SP-B) (Cederfur et al. 2012). This study indicated that galectins, which are implicated in inflammatory lung diseases, can be used to dissect the lung glycoprotein dynamics in asthmatics.

In another targeted approach, a global feasibility study of the BALF phosphoproteome was performed to begin to assay the role of PTMs in biofluids (Giorgianni et al. 2012). While not the first to establish the importance of PTMs in biomarker discovery of biofluids (Brasier et al. 2012a), this study is the first to examine the presence of phosphorylated proteins in BALF. The authors enriched their sample by immobilized metal affinity chromatography (IMAC), followed by LC-MS/MS. From their analysis of normal subjects, they were able to identify 36 phosphopeptides containing 26 different phosphorylation sites, which were mapped to 21 phosphoproteins. Among these are included vimentin, plastin-2, ferritin heavy chain, kininogen-1, filamin, and, notably with regard to LTE_4 downregulation of GR discussed above, HSP90α and β. Many of the proteins identified have precedence in the literature of lung perturbations due to disease, smoking, and other environmental stresses.

Clearly, although the perfect approach to analyzing the proteome of BALF remains to be established, the value of such analyses in illuminating functional pathways and in generating candidate biomarkers is well worth the effort, with an intriguing future.

14.2.4 Induced Sputum

While BALF represents a diluted sample of the ELF, induced sputum samples the secretions of lung airways. Sputum induction is used for the noninvasive assessment of chronic inflammatory lung diseases such as asthma, COPD, and cystic fibrosis. Sputum is induced by delivering nebulized saline (3 % saline) to the lung, which irritates central airways and results in coughing. The major components of induced sputum are cells and fluids rich in many proteins. Apart from its diagnostic application, in recent years induced sputum has received a considerable attention for its use in biomarker discovery through global proteomic investigations in lung disorders (for recent review, see ref. Nicholas and Djukanović 2009). Since 2009, there have been a few proteomic studies using IS to identify new biomarkers of asthma and COPD (Gharib et al. 2011; Terracciano et al. 2011).

Recently, Gharib et al. (2011) reported identification and quantification of protein abundance using a shotgun label-free proteomic approach of asthmatic and healthy IS, followed by functional annotation by gene ontology and pathway analysis by IPA. In all, 254 proteins were identified in healthy control and asthmatic patients. Of these 17 proteins S100A8, S100A9, SCGB1A1, and SMR3B were

reduced, while serpin A1 was elevated in asthmatics versus controls and permitted "robust" classification of healthy controls from asthmatic patients. Western blots confirmed the increase of serpin A1, and decreases in SCGB1A1 and SMR3B in asthmatics relative to controls. The top functional categories mapped to these proteins include defense response, protease inhibitor activity, immunity, inflammation and wound response, and complement activation. However, in attempting to extract principle components representing of protein abundance variation of asthma and control samples by PCA, the data did not exhibit any significant clustering that corresponded to the clinical phenotypes. As with all proteomic strategies with limited fractionation prior to MS, the authors observe that their shotgun approach is biased toward detection of larger, more abundant proteins.

In a more recent study, the peptidome of asthmatics was compared to patients suffering from COPD by fractionating and extracting peptides from mesoporous silica beads with variable selectivity coupled with MALDI-TOF MS (Terracciano et al. 2011). Because these beads are rationally designed nanoporous network, the smallest of which has a pore diameter of 2.7 nm, only peptides of <4,500 were detected. With this fractionation/MALDI-TOF approach—sharing striking similarities to surface-enhanced laser desorption ionization (SELDI)—the authors report detection of >400 peptides. Comparison of healthy, asthmatic, and COPD ISs yielded 6 *m/z* peaks corresponding to three α-defensins, and three peaks of unknown identity that allowed discrimination between the three states by virtue of *t*-test statistics ($p \leq 0.05$).

14.2.5 Exhaled Breath Condensate

Exhaled breath condensate (EBC) is relatively a new and simple airway sampling approach that has been applied in the study of asthma and COPD, with the attraction of its noninvasiveness and risk-free collection. Because of these features, samples can be collected from a wide range of subjects, from youth to the elderly. EBC is composed of water-saturated aerosols from the airway lining fluid (ELF) containing water soluble gases, leukotrienes, isoprostanes, hydrogen peroxide, and proteins (Montuschi 2007). It is typically collected when subjects breathe into a mouthpiece through a one-way valve connected to a cooled condensing collection device. This condensed liquid may then be recovered for analysis. Although simple in concept and in operation, however, unless saliva is simultaneously collected for comparison and detected proteins that are in common ignored, the final analysis may be confounded by this contamination.

In a relatively large-scale study of healthy ($n=30$) and asthmatic ($n=40$) children, EBCs samples were concentrated, trypsin digested, separated by reversed-phase LC, and peptides quantified by MALDI-TOF/TOF. Saliva contamination was judged by the presence of salivary α-amylase, and those samples were removed from the analytical pool. Support vector machine analysis was performed to identify

the minimal model necessary to correctly classify 100 % of the subjects, regardless of their status. Ten peptides were identified—five more abundant in the control and five in the patient group. Upon validation with new samples, only eight peptides were detected with a classification accuracy of 80 %. Despite the promise of the first relatively successful proteomics study of EBCs, a major fault in this study was the inability to identify any of these peptides due to their low abundance. Nevertheless, this preliminary study presages successful use of EBCs for proteomic analysis, one without patient discomfort or risk.

14.3 Analytical Procedures

Proteomic analysis entails several steps and the technologies employed vary according to sample types. As indicated in Sects. 14.2 above and 14.3.1 below, biofluids require special processing compared to tissue or cellular material. In general, separations may involve HPLC using reversed phase (RP) chromatography; two dimensional chromatography for greater depth of inquiry with tandem RP and strong cation exchange (SCIEX) supports; one dimensional, size-based electrophoresis with SDS; two-dimensional electrophoresis with isoelectric focusing (IEF), followed by size-based electrophoresis; and preparative electrophoresis, based on solution IEF, or size fractionation (for recent review, see Wiktorowicz et al. 2011). An extensive survey of the general analytical procedures used in proteomics is beyond the scope of this chapter, and the reader is referred to recently published books on proteomic technologies (Lovric 2011; Mishra 2010).

This section, however, focuses on sample preparation techniques that are uniquely applicable to the airway samples, namely BALF and induced sputum.

14.3.1 BALF

In many respects, BALF is similar to plasma since plasma leakage occurs through airway tissues during injury, inflammation, and disease. For proteomic investigations using BALF, this means that similar challenges exist as those using plasma (or serum).

Since targets for proteomic analyses may be proteins, peptides, or both—and they may be complexed with other more abundant, but less informative molecules—one strategy is to create individual protein and peptide pools from the same sample for subsequent separation and analysis by separation of urea-denatured BALF by size-exclusion chromatography (SEC) (Brasier et al. 2012a). Furthermore, since native peptides constitute about 12 % of the plasma “peptidome” (Richter et al. 1999), this approach permits discrimination between derived peptides (peptides

derived from proteolysis of circulating proteins) and native peptides—since native peptides will be uniquely present in the peptide pool, whereas derived peptides will also appear as part of a protein in the protein pool. Since samples to be compared cannot be multiplexed, reproducible fractionation and creation of protein and peptide pools can be accomplished by incorporating a fluorescently labeled internal standard (e.g., Alexa-488 thaumatin—MW = 23 kDa) into the sample, with SEC systems equipped with UV monitors that electronically trigger fraction collection upon detection of the internal standard. This fractionation-pooling approach uniquely sets the stage for the discovery of higher quality candidate biomarkers, where it has been used extensively in the NIAID Clinical Proteomics Center for Infectious Disease and Biodefense at UTMB (Brasier et al. 2012a, b).

After antibody depletion of the 14 most highly abundant proteins, the remaining proteins are then saturation fluorescence labeled (Pretzer and Wiktorowicz 2008; Tyagarajan et al. 2003) to yield highly quantitative and accurate protein abundance estimates ($CV < 0.08$), separated and quantified by 2-DE, and the picked proteins (abundance ratio $\geq |1.25|$, $p < 0.05$) identified *via* LC-MS/MS as described above.

The peptide pools to be compared are individually labeled with $^{16/18}O$ by trypsin-mediated exchange in the presence of heavy (control) or light (case) water for 24 h. Peptides are quantified and identified by a nanoLC tandem electrospray MS/MS. As stated previously, the identities of the proteins and peptides in the pools are then compared to eliminate peptides from the candidate status by their coincident appearance of their precursor proteins in the protein pool.

Procedure: Alexa-488 thaumatin is produced by mixing 200 μL 8 M urea–Tris buffer (pH 7.5) with 50 μg thaumatin (Sigma-Aldrich, St. Louis, MO). To this is added 0.8 μL TCEP (Sigma–Aldrich) and the mixture incubated for 30 min at room temperature. The whole solution is then added to a tube with ~0.34 μmol dried Alexa-488 (Invitrogen, Carlsbad, CA) and incubated in the dark at room temperature for 120 min. The reaction is quenched by adding 1.2 μL of 2-mercaptoethanol and incubated for 30 min at room temperature. After aliquoting the solution into microcentrifuge tubes, the labeled protein is stored at −80 °C until needed.

To 100 μL of concentrated BALF containing at least 200 μg of protein is added enough urea to make an 8 M solution. Also added is 50 μg of Alexa-thaumatin. This solution is pumped at a flow rate of ~20 mL/h into a 1.5 × 30 cm column packed with Superdex75™ (GE Healthcare, Piscataway, NJ) for which the void and exclusion volumes have been determined (GE Healthcare mfg recommendations). After the free dye has eluted, the fractions after the void volume to the end of the first thaumatin peak (~17 kDa) are pooled and termed the "protein pool." Fractions from the end of the thaumatin peak to the beginning of the free dye peak are pooled and termed the "peptide pool."

The protein pools may be analyzed by conventional 2D electrophoresis, while the peptide pools by either label-free LC-MS/MS or stable isotope labeled and analyzed by LC-MS/MS.

14.3.2 IS

Extraction of proteins or peptides from induced sputum is complicated by the glycoproteins MUC5B and MUC5AC, which impart viscosity to mucin. Most protocols decrease the viscosity by treatment with disulfide reducing agents and centrifugation to separate associated cells. Supernatants are then analyzed by conventional separations and MS.

Procedure: As a recently published example of a typical approach (Terracciano et al. 2011), IS samples were treated with PBS containing 5 mM DTT. After addition of a protease inhibitor cocktail at 22.5 mL/g of sputum, the samples were incubated for 30 min at room temperature. The samples were then filtered through a 70-μm mesh filter and centrifuged at 400×*g* for 10 min at 4 °C to pellet the cells. The supernatants were removed and recentrifuged at 12,000×*g* for 10 min at 4 °C, before being aliquoted and stored at −80 °C until needed for analysis.

14.4 Future Directions

Because of its widespread use and ease of administration and recovery, BALF provides the lion's share of samples for the study of asthma classifiers. However, since BALF samples the ELF, whose volume may be variable due to inflammation or other factors, dilution of ELF constituents may confound quantitative comparisons of BALF. A means for normalization is needed, and one might envision combining ELF microprobe estimation of ELF volume (Franciosi et al. 2011) accompanied by measurement of total protein. This value could then be used to normalize the protein constituents recovered in BALF from the same patient.

More generally, discovery proteomics remains a technology-driven discipline, whose promise is based on the ability of academic and industry researchers to address its very real and daunting challenges. In comparison to the genome, where the complexity largely understood, the complexity of the proteome—due to alternative splicing, posttranscriptional, and translational modifications—present in even a single cell type is still subject to speculation. Moreover, the challenge is further exacerbated by the enormous dynamic range of protein concentrations in biological samples, and not the least of which is requirement for high sensitivity to permit detection of hundreds, if not tens of copies of a given protein per cell from small numbers of cells (<10,000), to name a few of the pressing challenges. So, while great progress has been made, there is still a long way to go.

Nevertheless, promising technologies that exploit new developments in the fields of microfluidics, nanotechnology, and chemistry fuel the optimism that proteomics research will yield breakthroughs in therapeutic discovery and translational medicine of this heterogeneous disorder.

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Chapter 15
Measurement of the Innate Immune Response in the Airway

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Abstract Asthma is an idiopathic disease associated with episodic inflammation and reversible airway obstruction that is triggered by environmental agents. Allergic and infectious agents trigger asthmatic exacerbations through the innate immune response (IIR). The IIR is activated by sentinel cells in the airways to elaborate inflammatory cytokines and protective mucosal interferons whose actions are designed to limit the spread of the organism, as well as to activate the adaptive immune response. We address the structure of the IIR pathway in sentinel cells of the airway and describe observations on its dysregulation. The IIR is triggered in a cell-type specific manner by germline-encoded pathogen recognition receptors (PPRs) including plasma membrane Toll-like receptors (TLRs) and the cytoplasmic Retinoic Acid-inducible Gene (RIG)-I-like RNA helicases, and protein kinase R (PKR). Although their mechanisms of intracellular signaling differ, both pathways converge on a small group of transcriptional effectors, nuclear factor-κB (NF-κB), IFN regulatory factor (IRF), and signal transducer and activator of transcription (STAT). We describe several distinct techniques to quantitate the IIR including assays based on quantitative real-time PCR (Q-RT-PCR) of NF-κB and IRF3-regulated genes, multiplex bead-based analysis of secreted proteins/cytokines and more recent developments in targeted, quantitative selected reaction monitoring (SRM)-mass spectrometry (MS). Application of these methods for quantitation of the IIR will further our understanding of the role of the IIR in asthma and its contribution to disease heterogeneity.

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15.1 Introduction

Asthma is an idiopathic disease associated with episodic airway inflammation and reversible obstruction whose lifetime prevalence is as high as 10 % of the general population (Busse and Lemanske 2001). In patients with established atopy, these episodic exacerbations are characterized by acute symptoms of obstruction (wheezing, chest tightness, shortness of breath, and mucous production) because the primary reasons for emergency room visits and reductions in the quality of life (Johnston and Sears 2006). Here, asthmatic exacerbations are linked to specific environmental exposures, the most frequent being aeroallergen exposure or respiratory virus infection.

One mechanism how allergic and infectious agents trigger acute exacerbations of asthma occurs through stimulating the airway inflammatory response. Inflammation is a temporally coordinated, multicellular response that produces recruitment of inflammatory cell populations into the lung (Brasier et al. 2011a, b). This process involves an initiating event, for example, the presence of viral or bacterial products, whose presence are detected by sentinel cells of the airway to trigger inflammation.

The immune response is mediated through two distinct arms, referred to as the innate and adaptive immune responses. Innate immunity is a rapid (minutes–hours), nonspecific inflammatory response produced by a previously unrecognized pathogen encountering sentinel cells in the airways (airway epithelial cells, macrophages, and dendritic cells). The innate immune response (IIR) is a coordinated intracellular signaling pathway triggered by cytoplasmic and plasma membrane pattern recognition receptors (PRRs) encoded in the germline of the host organism. The IIR elaborates inflammatory cytokines and protective mucosal interferons whose actions are designed to limit the spread of the organism and regulate the nature and magnitude of the adaptive immune response. By contrast, the adaptive immune response is a slower event (over days–weeks), requiring genetic recombination to induce long-lived, microbe-specific antibody and cellular immunity.

How infectious stimuli shape the adaptive immune response is an important question in the field. Depending on the nature of the host–environment interactions, signals produced by the IIR in sentinel airway cells induce differentiation and polarization of specific populations of CD4 T helper (T_H) cells. These signals produce the differentiation and selection programs that result in phenotypically distinct T helper (H) type 1 (T_H1), type 2 (T_H2), or the more recently identified (T_H17) population. These T_H populations are distinct by immune regulatory function and cytokine secretion patterns; for example, T_H2 predominant inflammation is primarily associated with nonspecific atopy and eosinophilia, hallmarks of asthma (Busse and Lemanske 2001).

Because of the central role of the IIR in activating and shaping the adaptive immune response including subsequent atopy, much attention is being place on the IIR in asthma. This chapter addresses the structure and dynamics of the IIR pathway in the airway and describes methods for its measurement for studies to dissect its role in human disease and understanding sources of asthma heterogeneity.

15.2 The Structure of the Innate Immune Response Signaling Pathway

Alveolar macrophages, dendritic cells (DCs), and epithelial cells represent the primary sentinel cells of the naïve airways. These cells normally exist in a sterile environment and are activated when they encounter pathogen-associated molecular patterns. These pathogen patterns are signatures associated with broad classes of pathogens (e.g., dsRNA, lipoteichoic acids, mannans, flagellins, and others). This activation process is mediated by germline-encoded pattern recognition receptors (PRRs), receptors that have evolved to bind pathogen-associated molecular patterns. The major classes of PRRs include the plasma membrane Toll-like receptors (TLRs) and the cytoplasmic Retinoic Acid-inducible Gene (RIG)-I-like RNA helicases (RLH). Although their mechanisms of intracellular signaling differ, both can be thought of as triggering a coupled intracellular serine kinase–ubiquitin ligase cascade that produces protein complex formation, proteolysis of inhibitory proteins, and nuclear translocation of transcriptional effectors (Akira et al. 2006). Discussed in more detail below, the primary transcription effectors of the IIR are nuclear factor-κB (NF-κB), IFN regulatory factor (IRF), and signal transducer and activator of transcription (STAT). Upon posttranslational modifications, these effectors, in turn, activate the expression of inflammatory cytokine and type I interferon (IFN) genes. These genes encode paracrine factors that limit pathogen replication and activate the adaptive immune response.

15.2.1 The Toll-Like Receptors

The toll-like receptors (TLRs) are a family of 12 related cell surface-localized PRRs that bind diverse pathogen-associated molecular patterns including lipopolysaccharide (LPS), lipoteichoic acids, mannans, flagellins, dsRNA, and DNA oligonucleotides (O'Neill and Bowie 2007). Interestingly, different TLRs are expressed in different compartments of sentinel mucosal cells, whose location is dependent on cell type and dynamic, depending on state of cellular activation. In resting epithelial cells, TLR-9 is cell surface associated, TLR-3 is endosomal, and TLR-4 is absent. After viral infection, TLR3 expression is upregulated and redistributes to the cell surface (Liu et al. 2007). Interestingly, TLR-3 upregulation is a paracrine

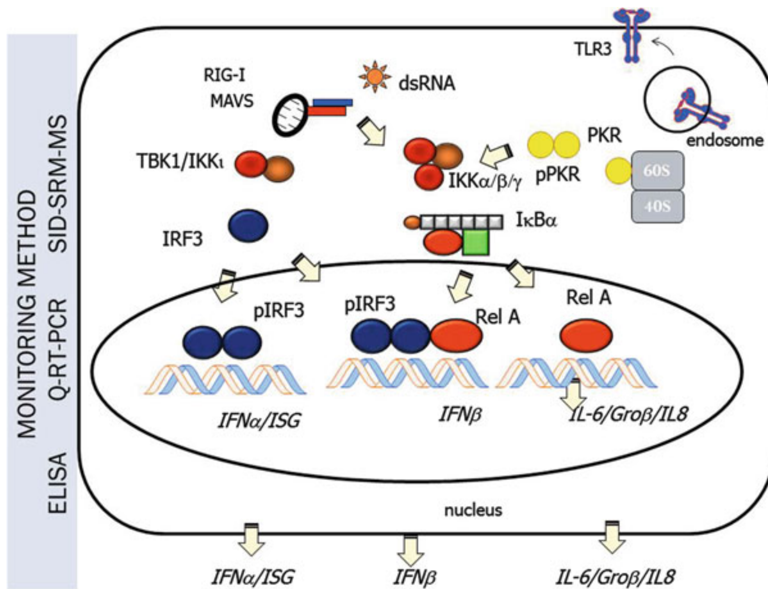

Fig. 15.1 Structure of the IIR. Levels of IIR for monitoring are shown. *Colored circles* indicate proteins for which SID-SRM assays have been developed

effect-dependent IFNβ signaling (Liu et al. 2007). In immune cells (dendritic cells and macrophages), TLR-1, -2, -4, -5, and -6 localize at the cell surface, whereas TLRs-3, -7, -8, and -9 are contained within the endosomal compartment.

TLR signaling is initiated upon binding to its cognate molecular pattern and activates downstream signaling pathways by inducing signaling complex formation by recruiting specific adapter proteins. The LPS receptor, TLR4, induces complex formation with CD14 and MD2. TLR3 is the primary receptor for dsRNA and activates innate signaling by recruiting the cytoplasmic adapter known as TRIF (Fig. 15.1).

15.2.2 The Retinoic Acid-Inducible Gene (RIG)-I Like RNA Helicases (RLH)

RIG-I and the melanoma differentiation-associated gene-5 (Mda5) are two members of the CARD-RNA RLHs that are essential PRRs of cytoplasmic double-stranded (ds) RNA (Kato et al. 2006; Yoneyama et al. 2004). RIG-I and Mda5 recognize different types of virus and dsRNAs, with RIG-I responding to most ssRNA viruses and short viral RNAs, whereas Mda5 responds to picornavirus RNA and longer dsRNAs. Activation of RIG-I/Mda5 involves conformational changes upon RNA binding, followed by its Lys 63-linked ubiquitylation, a nondestructive

posttranslational modification that promotes protein complex formation (Gack et al. 2007; Oshiumi et al. 2009). Subsequently, activated RLHs bind a common downstream adaptor known as mitochondrial antiviral signaling (MAVS). MAVS contains an NH2-terminal CARD domain, an essential motif that binds cognate CARD domains in activated RIG-I or Mda5, and a transmembrane domain critical for mitochondrial location (Seth et al. 2005). The formation of RIG-I/Mda5·CARD complex induces MAVS oligomerization and ubiquitylation of the IKKγ, TRAF, and RIP adapter proteins, also by formation of Lys 63-linked polymers to generate a functional mitochondrial signal generator complex (Chen 2005). This complex activates both primary effector arms of the IIR, nuclear factor-κB (NF-κB), and interferon response factor (IRF)-3.

15.2.3 Protein Kinase R

Another major intracellular PRR for dsRNA is protein kinase R (PKR). Activated PKR limits viral replication through phosphorylation of the α-subunit of the translation initiation factor eIF-2, which results in shutdown of cellular and viral protein synthesis (Williams 1999). PKR also mediates type I IFN production in response to specific types of virus infections, such as encephalomyocarditis virus, but not major airway pathogens, such as influenza or RSV (Schulz et al. 2010) and therefore is thought to be a minor contributor to innate immunity in the airways.

15.2.4 Downstream Transcriptional Effectors of the IIR

Mammalian cells contain NF-κB family members, RelA/p65, c-Rel, RelB, p105/p50, and p100/p52, all of which form homo- or heterodimeric-inducible cytoplasmic DNA complexes tethered in the cytoplasm by association with IκB inhibitors (Siebenlist et al. 1994). The well-known "canonical NF-κB pathway" is initiated by either activated TNF superfamily receptors or the cytoplasmic PRR, RIG-I, that converges on IκB kinase (IKK), the rate-limiting kinase controlling IκBα degradation (Fig. 15.1). Importantly, IKK activation absolutely requires the IKKγ regulatory subunit (Liu et al. 2009; Zhao et al. 2007), whose role is to mediate its oligomerization and recruitment to the activated TNF receptor, or the activated RIG-I·MAVS complex (Poyet et al. 2000; Zhao et al. 2007). By contrast, the "noncanonical NF-κB pathway," is activated by RIG-I in an IKKγ-independent manner to converge on the NF-κB inducing kinase (NIK)–IKKα complex that initiates NF-κB2/p100 processing to liberate both sequestered RelB/p52 and RelA complexes from cytoplasmic NF-κB2/p100 precursor (Basak et al. 2007; Liu et al. 2008). This latter arm has been termed the "cross-talk" pathway (Basak et al. 2007), because it is dependent on noncanonical NIK-IKKα kinases but liberates the canonical RelA transcriptional activator.

The IRF family of transcription factors is a distinct effector arm of the IIR that controls type I IFN expression in response to viral patterns. So far, nine human IRFs have been reported (IRF1–9); of these, IRF3, -1, and -7 are the key regulators of type I IFN gene expression (Hiscott 2007; Pitha et al. 1998). IRF3 is the major early signaling protein constitutively expressed in the cytoplasm. The molecular mechanisms by which RIG-I·MAVS activates IRF3 signaling are partly understood (Fig. 15.1). RIG-I-complexed MAVS recruits TRAF3, a signal adapter that binds to specific motifs on MAVS (Saha et al. 2006), that in turn mediates activation of the Tank Binding Kinase (TBK)-1–IKKε/ι, a complex representing the major rate-limiting kinases of IRF3 activation (Liu et al. 2009). Phosphorylation of IRF3 on COOH-terminal domain serine residues allows it to homodimerize, associate with p300/CBP, and translocate into the nucleus (Hiscott 2007). In this manner, phospho-IRF3 activates target genes containing interferon-stimulated response elements, such as IFNβ/IFNα4, and induces downstream IRFs-1 and -7. IRF-1 and -7 induce other type I IFNs, whose actions induce antiviral responses in neighboring cells (Marie et al. 1998).

15.3 How the IIR Couples to Adaptive Immunity

Activation of the IIR in sentinel cells produces the secretion of diverse profiles of cytokines, chemokines, defensins and type I IFNs that activate neighboring cells in the lung. These produce acute inflammation and initiate programs for long-term pathogen-specific (adaptive) immunity.

Although the individual roles for epithelial, dendritic, and monocyte/macrophages are not completely known, the epithelium is a major source of activating CC, CXC, and C-classes of chemokines (Zhang et al. 2001). In the presence of activating chemokines, specific classes of leukocytes are recruited to the airways via integrin–addressin interactions (Garrood et al. 2006). As a result, activated leukocytes invade the interstitial spaces and lumen of the airway. These activated leukocytes produce acute cellular inflammation by migrating down chemotactic gradients to the site of infection and are responsible for phagocytosis and antigen uptake.

Another sentinel cell of the airway is the dendritic cell (DC), a highly specialized cell type that coordinates the development of adaptive immunity. Through CCL20-MIP-1α chemokine signaling DC are recruited into the lung in response to PRR activation. In combination with microenvironmental cues, distinct classes of DCs play an important role in shaping the adaptive immunity by determining polarization of CD4 T helper (T_H) lymphocytes (Pulendran et al. 1999). One important microenvironmental signal of special relevance to asthma is the epithelial-derived thymic stromal lymphopoietin, an IIR-inducible cytokine-like molecule that enhances DC maturation and promotes T_H differentiation (Soumelis et al. 2002). Depending on microenvironmental cofactors and cell type interactions, T_H lymphocytes differentiate into phenotypically distinct subsets, known as T_H type 1 (T_H1), type 2 (T_H2), or the more recently identified (T_H17) cells; these types are distinguished

by immune regulatory function and cytokine secretion patterns (Busse and Lemanske 2001; Park et al. 2005). T_H1 cells produce IFNγ and mediate cellular immunity, whereas T_H2 cells produce interleukin (IL)-4, -5, and -13 that mediate humoral immunity, IgE production, and allergic responses. T_H17 cells express ILs-17 and -6, and although their role in asthma is not fully understood, T_H17 cells have been associated with chronic inflammation (Park et al. 2005). It is currently thought that T_H polarization events alter the underlying pathophysiology of the lung. For example, T_H2-polarized inflammation supports recruitment and activation of eosinophils and mast cells in the mucosa, making mild asthmatics sensitive to exacerbations induced by aeroallergen exposure that inducing IgE-dependent mast cell and eosinophil degranulation.

15.4 Viruses and Asthma Exacerbations

15.4.1 Bronchiolitis in Children

Infection of the small airways (bronchioles) is the most common cause of lower respiratory tract infections in children. The primary causative agent is the paramyxovirus Respiratory Syncytial Virus (RSV), and to a lesser extent, the more recently identified fastidious human Metapneumovirus (hMPV). In fact, RSV infects virtually all children by the age of 3 (Glezen et al. 1986). A recent prospective, population-based study of 5,000 children presenting for acute medical care estimated that 18 % of acutely ill children have acute RSV infection (Hall et al. 2009). Here, the presence of RSV infection produces three times the risk of subsequent hospitalization over that seen in infections with other common cold viruses, and in young children, hospitalization rates of 17 per 1,000 babies is estimated. Overall, approximately 120,000 hospitalizations for bronchiolitis are seen in the USA annually (Shay et al. 1999). These findings indicate that RSV is a significant cause of morbidity in children.

Bronchiolitis is typically seen in immunologically naïve children with first-time RSV infections (Zorc and Hall 2010), where it is associated with recurrent wheezing throughout childhood. This infection is thought to begin as an upper respiratory tract infection that spreads into the lower respiratory tract. In the case of RSV, propagation to the lower airway is via either cell-to-cell spread through intraepithelial cellular bridges or via the mucosal spread involving free virus binding to epithelial cilia (Zhang et al. 2002). RSV productively replicates in the airway mucosa, principally in epithelial cells, where RSV produces bronchiolar epithelial inflammation, producing epithelial necrosis, peribronchial monocytic infiltration, and submucosal edema (Aherne et al. 1970; Ferris et al. 1973). In the lower airways, plugs of necrotic epithelial and fibrin contribute to small airways airflow obstruction, thereby impairing gas exchange. Bronchiolitis is, therefore, a predominately small airways disease.

Bronchiolitis is associated with an intense airway inflammatory response and is a condition that has been linked to recurrent wheezing throughout childhood (Smyth and Openshaw 2006). Current research focuses on the role of RSV and hMPV in the potential etiology of asthma.

15.4.2 Viral Exacerbations of Asthma

Acute exacerbations account for the majority of morbidity and medical costs related to asthma (Johnston and Sears 2006). We now know that acute respiratory viral infections are associated with asthmatic exacerbations in both children and adults, where 70–80 % of exacerbations are suspected to be provoked by RNA viruses. RSV and hMPV are the major etiological agents of acute asthma exacerbations in children (Hall and McCarthy 1995; Hall et al. 2009; Shay et al. 2001). By contrast, rhinovirus (RV) infections are associated with the majority of asthmatic exacerbations in adults (Johnston 2007).

The presence of replicating RNA virus is a potent inducer of the IIR in sentinel cells of the airway. Here, 5′ phosphorylated RNA, dsRNA, and viral nonstructural proteins all serve as molecular patterns sensed by RIG-I in the epithelium and DCs (Liu et al. 2007). RLH activation triggers type I IFN production, a cytokine that works in a paracrine manner to upregulate TLR3 expression and stimulates its translocation to the cell surface in adjacent cells. In addition, TLRs-3, -4, -7/8, and -9 have been reported as RSV sensors in different sentinel cells. For example, in airway epithelial cells, TLRs-3 and -4 have been shown to interact with RSV, whereas TLR7/8 and TLR9 function in pDCs (Kurt-Jones et al. 2000; Liu et al. 2007; Rudd et al. 2005). Surprisingly, RSV clearance is apparently not reduced in $TLR3^{-/-}$ mice (Rudd et al. 2006); TLR3, therefore, may play a modulating, but nonessential, role in clearance of RSV infection.

Recent studies have indicated that epithelial cells isolated from atopic asthmatics show a relative defect in triggering the IIR. For example, RV replicates more efficiently in asthmatic bronchial epithelial cells than normal controls (Wark et al. 2005) and a defective type III IFN production was noted (Contoli et al. 2006). The mechanism for defective IIR has not been fully elucidated and will require more efficient and quantitative approaches to measurement of the IIR. There remains much to be discovered on the role and modulating effect of IIR on the genesis and exacerbation of asthma.

15.5 Quantitative Techniques for Measurement of the IIR

Detection of the activated state of the IIR can be done at several levels of its action, from the detection of genes under IIR control, measurement of cytokine secretion, or by quantification of activated intracellular proteins and kinases (Fig. 15.1).

15.5.1 Pathway Genes: Quantitative Real-Time PCR

Quantitative real-time PCR (Q-RT-PCR) is a robust and highly quantitative method for detection of relative changes of mRNA expression in airway cells. In this assay, measurement of inducible IIR genes can be used as indicator of pathway activation. Genes that are primary regulated by NF-κB (*IL6*, *Groβ*, *IκBα*, and *TNFAIP3/A20*), IRF3 (*ISG15*, *ISG54*, and *ISG56*), or both (*IFNβ* and *CCl5/RANTES*) can be quantified for monitoring different arms of the IIR (Fig. 15.1). The sources of cells for Q-RT-PCR can include airway cells from induced sputum or bronchoalveolar lavage, bronchial brushings, or tissue derived bronchial biopsies (for discussion of different fluid preparations, please see companion Chap. 14 by Wiktorowicz in this section).

In primer design for custom assays, we regularly use NCBI/Primer-BLAST in NCBI website for designing real-time PCR primer pairs that are typically 20–25 mers with a Tm range of 58–63 °C (maximum Tm difference 2 °C) and that amplify a product of 50–150 bp. The melting temperature is calculated based on the Nearest-Neighbor thermodynamic parameters. The GC content is set between 40 and 55 %.

For gene expression analyses, 1 μg of RNA is reverse transcribed using Super Script III in a 20-μL reaction mixture (Brasier et al. 2011a, b). One μL of cDNA product is diluted 1:2, and 2 μL of diluted product is then amplified in a 20-μL reaction mixture containing 10 μL of SYBR Green Supermix (Bio-Rad) and 0.4 μM each of forward and reverse gene-specific primers (Table 15.1). The reaction mixtures are aliquoted into Bio-Rad 96-well clear PCR plate and the plate is sealed by Bio-Rad Microseal B film before putting into PCR machine. The PCR plates are initially denatured for 90 s at 95 °C and then subjected to 40 cycles of 15 s denaturation at 94 °C, 60 s annealing at 60 °C, and 1 min extension at 72 °C in an iCycler (Bio-Rad). In new assays, we routinely subject PCR products to melting curve analysis to assure that a single amplification product was produced.

A melting curve analysis is conducted upon completion of the PCR assay. Here, the sample is gradually heated to detect the temperature at which the major product melts. This should produce a distinct, single spike, indicating the amplification of a single PCR species. If multiple peaks are seen, this result suggests that nonspecific PCR

Table 15.1 Validated primer sets for Q-RT-PCR of the human IIR

Q-RT-PCR	Forward (5′-3′)	Reverse (5′-3′)
hNFKBIA/IkBa	TCGCAGTGGACCTGCAAAAT	TGAGCTGGTAGGGAGAATAGC
hCXCL2/Groβ	AGGGGTTCGCCGTTCTCGGA	CCGCAGGAGCCGGGGATTG
hCXCL1/IL8	ACTGAGAGTGATTGAGA GTGGAC	AACCCTCTGCACCCAGTTTTC
hTNFAIP3/A20	TGGCTAGCCGGCGAGTGAG	CGGAGATTGATCCACTCTTGTCCCC
IL6	AAATTCGGTACATCCTCGACGG	GGAAGGTTCAGGTTGTTTTCTGC
hCCL5/ RANTES	CATCTGCCTCCCCATATTCCT	GCGGGCAATGTAGGCAAA
hGAPDH	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAATA

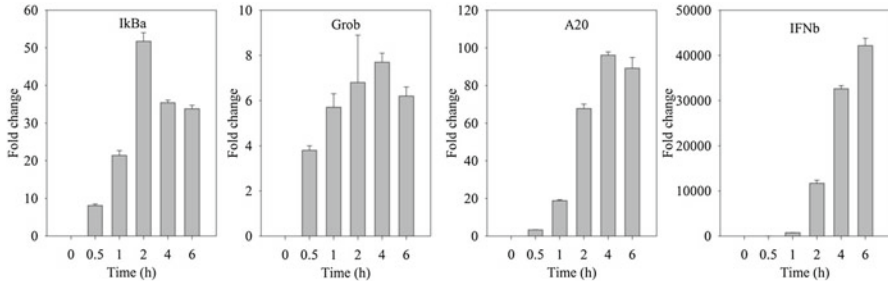

Fig. 15.2 Q-RT-PCR analysis of IRF3 and RelA downstream genes. A549 cells were treated with dsRNA for varying times. The mRNA expression level of NFKB/IκBα, CXLC2/Groβ, TNFAIP3/A20 and IFNβ were quantified by Q-RT-PCR. From Zhao et al. (2013)

amplification products are being produced. If this is observed, then the assay is redeveloped by either altering the annealing temperature or redesigning the PCR primers.

Quantification of relative changes in gene expression is calculated using the ΔΔCt method. The ΔCt value is calculated (normalized to Glyceraldehycle-3-phosphodehydrogenase, GAPDH) for each sample by: ΔCt = [Ct (target gene) − Ct (GAPDH)]. Next, the ΔΔCt is calculated by the formula: ΔΔCt = [ΔCt (experimental sample) − ΔCt (control sample)]. Finally, the fold differences between experimental sample and control sample were calculated using the formula 2^(−ΔΔCt). Quantification of absolute concentrations of the short and spliced RNA transcripts can alternatively be performed by estimating transcript number relative to serial dilutions of cDNA standards in Q-RT-PCR. As shown in Fig. 15.2, we used Q-RT-PCR to quantify the temporal profiles of NF-κB and IRF3 downstream genes in response to dsRNA stimulation (Zhao et al. 2013). The activation of the canonical NF-κB pathway and IRF3 pathway resulted in expression of distinct temporal waves of downstream genes. dsRNA strongly induced the parallel expression of NFKBIA/IκBα, CXLC2/Groβ, TNFAIP3/A20, and IFNβ with an initial increase in mRNA expression first detectable at 0.5 h. CXLC2/Groβ and TNFAIP3/A20 showed qualitatively similar profiles, with a gradual increase in mRNA expression until mRNA abundance peaked at 4 h, followed by a slightly decrease. The induction of mRNA expression of NFKBIA/IκBα reached an apparent maximum after 2 h of dsRNA stimulation and declined thereafter.

15.5.2 Pathway Proteins: Multiplex Bead-Based Analysis of Secreted Proteins/Cytokines

A similar approach to the Q-RT-PCR assay would be to monitor the secretion of cytokines activated by the IIR. Commercially available immunoassays based on luminex bead technologies have been widely applied for this purpose. A method that we have used is described below.

Bead-based immunoassays can be used to quantify cytokine concentrations in a number of biological fluids, cell supernatants, or tissue extracts (for discussion of

different fluid preparations, please see companion Chap. 14 by Wiktorowicz in this section). In these Luminex-based multiplex assays, panels of colored microspheres conjugated with capture antibodies are bound to the sample (each capture antibody is conjugated with a uniquely colored microsphere). For each sample, 50 μL is typically clarified by high-speed centrifugation (10,000× *g* for 3 min at 4 °C) before analysis.

The clarified supernatants are then analyzed using the appropriate cytokine panel. Panels of up to 25 human cytokines are widely commercially available; one common supplier is Invitrogen, Carlsbad, CA. Quantification is performed relative to recombinant standards supplied with the commercial assays. For this purpose, duplicate samples and serial dilutions of the cytokine standards (50 μL) are incubated with antihuman cytokine-coated beads in 96-well filtration plate (Millipore, Bedford, MA) for 30 min. The plates were vacuum-washed three times with 100 μL of wash buffer and incubated with 25 μL biotinylated antibody cocktail for 30 min. The immune reaction is then developed by adding 50 mL streptavidin–phycoerythrin for 10 min followed by three washes. The samples are then resuspended in 100 μL assay buffer, and 100 beads of each cytokine are acquired and analyzed.

For each cytokine, a standard curve is generated by using recombinant proteins to estimate protein concentration in the unknown sample. In our hands, these assays have a sensitivity comparable to enzyme-linked immunosorbent assay measurements, with a detection limit of 10–30 ng/L (depending on cytokine), low interassay variation (<10 %), and a dynamic range of as many as 3 orders of magnitude (Brasier et al. 2010, 2011a, b). In certain cases, the cytokine concentration can be normalized to total protein in the sample, or normalized for volume.

15.5.3 Pathway Activation: Selected Reaction Monitoring-Mass Spectrometry

Selected reaction monitoring (SRM)-mass spectrometry (MS) assays have been developed as a new "targeted" approach for the detection and accurate quantification of a predetermined set of proteins in a complex background (Gerber et al. 2003; Lange et al. 2008; Zhao et al. 2013). Specifically, SRM-MS has been used for studying the dynamics of signaling pathways (Bisson et al. 2011; Dong et al. 2007), and recently we have adapted this approach to the quantification of the IIR (Zhao et al. 2011, 2013; Zhao and Brasier 2013). We have developed a workflow for SID-SRM-MS assay design for quantification of low-abundance proteins (Zhao et al. 2011, 2013) and have optimized assays for the major effector arms of the IIR (Table 15.2). Our evaluation suggests that these SRM-MS are highly sensitive and specific and will enable quantification of temporal profiles of the IIR.

In an SRM-MS assay, one or two signature proteotypic peptides, unique to the protein of interest, are selected to stoichiometrically represent the protein. SRM-MS analysis of these signature peptides are performed on a triple quadrupole mass spectrometer (QQQ-MS), an instrument with the capability to selectively isolate precursor ions corresponding to the m/z of the signature peptides and to selectively monitor peptide-specific fragment ions (the combination of m/z of precursor ion and its

Table 15.2 SRM parameters of SRM assays of proteins for sample amount normalization. Masses listed are for the native forms of the peptides

Gene name	Swissprot No	Sequence	Q1 *m/z*	Q3 *m/z*	Ion type	CE (*V*)	Linear range	R^2	LLOQ (amol)
IKKa	O15111	IQLPIIQLR	547.358	529.345	y4	26	20,000	0.9996	50
			547.358	642.429	y5	23			
			547.358	739.482	y6	19			
			547.358	852.566	y7	18			
MAVS	Q7Z434	VSASTVPTDGSSR	632.312	719.331	y7	20	20,000	0.9995	250
			632.312	818.400	y8	16			
			632.312	919.447	y9	17			
			632.312	1,006.479	y10	20			
RelA	Q04206	TPPYADPSLQAPVR	756.396	867.504	y8	27	20,000	0.9990	250
			756.396	982.531	y9	27			
			756.396	1,053.568	y10	27			
			756.396	1,313.684	y12	30			
IRF3	Q14653	LVGSEVGDR	466.242	446.235	y4	24	100,000	0.9998	50
			466.242	575.278	y5	26			
			466.242	719.331	y7	26			
RIG-I	O95786	VVFFANQIPVYEQQK	905.480	891.457	y7	26	2,000	0.9989	2,500
			905.480	1,004.541	y8	24			
			905.480	1,132.599	y9	26			
			905.480	1,246.642	y10	27			
TBK1	Q9UHD2	TTEENPIFVVSR	696.362	817.493	y7	24	20,000	0.9988	250
			696.362	931.535	y8	24			
			696.362	1,060.578	y9	23			
			696.362	1,189.621	y10	23			

IKBA	P259633	LEPQEVPR	484.264	628.341	y5	20	20,000	0.9992	50
			484.264	725.394	y6	20			
			484.264	854.436	y7	20			
			484.264	967.520	y8	20			
IKKR	Q9Y6K9	AQVTSLLGELQESQSR	873.455	976.469	y8	27	10,000	0.9984	500
			873.455	1,033.490	y9	27			
			873.455	1,146.574	y10	27			
			873.455	1,259.658	y11	27			
			873.455	1,346.690	y12	27			
p52/p100	Q00653	DSGEEAAEPSAPSR	701.808	743.368	y7	20	20,000	0.9976	250
			701.808	814.405	y8	20			
			701.808	885.442	y9	20			
			701.808	1,014.484	y10	27			
p100	Q00653	ALLDYGVTADAR	632.83	689.357	y7	20	20,000	0.9985	250
			632.83	852.421	y8	20			
			632.83	967.447	y9	20			
			632.83	1,080.531	y10	21			

From Zhao et al. (2013)
CE collision energy, *Q* quadrupole, R^2 coefficient of determination

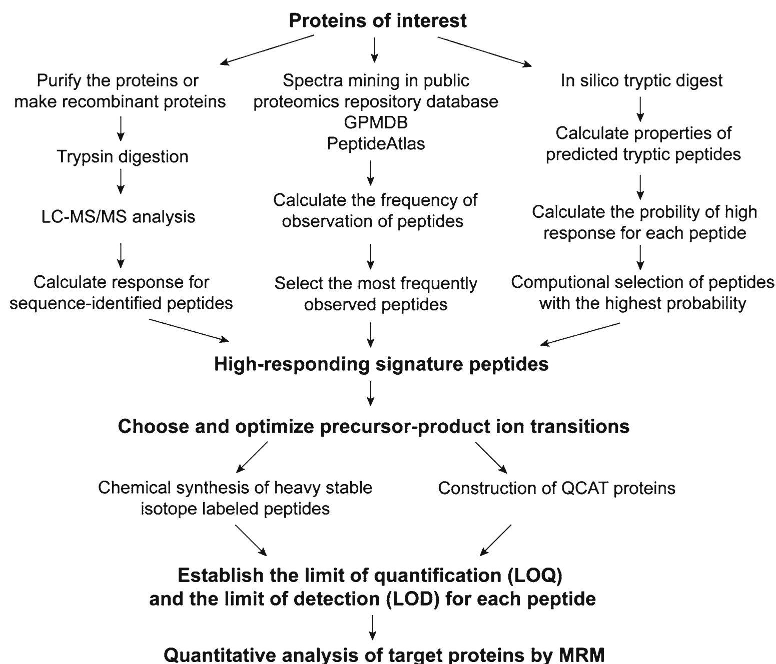

Fig. 15.3 Selection of proteotypic peptides. Workflow for selection of proteotypic peptides. From Zhao and Brasier (2013)

product ions are called precursor-product ion transitions). SRM assays do not require the generation of high-affinity antibodies and yet have lower limit of detection to pg/ml protein concentrations. In addition, SRM assays offer several attractive features. First, because only preselected precursor-product ion transitions are monitored in SRM mode, the noise level is significantly reduced and thereby SRM assays decrease the lower detection limit for peptides by up to 100-fold in comparison to a conventional full scan MS/MS analysis. Second, if the precursor-product ion transition of one proteotypic peptide is unique to the protein of origin, it is not only distinguishable from other MS signals in one LC run, but also it is a characteristic signature for the protein of interest. Therefore, two filtering stages in SRM mode result in near-absolute structural specificity for the target protein. Third, SRM-MS in combination with stable-isotope dilution (SID) is so far the most accurate approach for direct quantification of target proteins in a complex mixture (Gerber et al. 2003; Zhao et al. 2013).

To develop an accurate and robust SRM assay, we employ a workflow combining datamining in public proteomics repository databases and computational prediction to select the high-responding signature peptide candidates (Fig. 15.3). Candidate tryptic peptides are then filtered based on the following series of criteria:

1. The proteotypic peptides should be unique to the target protein.
2. The proteotypic peptides should be between 6 and 25 residues long.

3. There should be no missed cleavages.
4. Peptides containing Lys (K)-Pro (P) and Arg (R)-P should be excluded because these are not efficiently cut by trypsin.
5. Peptides with Cys or Met should be excluded.
6. Peptides with two neighboring basic amino acid (K,R) at either cleavage site of the peptide sequence should be excluded.
7. Peptides with known PTMs should be excluded (if the SRM is to detect total protein abundance).

The intensities of individual fragments derived from one precursor ion differ substantially. Selection of the most intense fragments for each signature peptide is therefore essential for developing a high-sensitivity SRM assay. The selection of fragment ions can be on the basis of MS/MS spectra of the peptide, where the highest intensity fragment ions in MS/MS are often selected to maximize the detection sensitivity. Major QQQ-MS instrument manufacturers have created software such as Pinpoint™ (Thermo Scientific), MRMpilot™ (Applied Biosystems), QuanOptimise™ (Waters), and MassHunter Optimiser™ (Agilent) to facilitate the selection of potential SRM transitions. The sensitivity of the assays can be further improved by optimizating mass spectrometric parameters such as collision energy (CE), the voltage of S-lens (TSQ series instrument), or declustering potential (QTRAP-type instrument).

For sample preparation in the SRM assays, the sample is first desalted using an Eksigent NanoLC-2D HPLC system (AB SCIEX, Dublin, CA). To perform this, a 5-μg (10 μL) aliquot of the tryptic digest is injected on a C18 peptide trap (Agilent, Santa Clara, CA) and desalted with 0.1 % formic acid at a flow rate of 2 μL/min for 45 min. Peptides are eluted from the trap and separated on a reverse-phase nano-HPLC column (PicoFrit™, 75 μm × 10 cm; tip ID 15 μm) packed in house using Zorbax SB-C18 (5-μm diameter particles, Agilent, Santa Clara, CA). Separations are performed using a flow rate of 500 nL/min with a 20-min linear gradient from 2 to 40 % mobile phase B (0.1 % formic acid-90 % acetonitrile) in mobile phase A (0.1 % formic acid), followed by 0.1-min gradient from 40 to 90 % mobile phase B and 5-min 90 % mobile phase B.

SRM-MS assays are performed on a TSQ Vantage QQQ-MS equipped with a nanospray source (ThermoScientific, San Jose, CA). The TSQ Vantage mass spectrometer is operated in high-resolution SRM mode with Q1 and Q3 set to 0.2 and 0.7-Da Full Width Half Maximum. All acquisition methods used the following parameters: 1,800 V ion spray voltage, a 275 °C ion transferring tube temperature, a collision-activated dissociation (CID) pressure at 1.5 mTorr, the CE of each transition uses the optimal voltage and the S-lens voltage uses the values in S-lens table generated during MS calibration.

Assay specificity: Once the SRM assays have been developed and optimized, the specificity of the assays should be evaluated on a real sample such as cell extract. With the use of crude, unpurified heavy peptides as internal standards, the specificity and accuracy of SRM assays in highly complex samples can be validated by manual

examination of the raw data. Native peptides and their heavy analogs have identical chromatographic retention time because of their physiochemical similarities. Under the same CE and CID pressure, the native and the heavy peptide dissociate to generate the same pattern of product ions, which differ only by *m*/*z* (reflecting addition of the stable isotope-labeled amino acids) and absolute intensity. Importantly, the relative intensities of the complement of product ions formed by each native peptide and its heavy analog are nearly identical. To test specificity in complex mixtures, we conduct an LC-SRM-MS analysis of native and stable isotope-labeled standard within the background of a cellular extract.

Four analyte-specific criteria—precursor ion *m*/*z*, product ion *m*/*z*, chromatographic retention time, and the relative product ion intensities—are used for evaluating the specificity of SRM assay and the certainty of peak assignment (Zhao and Brasier 2013). The native peptides and their heavy analogs should have the same chromatographic retention time (variance below 0.05 min) and relative product ion intensities (±20 % variance in the relative ratios for each fragment).

For quantification, the peak area in the extract ion chromatography of the native and SIS version of each signature peptide are integrated using Xcalibur® 2.1. The default values for noise percentage and base-line subtraction window were used. The ratio between the peak area of native and SIS version of each peptide are calculated. Beta-actin is used as loading control. All of the measured natives versus SIS standard peptide ratios are normalized by beta-actin.

15.5.4 Quantification of Activation of NF-κB and IRF3 Pathways Using SID-SRM-MS

The nuclear translocation of transcription factors (NF-κB/RelA, IRF3, and p52) and degradation of cytoplasmic NFKBIA/IκBα are the signatures of the activation of NF-κB and IRF3 pathways. SID-SRM-MS is an ideal approach to quantify the temporal and cellular compartment specific changes in the protein abundance. In a recent study, we used SID-SRM-MS to quantify the activation of NF-κB and IRF3 pathways in response to dsRNA by measuring the temporal and cellular compartment specific changes of NF-κB/RelA, IRF3, p52, IκBα, and other members of these two pathways (Zhao et al. 2013). As shown in Fig. 15.4, by this quantitative measure, IRF3 and RelA showed an increased amount in nucleus after dsRNA treatment. The accumulation of RelA and IRF3 was accompanied by gradual depletion of cytoplasmic RelA and IRF3. Meanwhile, SID-SRM-MS analysis of cytoplamic IκBα reveals that after stimulation of dsRNA for 30 min, the amount of IκBα in cytoplasm was dramatically decreased, becoming virtually undetectable 30 min after induction with dsRNA, which consists with the rapid degradation of IκBα and the initiation of RelA nuclear translocation. Taking together, these observations suggest that dsRNA activated canonical NF-κB pathway and IRF3 pathways. More importantly, our study revealed that the nuclear translocation of RelA and IRF3 was asynchronous despite of the common PRR-dependent activation of IRF3 and NF-κB translocation.

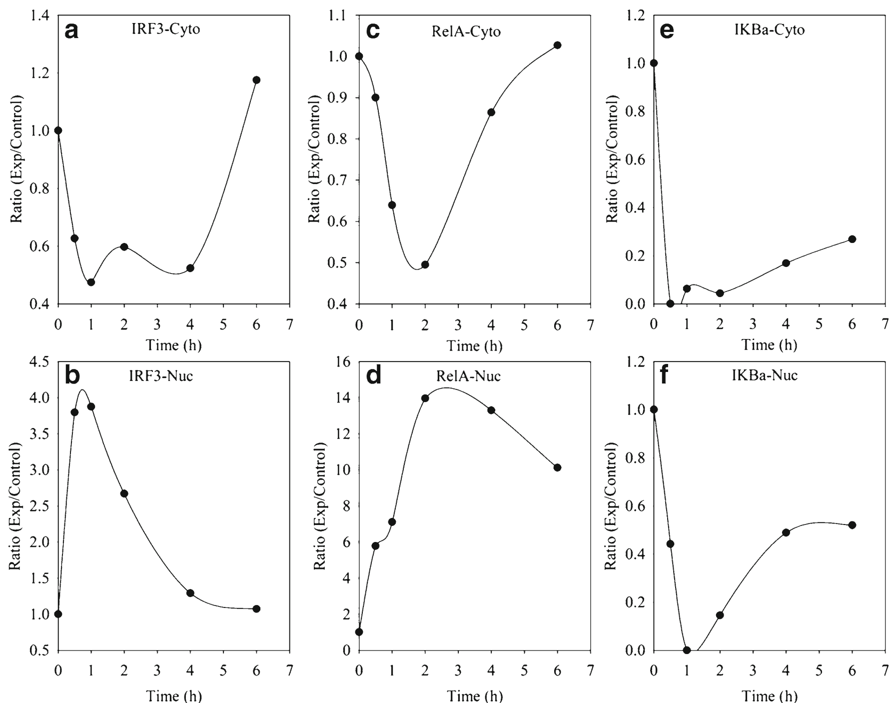

Fig. 15.4 Dynamics of innate immune response to the stimulation of dsRNA. The temporal profiles of cytoplasmic (Cyto) IRF3 (**a**) and nuclear (Nuc) IRF3 (**b**), cytoplasmic RelA (**c**) and nuclear RelA (**d**), cytoplasmic IκBα (**e**), and nuclear IκBα (**f**), derived from SID-SRM-MS analysis. Beta-actin was used to normalize between samples in SID-SRM-MS analysis. From Zhao et al. (2013)

SID-SRM-MS assays are also very useful in identifying how a pathway responses to different stimuli. For example, we used SID-SRM-MS to quantify p52 and p100 in dsRNA or TNF-treated cells (Zhao et al. 2013). Our data show that dsRNA treatment led to a time-dependent increase in the nuclear accumulation of p52. Meanwhile, the level of nuclear p100 was barely changed (Fig. 15.5). The accumulation of p52 suggests that p52 processing and nuclear translocation are induced by dsRNA. In the control experiment, p52 processing and nuclear translocation were not induced by TNF stimulation (Fig. 15.5). These data indicate that noncanonical NF-κB pathway was activated by dsRNA, but not by TNF.

Quantification of Activated NF-κB/RelA Complex Using SSDNA Aptamer-SID-SRM-MS

Another strategy for measurement of the activated IIR is to apply a method for quantification of the activated state of the NF-κB transcriptional effector. To accomplish this, we have discovered and characterized an ssDNA aptamer (termed P028F4) that binds to the activated (IκBα-dissociated) form of RelA with a K_D of

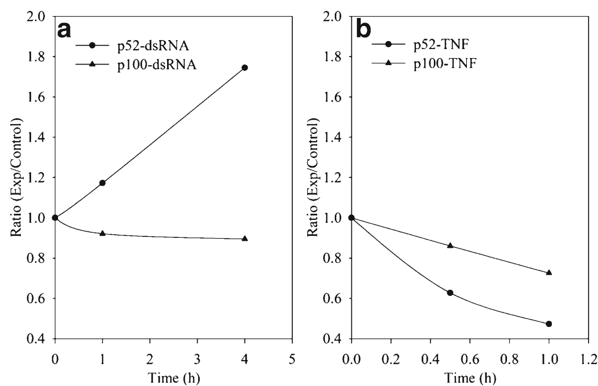

Fig. 15.5 Activation of noncanonical NF-κB pathway by the stimulation of dsRNA. A549 cells were treated with dsRNA and TNF, respectively. The nuclear proteins were extracted and subjected to SID-SRM-MS analysis. (**a**) The temporal profiles of nuclear p52 and p100 in the cells treated with dsRNA, derived from SID-SRM-MS analysis. (**b**) The temporal profiles of nuclear p52 and p100 in the cells treated with TNF, derived from SID-SRM-MS analysis. From Zhao et al. (2013)

6.4×10^{-10}. ssDNA P028F4 competes with cognate duplex high-affinity NF-κB-binding sites for RelA binding in vitro, binds activated RelA in eukaryotic nuclei, and reduces TNFα-stimulated endogenous NF-κB-dependent gene expression. This aptamer was used to develop an aptamer-SID-SRM-MS assay for quantifying the fraction of activated RelA in subcellular extracts (Zhao et al. 2011).

In this assay, aptamer P028F4 is synthesized as a biotin conjugate and incubated with subcelluar extract of airway cells. The aptamer-activated NF-κB/RelA complex is isolated by the addition of streptavidin-conjugated magnetic beads. The proteins are trypsinized on-beads, and RelA and its associated proteins were quantified with SID-SRM-MS (Fig. 15.6). In comparison to nonspecific aptamer, the single-step enrichment with P028F4 aptamer resulted in 14–30-fold enrichment of activated RelA in cytoplasmic and nuclear protein extracts. With the quantification of SID-SRM-MS, we found that, in unstimulated cells, about 5 % of total RelA proteins in the cytoplasm were activated, and only a small amount of activated (and total) RelA was in the nucleus. By contrast, after TNFα stimulation, the amount of activated RelA in CE increased about sixfold. We also observed a significant amount of activated RelA translocated into the nucleus, where the amount of activated RelA was the same as the total amount of nuclear RelA.

Compared to direct quantification of RelA and associated proteins from crude cell extracts, aptamer-based enrichment approach has several advantages. First, using a RelA-specific aptamer to enrich RelA and its associated proteins prior to SRM-MS analysis dramatically reduces the sample complexity and hence improves the signal-to-noise ratio of endogenous RelA 36-fold. Second, aptamer enrichment also reduces the likelihood of the interference of other analytes, which have isobaric (or very similar *m*/*z* values) to the target peptides. Therefore, the

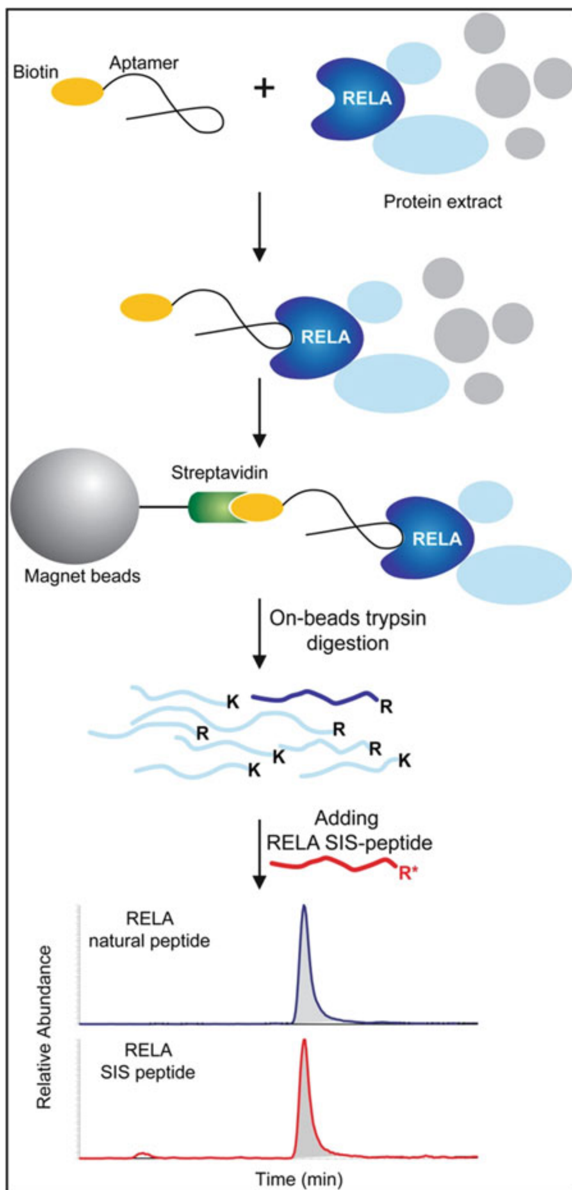

Fig. 15.6 Schematic diagram of the aptamer-SID-SRM-MS assay for quantification of activated NF-κB/RelA complex. From Zhao et al. (2011)

specificity and accuracy of SRM assay are significantly improved. Third, aptamer enrichment allows RelA to be isolated from a large amount of protein extract so that the amount of endogenous RelA peptide falls in the middle of the linear dynamic range of the calibration curve, which improves the accuracy and

reproducibility of quantification. This feature is especially attractive for quantification of low-abundance proteins, such as RelA or p300 transcription factors, in complex protein mixtures. Finally, because P028F4 selectively recognizes the IκBα-free, activated form of RelA, we are able to quantify the abundance of the activated form of RelA. This measurement cannot be accomplished by directly analyzing RelA abundance in crude cellular (or subcellular) extracts. In summary, the aptamer-SID-SRM-MS assay is a versatile tool for quantification of activated NF-κB/RelA and its associated complexes in response to IIR activation.

15.6 Conclusions and Perspectives

The IIR plays a central role in airway physiology and homeostasis. Because of its central role in activating and shaping the adaptive immune response, including allergic sensitization/atopy, much attention is being placed on dysregulation of the IIR in asthma. Here, we have described several quantitative techniques to profile the IIR, including Q-RT-PCR, multiplex bead-based analysis of secreted proteins/cytokines, and SRM-MS assays. Application of these methods for quantitation of the IIR will further our understanding of its role in asthma and contribution to heterogeneity.

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Chapter 16
Functional Proteomics for the Characterization of Impaired Cellular Responses to Glucocorticoids in Asthma

Konrad Pazdrak and Alexander Kurosky

Abstract In chronic airway inflammatory disorders, such as asthma, glucocorticoid (GC) insensitivity is a challenging clinical problem associated with life-threatening disease progression and the potential development of serious side effects. The mechanism of steroid resistance in asthma remains unclear and may be multifactorial. Excluding noncompliance with GC treatment, abnormal steroid pharmacokinetics, and rare genetic defects in the glucocorticoid receptor (GR), the majority of GC insensitivity in asthma can be attributed to secondary defects related to GR function. Airway inflammatory cells obtained from patients with GC-resistant asthma show a number of abnormalities in cell immune responses to GC, which suggests that there is a causative defect in GR signaling in GC-resistant cells that could be further elucidated by a functional and molecular proteomics approach. Since T cells, eosinophils, and monocytes play a major role in the pathogenesis of airway inflammation, most of the work published to date has focused on these cell types as the primary therapeutic targets in GC-insensitive asthma. We herein review several distinct techniques for the assessment of (1) the cellular response to GCs including the effect of GCs on cell viability, adhesion, and mediator release; (2) the functionality of GC receptors, including phosphorylation of the GR, nuclear translocation, and binding activities; and (3) the characterization of proteins differentially expressed in steroid-resistant cells by comparative 2DE-gel electrophoresis-based techniques and mass

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spectrometry. These comprehensive approaches are expected to reveal novel candidates for biomarkers of steroid insensitivity, which may lead to the development of effective therapeutic interventions for patients with chronic steroid-resistant asthma.

Keywords Steroid resistance • Glucocorticoids • Proteomics • Asthma • Mass spectrometry • Cell signaling pathways • Biomarkers • Eosinophils • Airway inflammatory cells • Phosphoproteomics

Abbreviations

7-AAD	7-Aminoactinomycin D
2DE	2-Dimensional electrophoresis
ADAM	A Disintegrin and metalloprotease domain family gene/protein
ASK1	Apoptosis signal-regulating kinase 1
CCL	C–C motif ligand
Cdk	Cyclin-dependent kinase
COPD	Chronic obstructive pulmonary disease
GC	Glucocorticoid
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HDAC	Histone deacetylase
HPLC	High-performance liquid chromatography
HSP90	Heat-shock protein 90
IFN	Interferon
IL	Interleukin
JNK	c-JUN N-terminal kinase
LPS	Lipopolysaccharide
MKP-1	MAP kinase phosphatase 1
MS	Mass spectrometry
PBMC	Peripheral blood mononuclear cells
PE	Phycoerythrin
PP5	Protein phosphatase 5
T2M	Type 2 myeloid cell
TNFα	Tumor necrosis factor α

16.1 Introduction

The administration of glucocorticoids (GCs) has long been a very effective anti-inflammatory therapy in clinical medicine and remains the cornerstone of anti-allergic and immunosuppresive treatment for bronchial asthma (Barnes and Adcock 2003). While the majority of patients with asthma respond favorably to

either inhaled or systemic steroid therapy, approximately 5–10 % of patients suffer from a severe asthma that is refractory to GCs resulting in a poor clinical outcome even to high doses of GCs (Kupczyk and Wenzel 2012; Bel et al. 2011; Wenzel 2012). Severe GC-refractory asthmatics have persistent symptoms, frequent exacerbations, and are at higher risk of mortality (Haselkorn et al. 2009). The lack of effective therapeutic alternatives for these patients poses not only a significant health problem but is also a financial burden to society as they account for more than 50 % of asthma-related health care costs (Godard et al. 2002; Heaney and Robinson 1976). The observed persistent airway obstruction in asthma is related to a cellular airway inflammatory profile characterized, in part, by an increased number of activated eosinophils observed in bronchoalveolar lavage fluid, bronchial biopsies, and induced sputum. There is also a significant increase in the CD4+/CD8+ ratio of T-cell infiltration in the airway mucosa, a thicker reticular layer of epithelial basement membrane, and increased T-helper type lymphocytes. T-helper lymphocyte-derived cytokines are known to regulate processes leading to mast-cell sensitization and the development of eosinophilic inflammation that characterizes allergic airways. While GCs affect virtually all primary and secondary immune cells during the treatment of bronchial asthma, their most beneficial effect strongly correlates with a decrease in the activation of T cells and lowering the number of both circulating and tissue eosinophils and macrophages (Louis et al. 2002; Quaedvlieg et al. 2009; Deykin et al. 2005). Although these three types of inflammatory cells have been considered to be critical targets of glucocorticoid therapy in asthma, several recent studies have also identified a contribution of airway epithelial cells, smooth muscle cells, and dendritic cells to steroid resistance (Bouazza et al. 2012; Gross et al. 2009; Lu and Cidlowski 2004, 2006). For example, airway smooth muscle cells exposed to proinflammatory cytokines showed impairment of both glucocorticoid receptor phosphorylation and glucocorticoid-dependent gene transcription (Tliba et al. 2008). Bronchial epithelial cells exposed to cytokines TNFα, IL-1β, or cigarette smoke exhibit attenuated GRE-dependent transactivating response to GC (Rider et al. 2011). There are at least eight functional GR isoforms, which occur as a result of translational mechanisms and it has been proposed that the glucocorticoid receptor in dendritic cells also has several transcriptional activities, which can potentially give rise to abnormal regulation and function that can cause chronic inflammatory diseases (Lu and Cidlowski 2004, 2005). This chapter describes the functional proteomic features that identify proteins that are differentially expressed and/or modified in GR-insensitive cells. Our laboratory has particularly focused on studies of blood-derived cells rather than cells obtained by bronchoscopy as a more practical approach for the identification of asthmatics with steroid resistance in clinical practice. Furthermore, the characterizations of those molecules involved in the mechanisms by which GCs fail to resolve inflammation in asthma will provide important insights into the pathogenesis of asthma, especially as it relates to the progressive deterioration of lung function.

16.2 Cellular Responses in Glucocorticoid-Resistant Asthma

16.2.1 Lymphocytes

Clinical studies have demonstrated that treatment of most patients with airway inflammatory disease such as asthma or COPD with GCs leads to decreased numbers of airway eosinophils and suppression of T-cell function (Keatings et al. 1996; Krouwels et al. 1996; Kelly et al. 2000). In allergic patients, GC treatment blocks the occurrence of the late-phase asthmatic response following allergen challenge. A significant feature of T lymphocytes in steroid-resistant asthma is their increased level of activation and unresponsiveness to GC treatment; T lymphocytes obtained from patients with steroid-resistant asthma also show increased proliferation in response to phytohemagglutinin with a relative inability of GCs to alter this process (Syed et al. 1998). GCs also induce the necrosis of lymphocytes and their removal by macrophages upon prolonged GC exposure (Cidlowski et al. 2000; Huang and Cidlowski 2002). This response contrasts with that observed in T cells from patients with steroid-responsive asthma, which typically shows a 60 % decrease in T-cell proliferation upon treatment with methylprednisolone (Kam et al. 1993). Peripheral blood T cells from GC-resistant asthmatics are also characterized by an increased expression of the CD25 (IL-2 receptor) activation antigen (Chakir et al. 2002). A significant clinical finding relating to steroid resistance was the increased expression of IL-2 and IL-4 in the airways of patients with steroid-resistant asthma (Kam et al. 1993). In this mechanism, the therapeutic, anti-inflammatory response to GCs was attributed to inhibition of cytokine secretion, mainly from IL-2 and IL-4 secreting T cells. Failure to decrease production of airway IL-2, IL-4, and IL-5 after GC therapy was further proposed as a strong indicator of a potentially poor clinical response to GC therapy (Leung et al. 1995).

16.2.2 Eosinophils

Increased recruitment of eosinophils into sites of allergic reaction and their prolonged cell survival are major mechanisms contributing to the expansion of eosinophil numbers within tissues. Normal, nonactivated eosinophils are extremely sensitive to GCs resulting in eosinophil apoptosis within hours of exposure to GCs in vitro and their subsequent uptake by macrophages (Schleimer and Bochner 1994; Brode et al. 2010). On the other hand, activated eosinophils (e.g., by IL-5 in vitro or in vivo) show prolonged viability (5–9 days) even in the presence of GCs, although even activated eosinophils eventually enter apoptosis at a faster rate in the presence of GCs when compared with activated cells not exposed to GCs (Brode et al. 2010). Another mechanism contributing to the action of GCs on airway eosinophilia, in addition to the induction of eosinophil apoptosis, is the inhibition of β2-integrin-mediated eosinophil adhesion as well as the activation and stimulation

of the noninflammatory phagocytosis of apoptotic cells by macrophages (Schleimer 1993; Schleimer and Bochner 1994). It is thought that a decreased eosinopenic response to GCs is due to impairment of all GC-induced proapoptotic and adhesion suppressing functions (Brightling et al. 2005; Green et al. 2002); however, the relative contribution of each of these processes and their molecular mechanisms are as yet unknown. Notably, the eosinophil count in airways is currently one of the few biomarkers available for predicting the GC response in asthma therapy (Brightling et al. 2005; Green et al. 2002).

16.2.3 Monocytes/Macrophages

Early studies demonstrated that monocytes release soluble factors that prime neutrophils for leukotriene production and this process is differentially affected by GC treatment of patients with GC-sensitive and GC-resistant asthma (Hallsworth et al. 1994; Lane et al. 1993). Furthermore, monocytes from GC-resistant asthmatics show persistent IL-8 and TNFα production even with GC treatment. Several studies with animal models of asthma involving monocyte-derived macrophages suggested a role for activated macrophages (M2) in asthmatic inflammation (Kurowska-Stolarska et al. 2009; Prasse et al. 2007); however, more recent translational studies did not support the existence of an M2 phenotype in human asthma as none of the M2 biomarkers (IL-10, CCL17, and CCL22) were differentially expressed (Staples et al. 2012). In the latter study, overexpression of CCL17 mRNA was found in patients with moderate asthma that correlated weakly with sputum eosinophilia but was not responsive to steroid therapy (Staples et al. 2012). More recent studies utilized differential gene expression in PBMCs to accurately predict nonresponders to inhaled steroids through analysis of severe asthma phenotypes (Goleva et al. 2012). Comparison of the mRNA profiles in monocytes from GC-resistant asthma revealed that monocytes had higher MKP-1, IL-8, and GCRβ mRNA levels than cells obtained from GC-sensitive asthma. All told, a set of 15 cytokine genes was identified that could predict nonresponders with 84 % accuracy; however, these results have yet to be confirmed by other investigators. One major limitation of this study was that selected subjects used in the study were naïve to steroids and 30 days after oral prednisone treatment there was no differences in the expression of some 15 cytokine genes previously observed.

16.2.4 Neutrophils

Several reports show that neutrophils are less sensitive to glucocorticoids than eosinophils and T cells, which explain their prominent role in the pathophysiology of COPD and severe asthma that involve neutrophilic inflammation. Early studies show that GC treatment prolongs neutrophils survival thereby enhancing airway neutrophilia (Cox

1995; Meagher et al. 1996). Moreover, a recent comparison of neutrophils with other blood leukocytes revealed no difference in the genomic response to glucocorticoids when compared with other blood leukocytes (Hirsch et al. 2012). Thus, the poor response to GCs observed in severe asthma or COPD is not due to a relative lack of inhibition of GCs on proinflammatory cytokines expression in neutrophils. One possible explanation may involve activation of neutrophils and monocytes due to infection and exposure to bacterial lipopolysaccharides (LPS) that trigger airway inflammation refractory to GC treatment (Bozinovski et al. 2005).

16.2.5 Other Airway Inflammatory Cells

GC therapy was also shown to reduce the numbers of mast cells in airway mucosa biopsies from mild atopic asthmatics. Since human mast cells and basophils are also significant sources of IL-4 and IL-5, several studies addressed the potential of GC therapy to modulate the release of these mediators (Schleimer et al. 1989; Yoshikawa et al. 1999). Short incubation of basophils with GCs inhibited the release of IL-4 while long preincubation with GCs inhibited the IgE-mediated release of histamine. In addition, steroid treatment of mast cells did not inhibit mast cell mediator release in vivo or in vitro. Interestingly, recent report of a new myeloid type 2 (T2M) cell indicated a possible role for this granulocyte in mediating steroid-resistant inflammation in the airway. In allergen-sensitized animals, these granulocytes were capable of releasing IL-4 and IL-13 in a steroid-independent and IL-25-dependent manner (Petersen et al. 2012). Moreover, an increased number of T2M was detected in patients with asthma (Petersen et al. 2012). In addition to inflammatory and immunoregulatory cells, structural cells were also shown to play a role in airway inflammation. Studies performed on bronchial biopsies from severe asthmatics showed an increased expression of eotaxin, CCL19, ADAM33, and ADAM8 in the airway smooth muscle layer and upregulation of these genes were resistant to GC therapy (Clarke and Dodson 2007; Tliba et al. 2008). Expression of these mediators correlated with the severity of asthma and could be replicated in vitro upon exposure of smooth muscle cells to TNFα and IFNγ. Of interest, this in vitro-induced state of GC resistance was accompanied by a suppression of GC receptor-mediated transactivation activities.

16.3 Glucocorticoid Receptor Signaling in Steroid-Resistant Asthma

The mechanism of steroid resistance in asthma remains unclear currently and is thought to be multifactorial (Durham et al. 2011; Poon et al. 2012). Once one excludes noncompliance of GC treatment, abnormal steroid pharmacokinetics, and rare genetic defects in the GR, the majority of GC insensitivity in asthma can be

attributed to secondary defects in GR expression and/or function. Under GC-responsive conditions, the anti-inflammatory effects of GCs are mediated via the GR, which is a ligand-dependent transcription factor (Rhen and Cidlowski 2005). GCs interact with the GR primarily in the cytoplasm resulting in the phosphorylation and translocation of the hormone–receptor complex into the cell nucleus. Three of the six serine residues in the GR molecule are known to be involved in the transcriptional activity of the GR and are substrates for Cdk2 (S203, S211), p38 (S211) and JNK (S226) (Galliher-Beckley et al. 2008; Ismaili and Garabedian 2004; Wang et al. 2002). GR dephosphorylation of S203 and S226 is regulated by Protein Phosphatase 5 (PP5), whereas the phosphatase that dephosphorylates S211 is as yet unidentified (Chen et al. 1996; Silverstein et al. 1997). PP5 also binds to HSP90 and ASK1 and participates in GR nucleocytoplasmic shuttling. Separate studies support the concept that GC-insensitive asthma correlates with an increased expression of the proinflammatory cytokines, IL-2, IL-4, IL-13, and TNFα in the airways (Leung et al. 1995; Naseer et al. 1997; Spahn et al. 1996; Kam et al. 1993; Bhavsar et al. 2008); however, the molecular mechanism responsible for impaired GC signaling associated with these cytokines is not well understood. Several studies have pointed to a defect in GR–ligand binding (Cho and Lee 2003), diminished GR nuclear translocation (Matthews et al. 2004), GR–DNA binding (29, 31, 33), and competition for binding with splice variants of GR (Leung et al. 1997). GR signaling abnormalities were investigated in the context of nuclear signaling, aberrant GR expression, and suppression of GC-inducible genes in fibroblasts, epithelial cells, smooth muscle cells, and mononuclear cells, with little or no investigations of posttranslational modifications of the GR in cells undergoing apoptosis upon GC treatment (11, 16, 57, 60). Additionally, the diminished expression of proteins involved in the suppression of inflammatory genes (e.g., histone deacetylase 2, HDAC2) or overexpression of proinflammatory transcription factors (e.g., NF-κB), and overexpression of the decoy GC receptor (GCRβ) were also proposed as contributing to steroid resistance in chronic inflammation (Ref). More recent studies, however, failed to confirm the differential expression of GRβ or HDAC2 in the airways of patients with severe asthma (Butler et al. 2012).

16.4 In Vitro Cellular Models of Steroid Resistance

Since GC-resistant patients with asthma showed an increased number of cells expressing IL-2, IL-4, and IL-13 mRNA, these finding were used to create in vitro models of steroid resistance (Kam et al. 1993; Zubiaga et al. 1992; Goleva et al. 2006, 2009). These models showed that the combination of IL-2 and IL-4 or IL-13 alone induced a reduction in the binding of GC to its receptor, suppressed GR nuclear translocation, and reduced the affinity of nuclear GRs in peripheral blood mononuclear cells. Similar findings were reported for T cells, monocytes, and fibroblasts and in several studies an increased expression of the inactive GRβ isoform was reported (Kam et al. 1993; Zubiaga et al. 1992). The increased expression of

IL-5 was indicated in mediating resistance of eosinophils to GC-induced apoptosis (Blom et al. 1994). As mentioned above, airway smooth muscle cells became insensitive to GCs upon incubation with TNFα and IFNγ; this model showed impaired phosphorylation of GR in steroid-resistant cells correlated with cytokine activation (Tliba et al. 2008).

16.5 Cell Function Assays Related to Steroid Resistance

16.5.1 GC-Induced Apoptosis

Eosinophils respond to GCs with increased apoptosis. Survival of T cells is also diminished upon exposure to GCs. An Annexin V^{PE} apoptosis detection kit (BD Biosciences) is commonly used to quantitatively determine eosinophils undergoing apoptosis by virtue of their ability to bind Annexin V and exclude 7-aminoactinomycin D (7-AAD) (Pazdrak et al. 1998, 2008). This assay detects viable cells (Annexin V-negative/7-AAD–negative), cells undergoing early apoptosis (Annexin V-positive/7-AAD–negative), and dead cells (Annexin V-positive/7-AAD–positive). Eosinophils (2×10^5) suspended in RPMI 1640 supplemented with 10 % FBS are collected 24 or 48 h after exposure to dexamethasone at concentration ranging from 10 nM to 1 mM. Cells are stained with Annexin V and 7-ADD according to the manufacturer's instructions. Data are acquired on a FACScan instrument (BD Biosciences) and analyzed using CellQuest software (BD Biosciences) with acquisition of 10,000 events per sample. About 20–30 % of nonstimulated eosinophils enter apoptosis after 24 h of culture and with dexamethasone treatment apoptic cells increases of up to 70–90 %. Prosurvival cytokines such as IL-5, IL-2 or IL-4 largely inhibit eosinophil apoptosis although longer culture times can result in dexamethasone-induced apoptosis.

16.5.2 Upregulation of Integrins

Integrins such as CD11b/CD18 ($\alpha_M\beta_2$) are constitutively expressed on human monocytes, macrophages, and eosinophils and their increased expression correlates with cell activation and response to steroid therapy (Brode et al. 2010; Sale et al. 2004). Moreover, enhanced expression of CD11b on PBMCs was reported to correlate with steroid insensitivity (Brode et al. 2010; Goleva et al. 2012). Measurement of the expression of CD11b on the surface of eosinophils can be performed after staining cells with PE-conjugated anti-$\alpha_M\beta_2$ mAb (Santa Cruz Biotechnology). For each population, a matched isotype control mAb is used to define CD11b negative cells. Eosinophils are analyzed using a FACSAria instrument (BD Biosciences), with at least 30,000 events collected per sample. The data are further analyzed using CellQuest software.

16.5.3 Mediator Release

For cytokine measurements we typically use ELISA or bead-based immunoassays. Eosinophils suspended at a concentration of 1×10^6cells/ml are cultured for at least 24 h in the presence of IL-5 or combination of IL-2/IL-4 in the absence or presence of dexamethasone followed by collection of the supernatants (200 ml) and cell debris clarification by centrifugation at $300 \times g$ for 10 min at 4 °C. Alternatively, bead-based assays using Bioplex instrumentation (Bio-Rad) can be used to detect panels of up to 25 human cytokines (Invitrogen, Carlsbad, CA). Quantification is performed relative to recombinant standards supplied with the commercial assay kit. For this purpose, duplicate samples and serial dilutions of the cytokine standards (50 μl) are incubated with antihuman cytokine-coated beads in 96-well filtration plate (Millipore, Bedford, MA) for 30 min.

16.6 Glucocorticoid Receptor Function

16.6.1 GR Phosphorylation and Nuclear Translocation

Several reports strongly suggest that the phosphorylation of the GC receptor on specific residues is impaired in cells with cytokine-induced steroid resistance (Bouazza et al. 2012; Ismaili and Garabedian 2004; Wang et al. 2007). The phosphorylation of GR on residues Ser203, Ser211, and Ser226 can be assessed by Western blot analysis with commercially available phosphospecific antibodies (Abcam, Cambridge, MA). Activated and control eosinophils are stimulated with dexamethasone for 0.5–1 h followed by centrifugation and subcellular fractionation using Calbiochem's ProteoExtract® Subcellular Proteome Extraction kit according to the manufacturer's protocol. Cytoplasmic and nuclear fraction are further resolved on SDS-PAGE and subjected to Western blot analysis with phosphospecific anti-GR antibodies. Nuclear translocation can be investigated by detecting the distribution of the GC receptor in the cytoplasmic and nuclear fractions of eosinophils providing the relative determination of impaired phosphorylation as a result of nuclear translocation.

16.6.2 DNA Binding Activity

Nuclear extracts obtained from eosinophils stimulated with dexamethasone at 1 mM for 1 h are used for this assay. Oligonucleotides containing GRE sequences for the MKP1 promoter modified with 3′ terminal biotinylation and its complementary strand are commercially synthesized (BioSource International, Camarelo, CA). After annealing the two single-stranded oligonucleotides, the double-stranded

oligonucleotides are incubated with streptavidin-conjugated agarose beads (Pierce, Rockford, IL) for 1 h at 4 °C and washed twice with cell lysis buffer [20 mM Tris–HCl (pH 7.5), containing 2 mmol EDTA, 2 mmol ethylene glycol-bis (-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10 mmol benzamidine, 5 mmol DTT, 1 mmol PMSF, 50 mmol NaF, 5 mmol $Na_4P_2O_7$, 1 mmol Na_3VO_4, 100 μg/ml aprotinin, 100 μg/ml leupeptin, and 1 % Nonidet P-40]. A nuclear extract (20 μg) suspended in 300 μl of cell lysis buffer is precleared with agarose beads for 1 h at 4 °C to remove any nonspecific binding to the beads. The lysates are then incubated with streptavidin-conjugated beads for 1 h at 4 °C. The beads are then washed three times with cell lysis buffer, and the adsorbed DNA is then eluted by boiling in Laemmli buffer for 4 min at 95 °C and subjected to Western blot analysis with anti-GR antibodies.

16.7 Comparative Proteomic Analysis of Steroid-Resistant and Sensitive Cells

Advances in deep profiling of the cellular proteome offer an opportunity to profile the proteome of cells differentially expressing proteins and their modifications that display impaired responses to GCs. An important outcome of such comparative expression studies is the discovery and validation of biomarkers that would indicate the state of cell activation and the patient's prognosis to GC therapy. Moreover, such an initiative would define altered GR signaling pathways associated with cell activation in the course of airway inflammation. Proteomics technologies available are well described (Straub et al. 2009, 2011).

16.7.1 Phosphoproteomic Analysis

Routinely two complementary and partially overlapping methods are needed to identify phosphoproteins that convey signals for steroid resistance. The first method uses a comparative proteomics approach employing phosphospecific ProQ Diamond fluorescent staining after 2DE gel electrophoresis to visualize phosphorylated proteins. The second method utilizes metal affinity chromatography, e.g., TiO_2 affinity, for phosphopeptide enrichment (ProteoExtract Phosphopeptide TiO_2 Enrichment Kit, Calbiochem), followed by analysis with a C18 LC system coupled online with a linear ion trap (LTQ)-Orbitrap Velos mass spectrometer (ThermoFinnegan). This comparative approach can identify differentially phosphorylated and unphosphorylated proteins in steroid-resistant cells from patients with asthma or in cells with in vitro induced steroid resistance and can be applied for the identification of potential protein biomarkers of steroid resistance. Moreover, these cells can also be analyzed after stimulation with dexamethasone to identify dexamethasone-regulated proteins. An identified set of up or down expressed proteins and phosphoproteins may

be subsequently compared with GC-sensitive and GC-resistant cells to identify potential defects on GC signaling and treatment response. Cells are typically stimulated (eosinophils or PBMCs) with nine doses of dexamethasone (1 μM) for 24 h. Cells are then collected and lysed in DeStreak rehydration buffer and frozen (−70 °C) before 2DE gel analysis. Whole cell lysates are subsequently fractionated over an isoelectric focusing range of 3–11 in the first dimension followed by SDS-PAGE as described previously (Straub et al. 2009). The resultant 2DE gels are then stained with ProQ Diamond, imaged, and then stained with Sypro Ruby and reimaged. The images of stained phosphoproteins are acquired by a Fuji 5100 imager and are then subjected to comparative analysis using Nonlinear SameSpots software (Nonlinear, USA Inc., Durham, NC). The alternative method of phosphoprotein detection and identification utilizes TiO_2-phosphopeptide enrichment (ProteoExtract Phosphopeptide TiO_2 Enrichment Kit, Calbiochem), followed by MS analysis using a LTQ-Orbitrap Velos mass spectrometer. Database searching for collected MS and MS2 data is carried out using a MASCOT search engine against the concatenated forward and reverse human genome sequence database. The fragmentation spectra of potential phosphopeptides are manually verified for both the presence of a phosphate group and the peptide sequence using previously described criteria (Macek et al. 2008).

16.7.2 Differential Protein Expression

After staining gels with ProQ Diamond for detection of phosphoproteins, the identical gels are stained with Sypro Ruby for detecting total protein abundance. The acquired images (Fuji 5100) are subjected to comparative analysis using SameSpots software. For each 2DE gel analysis, the change (the ratio of the mean normalized protein spot volume of control vs. activated cells) and paired *t*-test are calculated (adjusted for multiple-hypothesis testing using the Bonferroni correction) for spots identified in samples from each analyzed group (Horgan 2007). We routinely focus on identifying proteins whose activation-related changes are >2-fold vs. proteins protein expressed in eosinophils from healthy donors, and whose corrected *t*-test statistics are <0.05. To determine whether 2DE gel protein expression data extracted from the analyzed groups reflect differences due to eosinophil activation or treatment with inhibitors, the spots from the control and stimulated cells are matched, and the normalized spot volumes are subjected to hierarchical clustering. In this technique, the protein expression profile for an individual 2DE gel is grouped to its nearest neighbor based on the mathematical proximity of the two gels. The gels are combined into a single node, and the node is then grouped with the next-nearest gel. This process is repeated iteratively until all gels are contained within one dendrogram. This clustering algorithm detects major groups of protein spots reflecting reproducible differences representative of the effect of cell stimulation or inhibition. Database interrogation for collected MS and MS2 data are routinely carried out using a MASCOT search engine against the concatenated forward and reverse

human genome sequence database. MASCOT is configured to allow for the posttranslational modification of Met, Ser, Thr, and Tyr residues. The fragmentation spectra of potential phosphopeptides are manually verified for both the presence of a phosphate group and the peptide sequence using the previously described criteria (Macek et al. 2008).

16.8 Conclusions and Perspectives

Airway inflammatory pathways that are repressed by GR signaling are strongly implicated in asthma pathogenesis and therapeutic responses to glucocorticoids. Clarifying the key molecular events regulating GC signaling during inflammatory processes should prove invaluable in designing therapeutic strategies for decreasing the inflammatory impact on intractable, GC-insensitive asthma. Inflammatory cells that play an important role in asthma usually respond to GCs with apoptosis and suppression of effector functions in a manner that parallels the clinical response to GC therapy. Here we have described several functional and discovery techniques to identify changes in the cell proteome that are linked to an impaired cell response to GC. Comparative proteomic techniques are especially useful for the identification of possible biomarkers of steroid resistance. Moreover, correlation of differentially expressed or phosphorylated proteins with impaired cell function may reveal novel signaling pathway regulating cell response to GC. This comprehensive approach has the potential of revealing novel components of signaling networks in steroid cells that may support future innovations and provide answers to deeper mechanistic questions.

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Part IV
Modeling Complex Data in Asthma

Suresh K. Bhavnani

Introduction

The complex multivariate nature of asthma requires powerful analytical methods for the identification, comprehension, and prediction of asthma phenotypes, in addition to aiding researchers in the inference of biological mechanisms involved in those phenotypes. This section focuses on two broad approaches to conduct such analyses: supervised learning and unsupervised learning.

Chapter 17 provides a broad overview of the motivation and methods related to supervised learning. In general, such methods are used to develop a predictive model of some outcome (e.g., severe versus non-severe asthma patients) based on some mathematical combination of the variables in the data (e.g., cytokine expression). These methods typically begin with an a priori classification of the data points (e.g., dividing patients into severe and non-severe classes), which by using appropriate statistical methods, is used to rank the variables based on the degree to which they are significantly different between the classes. This ranking of variables is used to remove variables that do not distinguish between the two or more classes, resulting in a smaller set of variables that carry strong information about the disease. As this ranked list could contain many variables that are highly correlated with each other and therefore redundant for prediction, classification methods attempt to find the smallest subset of uncorrelated variables which, in some mathematical combination, can together explain the maximum variance in the data. The resulting model is examined to make inferences about the biological mechanisms involved. This chapter concludes with a demonstration and comparison of such methods in the analysis of asthma patients and their molecular and clinical variables.

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While the above supervised methods are powerful for developing predictive models, they are dependent on the veracity of the a priori classification. Furthermore, because variables that are part of a biological pathway tend to be highly correlated, dropping correlated variables from a model could compromise inferring known or novel pathways underlying the disease.

In contrast to supervised methods, unsupervised methods do not begin with an a priori classification of the data. Instead, the goal of unsupervised methods is to reveal an unbiased structure that underlies the data. Because such methods do not use a priori classifications, they often help to identify clusters of patients that are more similar to each other compared to others and therefore suggest new phenotypes of the disease. Furthermore, because they do not drop highly correlated variables, the methods preserve critical information that aid in the inference of known and novel biological pathways.

Although unsupervised methods have been developed and used for many years, the emerging field of visual analytics tightly integrates these methods with powerful interactive visualizations designed to enable comprehension of complex patterns. Chapter 18 provides a broad overview for the motivation and methods of visual analytics and focuses on one of the most developed methods called network analysis. Because network analysis is based on graph theory, it enables both a visual and a quantitative analysis of the data using a unified representation. This chapter describes the typical steps of performing a network analysis of diseases such as asthma consisting of (1) exploratory visual analysis to identify emergent patterns in the data such as phenotypes, (2) quantitative verification of the patterns using methods suggested by the visualization, and (3) inference of biological mechanisms involved across the emergent phenotypes. A demonstration of this approach in the analysis of asthma molecular and clinical variables sheds light on the strengths and limitations of network analysis. The chapter concludes by proposing the need for future research which combines the strengths of supervised and unsupervised learning methods in an integrated approach with the goal of enabling more effective identification, comprehension, and prediction of asthma phenotypes. Such an approach could aid researchers in the inference of biological mechanisms involved in specific phenotypes and accelerate the translation of discoveries into clinical practice.

Chapter 17
Analysis and Predictive Modeling of Asthma Phenotypes

Allan R. Brasier and Hyunsu Ju

Abstract Molecular classification using robust biochemical measurements provides a level of diagnostic precision that is unattainable using indirect phenotypic measurements. Multidimensional measurements of proteins, genes, or metabolites (analytes) can identify subtle differences in the pathophysiology of patients with asthma in a way that is not otherwise possible using physiological or clinical assessments. We overview a method for relating biochemical analyte measurements to generate predictive models of discrete (categorical) clinical outcomes, a process referred to as "supervised classification." We consider problems inherent in wide (small *n* and large *p*) high-dimensional data, including the curse of dimensionality, collinearity and lack of information content. We suggest methods for reducing the data to the most informative features. We describe different approaches for phenotypic modeling, using logistic regression, classification and regression trees, random forest and nonparametric regression spline modeling. We provide guidance on post hoc model evaluation and methods to evaluate model performance using ROC curves and generalized additive models. The application of validated predictive models for outcome prediction will significantly impact the clinical management of asthma.

Keywords Multivariate analysis • Supervised learning • False discovery rate • Feature reduction • Significance of microarrays (SAM) • Receiver operating characteristic (ROC) curve • Logistic regression • Random forest • Multivariate adaptive regression splines (MARS) • Generalized additive models (GAMs)

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17.1 Introduction

The application of multidimensional profiling is a disruptive technology that enables identification of molecular phenotypes of asthma (Bhavnani et al. 2011; Brasier et al. 2008, 2010). When properly conducted and interpreted, multidimensional measurements of proteins, genes, or metabolites can identify subtle differences in the pathophysiology of asthma in a way that is not otherwise possible using physiological or clinical assessments. The molecular profiling approach holds promise for revolutionizing the diagnosis and management of asthma because it can identify robust objective, measurable, and quantitative features associated with disease phenotypes, disease progression, and treatment response.

Currently, the diagnosis and management of asthma is based on what could be considered coarse physiological assessments. Phenotypic classifications of asthma using age of onset, exacerbating factors (aspirin sensitivity, exercise induced, and occupational) cellular type of inflammation (eosinophilic vs. noneosinophilic), pattern of severity, or lack of response to conventional therapy provide little insight into mechanisms of disease or strategies for its management. In particular, the conventional ATS criteria for the diagnosis of severe asthma is based on a constellation of findings and clinical features that is better considered a syndrome than a specific disease diagnosis (ad hoc writing committee of the Assembly on 2000; Wenzel and Busse 2007). In the absence of biochemical marker measurement, the multiple underlying pathological processes that contribute to the clinical syndrome of asthma, such as allergy, inflammation, airway remodeling, smooth muscle hypertrophy, and neurogenic dysregulation, are not considered. This situation leads to the obvious result that patients carrying the diagnosis of asthma will exhibit heterogeneous clinical courses and variable medication responses. As a result, there is no reliable method for early recognition of treatment-resistant subtypes. Precise quantification of these processes at the biochemical level would significantly impact clinical management.

The ability to use precise molecular measurements to identify subtypes of disease is an exciting arena that opens the door for applications of personalized medicine. Approaches for the analysis of complex molecular profiling can be broadly broken into two distinct disciplines that have complementary, but distinct, outcomes. Supervised learning, or classification, is an analysis in which profiling is conducted in an experimental design where the outcome (phenotype) is known. Classification employs analytical techniques that use the outcomes of test cases for the generation of predictive models that classify outcome of unknown cases. One example of supervised learning would be to predict the risk of disease, or response to treatment. Unsupervised learning, by contrast, is conducted when the phenotype is not known and employs techniques to discover emergent characteristics of the data. An example of unsupervised learning would be hierarchical clustering, where natural groupings in the data are sought. This chapter focuses on the approach of classification (supervised learning). The accompanying chapter by Bhavnani et al. (Chap. 18) will treat the issue of unsupervised learning.

Here, we describe approaches for how high-dimensional data sets can be analyzed and used to develop models that link analyte measurement to discrete (categorical) outcomes. We consider problems inherent in "wide" (small *n* and large *p*) high-dimensional data, including the curse of dimensionality, collinearity, and lack of information content. We suggest methods for reducing the data to the most informative features. We describe different approaches for molecular phenotyping using logistic regression, classification and regression trees, random forest and nonparametric spline modeling. We provide guidance on post hoc model evaluation and discuss advantages and limitations of this approach.

17.2 Analytic Approach

17.2.1 Data Visualization

The focus of this discussion is how to develop robust models of physiological phenotypes. Analytic approaches for high-throughput data should recognize some of the unique features of high-dimensional data. Prior to the application of any modeling technique, high-dimensional data sets should be carefully analyzed by visualization and conventional statistical approaches to ensure data quality, detect missing data, identify outliers, determine data distributions and suggest batch effects (Spratt et al 2012). This approach is schematically diagrammed in Fig. 17.1.

17.2.2 Data Preprocessing

Data preprocessing is a method to manage high-dimensional data prior to classification approaches. High-dimensional data frequently may contain missing values, exhibit measurements with a broad dynamic range, and exhibit skewed population distributions.

The presence of missing data is characteristic of high-throughput data sets. Missing data frequently arises in mass spectrometry, for example, where observations are not made in every sample due to sampling bias, or in immunoassays, where the analyte concentration is below the limit of detection. The presence of a significant amount of missing data complicates many statistical analytic and machine learning approaches. If a few data points are missing, imputation can be used, where missing values are substituted with reasonable numerical values. However, if significant amount of data is missing, we frequently will remove the analyte from consideration, because imputation then can introduce bias.

Several different approaches can be used for imputing missing values, the selection of which depends on the data type and understanding of why the data is absent. In highly quantitative assays, such as immunoassays, values may be missing because

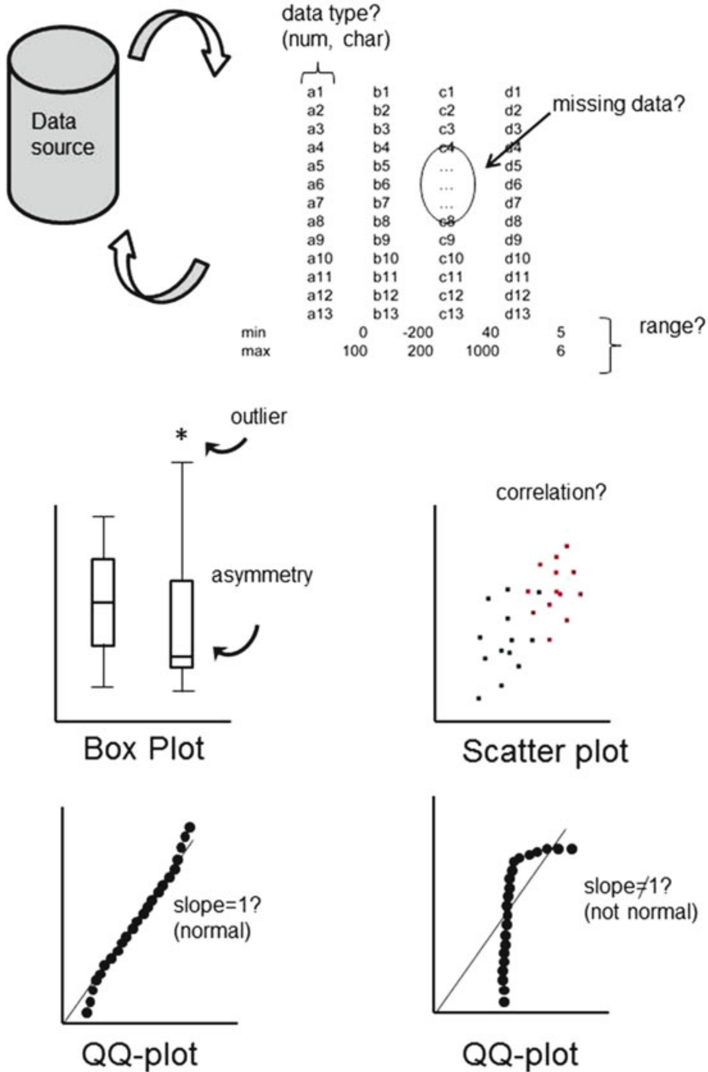

Fig. 17.1 Data preprocessing. A schematic for the general approach to the analysis of a high-throughput data set. Shown is an example of a spreadsheet example with common problems identified by data visualization. The spreadsheet contains missing values (…). Identification of data type, and distribution of data can be performed using min and max analysis, or box-plot determinations. In a box-plot determination, the 25–75 % quartile is shown as well as the mean. Inspection of the *box-plot* suggests nonnormally distributed data sets. Also illustrated are *q–q* plots that suggest data that is normally distributed (*left*) and data that is not (*right*)

a measurement is below the limit of detection. In this case, an appropriate imputation method could involve substitution of a value below the assay's lower limit of quantification. In earlier studies of bronchoalveolar lavage cytokines, we evaluated the effect of substituting a value of 1/10th of the lower limit of detection for each assay. This conservative estimate of the analyte measurement has been widely used (Brasier et al. 2008, 2011). Another imputation technique, appropriate for missing values that could be any value, is *K* nearest neighbors (KNN); KNN is a technique where values are imputed by an algorithm in which neighbors are the variables of interest and the distance between neighbors is based on the correlation between them (Troyanskaya et al. 2001) (KNN implementations are available in R package and Matlab program).

Another issue in data preprocessing for classification is how to treat data that fails analysis of normality. Typical normality tests would include box-plot or *q*–*q* graphical analysis (Spratt et al. 2012). This point is important because comparing feature expression in a nonnormally distributed data set requires the use of less powerful nonparametric analytical approaches. Because parametric testing is more powerful, where possible, we will try to employ statistical manipulations to make the data set behave more like normally distributed data. Popular data transformations include taking the logarithm of the data (log base 10 or natural logarithm) or taking the square root of the data. Log transformation is an approach is widely used in gene expression analysis and protein abundance estimation.

17.2.3 Feature Reduction Strategies

A characteristic of complex, high-dimensional data sets is that many analytes are identified, only some of which are informative features, whereas others only contribute noise. The measurement of multiple features leads to the "curse of dimensionality," where the presence of many features often leads to poorer, rather than better, classifier performance. One potential solution to this problem is to reduce the dimensionality and simplify model complexity. This approach is frequently referred to as feature reduction and schematically diagrammed in Fig. 17.2.

Feature reduction seeks to reduce dimensionality by selecting an informative subset from the existing set of analytes. In practice, feature reduction can be accomplished by first selecting a subset of the most informative features that distinguish the two groups. This selection can be done by rank-ordering analytes by statistical significance based on groupwise comparison, by calculating the fold change between the two comparison groups (ratio of group means), by a statistical analysis of microarrays (SAM), Bayes methods or heuristics (biological intuition of the investigator). Selection of the feature reduction method is a decision made on the basis of the data set characteristics, the goals of the analysis and/or heuristics. Some considerations for this decision are shown in Table 17.1.

Statistical methods for feature reduction is approached by comparing different group means either using the two sample *t*-test or Wilcoxon rank sum test at a given appropriate level of significance. The Wilcoxon rank sum test is an alternative

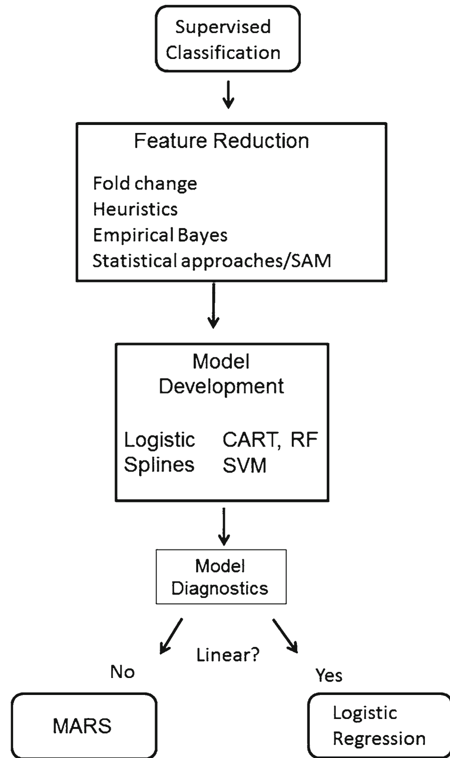

Fig. 17.2 Model development and assessment. A schematic for the use of feature reduction and downstream supervised or unsupervised classifications. Abbreviations: *CART* classification and regression trees, *RF* random forests, *SVM* support vector machines, *MARS* multivariate adaptive regression splines

Table 17.1 Selection of feature reduction techniques

Technique	Considerations
Rank ordered *t*-test	• Useful for large sample number (>30) • Sensitive to features with small group mean variance
Fold change (FC) cut-off	• Cut-off value selection can be seen as arbitrary • Sensitive to error if class is unmeasureable
SAM	• Appropriate for small sample number or correlated measurements • Enables FDR estimation • Must specify comparator for group differences
Empirical Bayesian	• Model uncertainty; model misspecification • Limited available software options (R or Winbugs); long time to compute; data structure dependent
PCA	• Good for large feature set; • Correlated data; • Unreliable if first three principal components do not account for at least 80 % of the variability
Heuristics	• Understand biological relevance or interpretation • Subject to bias

approach to the Student *t*-test in two-sample comparisons that does not require a parametric distribution. Accommodation for effects of multiple hypothesis testing should be routinely applied to high-dimensional data that contains a large number of analyte measurements on a small number of samples [small *n*–large *p*, or wide data set (Hastie et al. 2009)]. In using this approach, it is important to recognize that simultaneous testing of more than one hypothesis leads to inflated numbers of false positives. Common methods for reducing errors from multiple hypothesis testing are to employ family-wise error rate corrections such as the Bonferroni correction or the false discovery rate (FDR). Because the Bonferroni correction is extremely conservative and leads to the rejection of true positives, FDR procedures have been developed. FDR use a resampling method and have improved ability to reject false hypotheses compared to the Bonferroni correction (Hastie et al. 2009). The FDR analysis results in a quantile (*q*)-value, a number that indicates the proportion of false positives. For example, if a feature has a *q*-value of 0.05, it means that 5 % of features that show *p*-values at least as small as that are false positives. Feature reduction can also be based on a fold change value (values of 1.5 or 2 are often used) or statistical significance of mean difference of two groups (a *q*-value threshold of 1 or 5 % are often used). The fold change threshold will change the number of significant features when the analysis is followed by the use of a parametric test with multiple testing corrections.

Significance analysis of microarrays (SAM) is a widely used permutation-based approach to identifying differentially expressed features in high-dimensional data sets. One of the advantages of SAM is that it is able to assess statistical significance using FDR adjustment (Tusher et al. 2001). In the SAM analysis, each feature is assigned a score on the basis of its change relative to the standard deviation of its repeated measurements. SAM accounts for feature-specific fluctuations in signals and adjusts for increasing variation in features with low signal-to-noise ratios. Data are presented as a scatter plot of expected vs. observed relative differences, where significant deviations that exceed a threshold from expected relative differences are identified and considered “significant.”

An alternative approach for feature reduction is an empirical Bayesian technique (Efron et al. 2001). This approach uses noninformative priors and derives the posterior probability difference for each predictor. An advantage of empirical Bayes is that it does not rely on initial feature selection using *t*-tests or Wilcoxon tests to identify which features are differentially expressed. However, there are limited software implementations of this method and it requires significant computational time.

Principal component analysis (PCA) is a classical method for reducing the dimensionality of data to identify informative features. Here, features are transformed into orthogonal linear representation to identify uncorrelated components that most contribute to the variability in the experimental data. The features employed in the major principal components are then used in order of the magnitude of their corresponding eigenvalues.

Finally, intuition of the biological system (heuristics) can be used to select features based on their plausibility or prior knowledge about the system under investigation. Heuristics is also frequently used in combination with various

statistical methods (Table 17.1), where, for example, known biological relevance to disease pathophysiology would be used as a criterion to select some analytes over others.

17.3 Classification Approaches

17.3.1 Logistic Regression

Logistic Regression (LR) seeks to model the relationship between a set of continuous, categorical, or dichotomous variables and the probability of a dichotomous outcome as a “logit” function. Stated in another way, logistic regression is the method used for a binary, rather than a continuous outcome. The logistic regression model does not necessarily require the assumptions of some other regression models, like assuming that the variables are normally distributed in linear discriminant analysis. Maximum likelihood estimation is the method used to solve for the logistic regression equation. Recent techniques, penalized shrinkage and regularization estimation, lasso-type regularization logistic regression models have been developed to improve prediction accuracy in classification (Hastie et al. 2009).

17.3.2 Variable Selection with the Lasso

Regularized and shrinkage estimation methods, such as the least absolute shrinkage and selection operator (lasso) estimator (Tibshirani 1997), are methods of choice to address the problems of variable selection and multicollinearity. The lasso utilizes the L1 penalty to perform simultaneous continuous feature set shrinkage and automated variable selection. For binary response problems, the lasso estimator is estimated by penalized the negative log-likelihood with the L1-norm. The penalty term is chosen by a cross-validation technique for evaluating the out-of-sample negative log-likelihood. The elastic net (Zou and Hastie 2005) combines both L1 and L2 penalizing terms and possess a grouping effect, i.e., if there is a set of variables which have high pairwise correlations, the elastic net groups the correlated variables together. Zou proposed the adaptive lasso (Zou 2006), an approach that permits different weights for different parameters. The adaptive lasso also has been shown to have the oracle property (consistency in variable selection and asymptotic normality).

17.3.3 Classification and Regression Trees

Classification and Regression Trees (CART) is a nonparametric modeling algorithm for building decision trees (Steinberg and Colla 1997). Decision trees have a human-readable split at each decision point that represents a binary response of an informative feature. CART is highly useful for because it is one of the modeling techniques that does not require initial variable selection. Some other advantages of CART are that it can easily handle data sets which are complex in structure, it is extremely robust and not very effected by outliers, it can use a combination of both categorical and continuous data, missing data values do not pose an obstacle to CART classification.

The three main steps in CART modeling are creating a set of rules for splitting each node in a tree, deciding when a tree is fully grown, and assigning a classification to each terminal node of the tree. The basic algorithm for building the decision tree seeks some feature of the data that maximizes the difference between the classes contained in the parent node. Once CART has decided on an appropriate split resulting in two child nodes, the child nodes then become the new parent nodes, and the process is carried on down the branches of the tree. Cross-validation is frequently used to determine decision tree accuracy.

17.3.4 Random Forests

Random forests (RF) is an ensemble of CART models that offers several unique and extremely useful features which include built-in estimation of prediction accuracy, measures of feature importance, and a measure of similarity between sample inputs (Breiman 2001a, b). Decision trees are known for their ability to select the most informative descriptors among many and to ignore irrelevant ones. The RF algorithm is very efficient, especially when the number of descriptors is very large. This efficiency over traditional CART methods arises from two general areas: the first is that CART requires some amount of pruning of the tree to reach optimal prediction strength; RF, however, does not do any pruning, which reduces performance time. Second, RF uses only a small number of descriptors to test the splitting performance at each node instead of doing an exhaustive search as does CART.

RF thus builds many trees and determines the most likely splits based upon a comparison within the ensemble of trees. The RF modeling uses first a bootstrapped sample from the training data set. Then, for each bootstrapped sample, a classification tree is grown. Here, RF modifies the CART algorithm by randomly selecting from a subset of the descriptors, instead of choosing the best split among all samples. This procedure is repeated until a sufficiently large number of trees have been computed, usually 500 or more.

RF performs a bootstrapping cross-validation procedure in parallel with the training step of its algorithm. This method allows some of the data to be left out at each step and then used later to estimate the accuracy of the classifier after each instance (i.e., tree) has been completed.

17.3.5 Multivariate Adaptive Regression Splines

Multivariate adaptive regression splines (MARS) is a robust nonparametric modeling approach for feature reduction and model building (Friedman 1991). MARS is a nonparametric, multivariate regression method that can estimate complex nonlinear relationships by a series of spline functions of the predictor variables. Regression splines seek to find thresholds and breaks in relationships between variables and are suited for identifying changes in the behavior of individuals or processes over time. Some of the advantages of MARS are that it can model predictor variables of many data types, continuous or categorical, and it can tolerate large numbers of input predictor variables. As a nonparametric approach, MARS does not make any underlying assumptions about the distribution of the predictor variables of interest. This characteristic is extremely important in high-dimensional modeling because many of the cytokine and protein expression values are not normally distributed, as would be required for the application of classical modeling techniques. The basic concept behind spline models is to model using potentially discrete linear or nonlinear functions of any analyte over differing intervals. The resulting piecewise curve, referred to as a spline, is represented by basis functions.

Each MARS basis function takes one of the following three forms (1) a constant, where there is just one such term, the intercept; (2) a hinge function. A hinge function has the form $\max(0,xi-ci)$ or $\max(0,ci-xi)$. MARS automatically selects variables and values of those variables for knots of the hinge functions; or (3) a product of two or more hinge functions. These basis functions can model interaction between two or more variables.

The MARS algorithm has the ability to search through a large number of candidate predictor variables to determine those most relevant to the classification model. The specific variables to use and their exact parameters are identified by an intensive search procedure that is fast in comparison to other methods. The optimal functional form for the variables in the model is based on regression splines called basis functions. MARS uses a two-stage process for constructing the optimal classification model. The first half of the process involves creating an overly large model by adding basis functions that represent either single variable transformations or multivariate interaction terms. The model becomes more flexible and complex as additional basis functions are added. The process is complete when a user-specified number of basis functions have been added. In the second stage, MARS deletes basis functions in order of least contribution to the model until the optimum one is reached.

Depending on the modeling assumptions, method for model selection and acceptance of interaction terms, MARS models are sensitive to overfitting. Overfitting is a phenomenon where the model describes noise in the data set as well as the underlying relationships, and as a result, will not generalize to other data sets. As with the other modeling techniques, MARS uses cross-validation avoid overfitting. The optimal end result is a classification model based on single variables and interaction terms which will optimally determine class identity.

17.4 Methods for Model Evaluation

In practice, it is often customary for a supervised analysis to be conducted using several modeling approaches. The investigator will then examine the model performance using a variety of criteria, as well as look for convergence of informative features.

A widely accepted approach in model evaluation is to evaluate the area under the receiver operating characteristic (ROC) curve (Hanley and McNeil 1982). The area under the ROC curve (AUC) measures indicate the ability of a model to discriminate amongst the outcome groups. An AUC score of 0.5 indicates that a model has no discriminatory ability and a score of 1 indicates the two groups are perfectly discriminated.

Model performance of machine learning algorithms can also be examined using a confusion matrix. A confusion matrix shows the actual number of cases within each group, as well as the number of cases predicted by the model to lie within each group. This analysis allows the modeler to determine model performance for prediction of each class. In addition, the overall percent correct prediction is also evaluated.

17.4.1 Classifier Performance in Asthma Phenotypes

Recently, we have evaluated the performance of various predictive models using multiplex BAL cytokine measurements in bronchoalveolar lavage (BAL) to predict intermediary phenotypes of atopic asthma (Brasier et al. 2010). In our preliminary analysis of 41 severe, and 43 nonsevere asthmatics from the Severe Asthma Research Program, we observed that cytokine concentrations could be used as molecular fingerprints of asthmatic subtypes using unsupervised clustering. Unsupervised classification using hierarchical clustering suggested that subjects could be segregated into groups that showed statistically significant differences in type of BAL cellularity, including eosinophils and lymphocytes, as well as measures of static and dynamic pulmonary lung function (Brasier et al. 2008). Our aim was, therefore, to compare classification methods to identify the best methods that would allow predictive models of these distinct clinical phenotypes.

We conducted this systematic comparison of distinct statistical learning approaches because currently it is not possible presently to select a priori the machine learning tool that performs best for any given data set. All statistical learning approaches are sensitive to the underlying data structure. To evaluate the performance of these distinct classifiers, we performed supervised classification using BAL cytokines as input, and clinical phenotype of methacholine sensitivity ("hyper-responders") as an outcome (as well as others).

We observed that an LR model constructed using the AIC information criterion, a method equivalent to cross-validation (Stone 1977), produced a good fit with the

Table 17.2 Model performance comparisons

Phenotype	Classifiers	Accuracy (%)	AUC	Sensitivity (%)	Specificity (%)
High eosinophils	LR	85	0.84	53	94
	MARS	85	0.89	46	93
	CART	67	0.48	30	74
	RF	49	0.40	46	49
High neutrophils	LR	97	0.76	60	98
	MARS	93	0.77	20	98
	CART	83	0.51	20	88
	RF	66	0.47	40	68
Bronchodilators	LR	76	0.71	23	85
	MARS	88	0.85	23	100
	CART	75	0.69	53	81
	RF	61	0.65	54	64
Hyper-responders	LR	85	0.85	53	94
	MARS	90	0.88	73	94
	CART	79	0.68	53	86
	RF	70	0.77	73	70

Shown is the model performance comparisons for the machine learning approaches for accuracy, AUC, sensitivity and specificity. Abbreviations: *AUC* area under the ROC curve, *LR* logistic regression, *MARS* multivariate adaptive regression splines, *CART* classification and regression trees, *RF* random forests. Reprinted with permission from (Brasier et al. 2010)

outcome. For the methacholine "hyperresponders" IL-1Ra, Eotaxin, IL-4, and IL-2R were selected (Table 17.2). MARS produced a slightly more accurate model than did LR with 90 % accuracy (Table 17.2, Fig. 17.3). Both of these models outperformed the CART and RF. The one cytokine with the greatest χ^2 score statistic in the LR and the greatest variable importance in the MARS was IL-1Ra. IL-1Ra was also highly statistically significant in the groupwise comparison of cytokines in the hyperresponders vs. nonhyperresponder groups.

The ROC curves for LR MARS, CART, and RF prediction of methacholine hypersensitivity are shown in Fig. 17.3. Note that MARS and LR show various degrees of leftward shift from the line of no discrimination (AUC of 0.88 and 0.85, respectively), indicating that these models perform better than random guessing. Finally, LR resulted in a model with 85 % overall accuracy, whereas MARS produced a model with 90 % overall accuracy.

17.5 Post Hoc Model and Feature Evaluation

We have found that post hoc model diagnostics is an important procedure to assess adequacy of machine learning predictors. In this regard, several techniques are used to examine model performance. In LR, often the interest is to assess the linear or nonlinear association of binary responses on its covariates (features). When we use an LR model, we assume that the logit of the outcome is a linear combination of

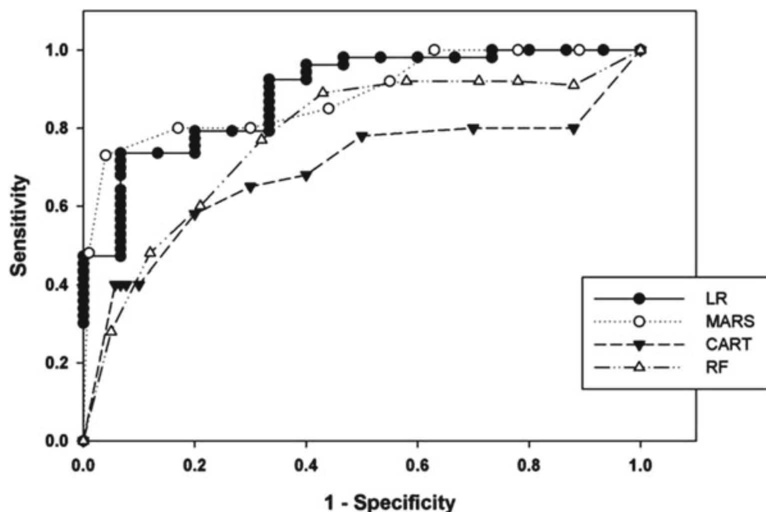

Fig. 17.3 Comparison of model performance in predicting asthma phenotype. Receiver operating characteristic (ROC) curve of methacholine hyperresponder models. The corresponding AUCs for each curve are presented. Reprinted with permission from (Brasier et al. 2010)

predictors. If the model assumptions are not satisfied, the model may contain biased coefficient estimators and very large standard errors for regression coefficients, and consequently not generalize well to other cases.

17.5.1 Generalized Additive Models

In practice, we examine whether our model has all relevant predictors and if the linear association with outcome is appropriate. One method for assessing this relationship is to use Generalized Additive Models (GAMs). GAMs are regression models which can evaluate complex nonlinear predictors on the outcomes in a generalized linear model setting (Hastie and Tibshirani 1995). A GAM plot is represented by the value of the covariate (feature) on the *X*-axis, and the partial residual represents the difference between the estimated and observed value on the *Y*-axis (Fig. 17.4). The relationship of the covariate with the outcome is indicated by a smoothed piecewise polynomial relationship contained within 95 % confidence limits.

We evaluate the partial residual plot as a diagnostic graphical tool for identifying nonlinear relationships between the response variable and covariates for generalized additive models. In the case of the IL-1Ra covariate, the piecewise polynomial relationship is not a linear relationship, and therefore is best estimated using a non-parametric modeling approach (Fig. 17.4). For linear regression models, we also use

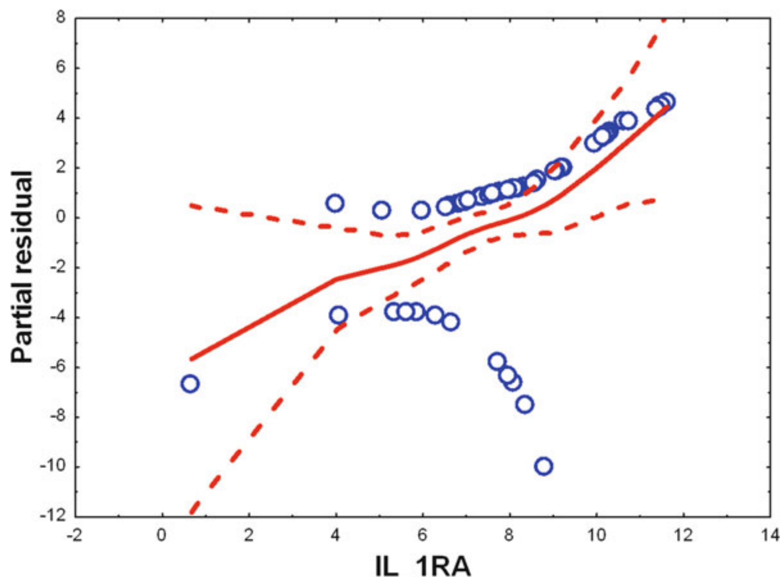

Fig. 17.4 Generalized Additive Model (GAM). In a GAM graph, the *Y*-axis is the partial residual and the *X*-axis is the value of the covariate (feature). *Dashed lines* are 95 % confidence intervals. *Open circles* are data points. Shown are the partial regressions for IL-1Ra in prediction of PC_{20} methacholine hyperresponsiveness, demonstrating a nonparametric relationship with outcome. In this case, the partial residual plot deviates from a *straight line*

the projection (hat) matrix and Cook's distance to evaluate the effect of outliers and influential points on the model (Spratt et al. 2012).

17.6 Strengths and Limitations of Supervised Approaches to Profiling

Supervised approaches can be extremely powerful to predict specific populations from unknown cases based on molecular profiling measurements. Informative analytes may provide information on potential mechanisms of dysregulation of disease; informative analytes are identified from statistical (between-group comparisons) and model analysis, where systematic relationships between analyte values a phenotype can be revealed. Additionally, the model may also suggest potential interfeature interactions that may further extend understanding of the pathophysiology of disease.

Supervised analysis critically depends on a method for analyte measurement that is quantitative and reproducible across the population. Moreover, an objective, reproducible phenotypic characterization of subjects in the data from which the model is developed is critical for success. It is obvious that if there is inconsistency

in outcome classification, the resulting model will not generalize (have wide applicability). For this reason, a parallel analysis of the data using unsupervised classification methods such as those described in the following chapter is advisable.

17.7 Conclusions and Future Directions

Supervised classification is critically dependent on the characteristics of the analyte distribution and the number of analytes being considered. These factors have an important contribution to the performance of different classifiers. For example, LR is a parametric approach that identifies main effects of candidate cytokines and assumes a global linear relationship between the independent (cytokine) and the dependent (asthma phenotype) variables. Although LR performs well if the relationship between the dependent and independent variables is well described, the performance of LR is reduced with high-dimensional data or when there are multiple interactions between independent variables. By contrast, MARS is a nonparametric, piecewise linear approach that can establish relationships over smaller intervals of independent variables, as well as detect interactions between independent variables.

Our experience so far shows that LR and MARS result in quite similar accuracy for relating proteomic profiles to clinical phenotypes in asthma, with both clearly outperforming CART and RF classifiers (Brasier et al. 2010). Interestingly to us, for many of the best performing models developed in LR and MARS, the cytokine features important for the model are different. The reasons for these discrepancies will require further investigation, but may be a consequence of the underlying data structure affecting feature selection.

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Chapter 18
The Role of Visual Analytics in Asthma Phenotyping and Biomarker Discovery

Suresh K. Bhavnani, Justin Drake, and Rohit Divekar

Abstract The exponential growth of biomedical data related to diseases such as asthma far exceeds our cognitive abilities to comprehend it for tasks such as biomarker discovery, pathway identification, and molecular-based phenotyping. This chapter discusses the cognitive and task-based reasons for why methods from visual analytics can help in analyzing such large and complex asthma data, and demonstrates how one such approach called network visualization and analysis can be used to reveal important translational insights related to asthma. The demonstration of the method helps to identify the strengths and limitations of network analysis, in addition to areas for future research that can enhance the use of networks to analyze vast and complex biomedical datasets related to diseases such as asthma.

Keywords Asthma • Phenotypes • Visual analytics • Network analysis • Visualization • Bipartite networks • Multivariate analysis • Exploratory visual analysis • Quantitative verification • Emergent clusters • Inference of biological pathways • Molecular-based classification • Phenotyping • Biomarker discovery

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18.1 Introduction

The explosion of molecular information generated by multidimensional measurements of proteins, genes, and metabolites, coupled with digital access to patient clinical records has created unprecedented opportunities for a more comprehensive understanding of asthma. However, this explosion of information has also created a challenge for researchers, especially those in multidisciplinary translational science teams, to comprehend and integrate such disparate and large amounts of information. For example, the identification of molecular pathways involved in different asthma phenotypes requires an interdisciplinary understanding of (1) biomarkers that are co-expressed across different groups of patients, (2) clinical characteristics of the patient groups across the biomarkers that are co-expressed, and (3) known and novel molecular pathways suggested by the patterns related to molecular and clinical patient profiles.

One approach to integrate and comprehend such complex information is through methods being developed in the new field of visual analytics. In this chapter we begin by presenting an overview of the evolving theoretical foundations for visual analytics, and the motivations to use methods from this field to analyze asthma data. Next, we focus on one form of visual analytics called networks which are particularly useful for analyzing complex molecular and clinical data. In contrast to the supervised learning methods (discussed in the last chapter) that use a priori information (e.g., cases and controls) to build predictive models, networks are considered to be an exemplar of unsupervised learning methods which do not use a priori information (e.g., cases and controls). We will demonstrate how this approach can be been used to identify asthma phenotypes and infer the molecular pathways involved in those phenotypes. These analyses reveal the strengths and limitations of the method, which are used to define a research agenda for advanced methods to enable in the future, comprehension of complex relationships in ever-increasing and complex asthma data.

18.2 Visual Analytics: Definition, Motivation, and Theoretical Foundations

Visual analytics is defined as the science of analytical reasoning, facilitated by interactive visual interfaces (Thomas and Cook 2005). The primary goal of visual analytics is to augment cognitive reasoning by translating symbolic data (e.g., numbers in a spreadsheet) into *visualizations* (e.g., a scatter plot) which can be manipulated through *interaction* (e.g., highlight only some data points in the scatter plot). As discussed below, visualizations, and interaction with those visualizations, are powerful for helping analysts comprehend complex relationships in asthma data because of the nature of human cognition, and the nature of tasks performed by analysts.

18.2.1 Motivation for Visualizations

Visualizations of data are often powerful because they leverage the massively parallel architecture of the human visual system consisting of the eye and the visual cortex of the brain (Card et al. 1999). This parallel cognitive architecture enables the rapid comprehension of multiple graphical relationships simultaneously, which often leads to insights about relationships in complex data such as similarities, trends, and anomalies (Thomas and Cook 2005). For example, the detection of an outlier in a scatter plot is fast because the graphical relationships between the outlier and the rest of the points can be processed in parallel by the visual cortex. Such parallel processing is independent of the number of non-outlying points and therefore scales up well to large amounts of data. In contrast, finding an outlier in a spreadsheet of numbers involves numerical comparisons to identify the outlier, which is dependent on the much slower symbolic processing areas of the human brain. Such symbolic processing is serial in nature, and therefore highly dependent on the number of data points, which when large can quickly overwhelm an analyst. Data visualizations therefore help to shift processing from the slower symbolic processing areas of the human brain, to the faster graphical parallel processing of the visual cortex enabling processing of large and complex datasets such as those currently available for asthma.

However, not all data visualizations are effective in augmenting cognition. For example, an organizational chart of employee names and their locations laid out in a hierarchy based on seniority is not very useful if the task is to determine patterns related to the geographical distribution of the employees. Similarly, if a chart lacks a legend or axes labels, the visualization is difficult to comprehend because it cannot be mapped to concepts in the data. Finally, a road map pointing south is not very useful to a driver who is facing north because it requires a mental rotation of the map before it can be useful for navigation. Therefore visualizations need to be aligned with tasks (Norman 1993), data, and mental representations of the user (Tversky et al. 2002), before those visualizations can be effective in augmenting cognition.

18.2.2 Motivation for Interactivity

While static visualizations of data can be powerful if they are aligned with tasks, data, and mental representations, they are often not sufficient for comprehending complex data. This is because data analysis typically requires many different tasks performed on the same data such as discovery, inspection, confirmation, and explanation (Bhavnani et al. 2012), each requiring different views of the data. Furthermore, when analysis is done in teams consisting of different disciplines, each member often requires a different representation of the same data. For example, a molecular biologist might be interested in which cytokines are co-expressed across patients, whereas a clinician might be interested in the clinical characteristics of patients with similar

cytokine profiles, and later how they integrate with the molecular information. To address these changes in task and mental representation, visualizations require interactivity or the ability to transform parts, or the entire visual representation.

18.3 Theories Related to Visual Analytics

Although the field of visual analytics has drawn on theories and heuristics from different disciplines such as cognitive psychology, computer science, and graphic design, the development of theories and taxonomies for visual analytics are still in early stages of development (Thomas and Cook 2005). For example, there are a number of attempts to define heuristics for the design of effective visualizations (e.g., Tufte 1983), and to classify visual analytical representations (e.g., Heer et al. 2010; Shneiderman 1996), and interaction methods at different levels of granularities and tasks (Yi et al. 2007). One such classification attempt categorizes visual analytical representations into (1) time series (e.g., line graphs showing how the expression of different cytokine change over time), (2) statistical distributions (e.g., box-and-whisker plots), (3) maps (e.g., pie charts showing percentages of different races at different city locations on the US map), (4) hierarchies (e.g., top-down tree showing the management structure of an organization), and networks (e.g., a social network of how friends connect to other friends such as on Facebook). Once these visualizations are generated, they are considered visual analytical if they enable interaction directly or indirectly with part, or all of the information being represented. Examples for such interactivity include transforming a top-down tree into a circular tree, coloring nodes in the tree based on specific properties such as gender, or dragging a node in the tree to swap its location with another sibling node.

It is important to note that visual analytics has considerable overlap with the fields of scientific visualization (focused on modeling real-world geometric structures such as earthquakes), and information visualization (focused on modeling abstract data structures such as relationships). However, visual analytics places a large emphasis on approaches that facilitate reasoning and making sense of complex information individually and in groups (Thomas and Cook 2005), which makes this approach particularly pertinent for tasks such as inferring biological pathways from molecular and clinical information in translational teams.

18.4 Network Visualization and Analysis: Making Sense of Asthma Molecular and Phenotype Information

Networks (Newman 2010) are one of the most advanced forms of visual analytics because they enable not only an interactive visualization of complex associations, but because they are based on a graph representation, also enable the quantitative analysis and validation of the patterns that become salient through the

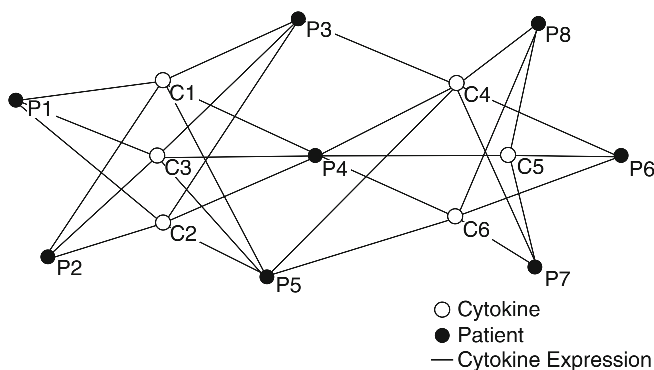

Fig. 18.1 A sample bipartite network where edges exist only between two different types of nodes. In this case, nodes represent either patients (*black*) or cytokines (*white*), and edges connecting the two represent cytokine expression

visualization. Networks are increasingly being used to analyze a wide range of molecular measurements related to gene regulation (Albert 2004), disease–gene associations (Goh et al. 2007), and disease–protein associations (Ideker and Sharan 2008). A network (also called a graph) consists of a set of nodes, connected in pairs by edges; nodes represent one or more types of entities (e.g., patients or cytokines). Edges between nodes represent a specific relationship between the entities (e.g., a patient has a particular cytokine expression value). Figure 18.1 shows a sample bipartite network where edges exist only between different types of entities (Newman 2010), in this case between patients and cytokines.[1]

Network analysis of biomedical data typically consists of three steps: (1) *exploratory visual analysis* to identify emergent bipartite relationships such as between patients and cytokines; (2) *quantitative analysis* through the use of methods suggested by the emergent visual patterns; (3) *inference* of the biological mechanisms involved across different emergent phenotypes. This three-step method used across our earlier studies (Bhavnani et al. 2007, 2010a, b) have revealed complex but comprehensible visual patterns, each prompting the use of quantitative methods that make the appropriate assumptions about the underlying data, which in turn led to inferences about the biomarkers and underlying mechanisms involved. Below we describe the methods used in each step, and their application to analyze a dataset of asthma patients and their cytokine expressions.

[1] Researchers have explored a wide range of network types including unipartite, directed, dynamic, and networks laid out in three dimensions to analyze complex data. As this wide range is beyond the scope of this chapter, we suggest other excellent sources (Newman 2010) for such information.

18.4.1 Exploratory Visual Analysis

Network analysis typically begins by transforming symbolic data into graphical elements in a network. To achieve this, the analyst needs to decide which *entities* in the data represent the nodes in the network, in addition to how other useful information can be mapped onto the node's shape, color, and size. Similarly, the analyst needs to decide which *relationships* between the entities in the data are represented by the edges in the network, in addition to how to map other useful information to the edge's thickness, color, and style. These selections are made based on an understanding of the kinds of relationships that are needed to be explored, and is often an iterative process based on an understanding of the domain and the nature of the data.

Once the symbolic data has been mapped to graphical elements, the resulting network is laid out so the nodes and edges can be visualized. The layout of nodes in a network can be done where either the distances between nodes has no meaning (e.g., nodes laid out randomly or along a geometric shape such as a line or circle) or where the distance between nodes represents a relationship such as similarity (e.g., similar cytokine expression profiles). Layouts where distance has meaning are typically generated through force-directed layout algorithms (Newman 2010). For example, the application of the *Kamada–Kawai* layout algorithm (well suited for small- to medium-sized networks in the range of 50–1,000 nodes) (Kamada and Kawai 1989; Nooy et al. 2005) to a network results in nodes with a similar pattern of connecting edge weights to be pulled together and those with different patterns to be pushed apart.

Figures 18.2–18.6 show the steps that were used to generate a bipartite network of 83 asthma patients, and 18 cytokines. Figure 18.2 shows how asthma patients, were represented as black nodes, and cytokines (molecules involved in intercellular signaling) were represented as white nodes. Furthermore, normalized cytokine expression values were represented as edges connecting each patient to each cytokine. These nodes were laid out equidistantly around a circle. Figure 18.3 shows the same network but where the edge thicknesses are proportional to the normalized cytokine expression values. Therefore, thick edges represent higher cytokine expression values compared to thin edges. Furthermore, the size of the node was made proportional to the total expression value of the connecting edges. Therefore, large patient nodes have overall higher aggregate cytokine expression values compared to smaller patient nodes.

Although the patients, cytokines, and the cytokine expression have been visually represented, the distances between the nodes have no meaning. To better comprehend the data, the patients who have higher cytokine expression value for a particular cytokine should be spatially closer to that cytokine compared to those who have lower cytokine expression value. This approach of using short distances between entities to show similarity, and long distances between entities to show dissimilarity is typical across clustering algorithms. As shown in Fig. 18.4 and reported in (Bhavnani et al. 2011a), application of the force-directed algorithm Kamada–Kawai to the circular layout results in nodes that have a similar pattern of cytokine

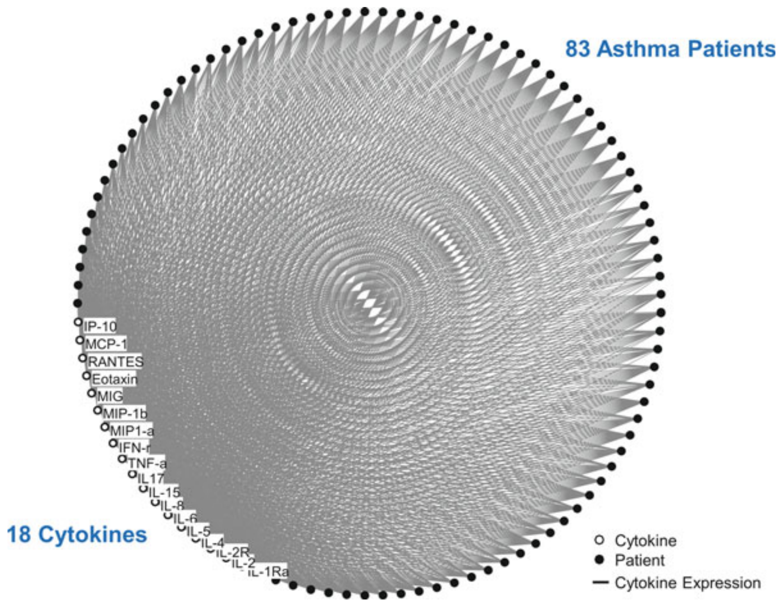

Fig. 18.2 A bipartite network showing patient nodes (*black*) and cytokine nodes (*white*) connected in pairs by edges which represent normalized cytokine expression. Patient and cytokine nodes were separately grouped and randomly laid out equidistantly around a *circle*

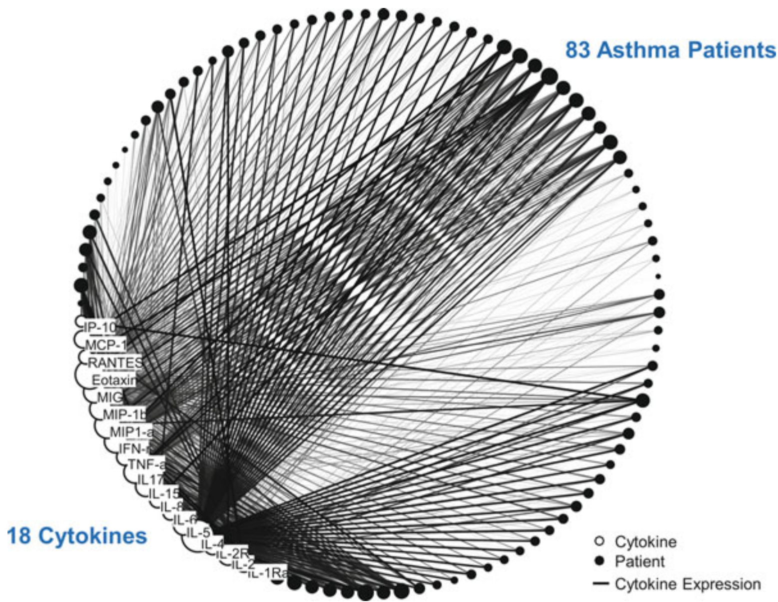

Fig. 18.3 The same network as in Fig. 18.2 but where edge thickness is proportional to the normalized cytokine expression value and the size of each node is proportional to the total expression values of the connecting edges. *Thick edges* represent higher cytokine expression values compared to *thin edges*. Similarly, larger patient nodes have higher aggregate cytokine expression values compared to smaller patient nodes

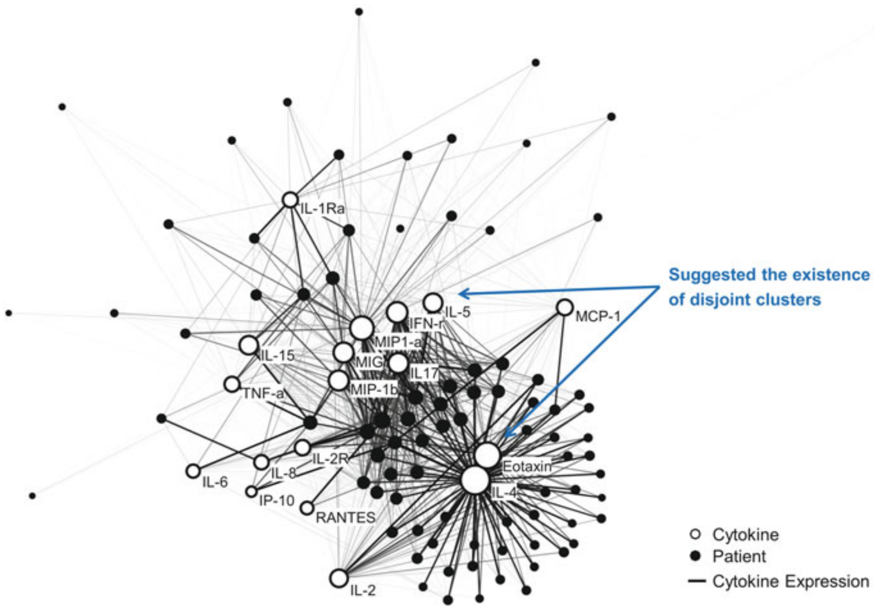

Fig. 18.4 Application of Kamada–Kawai, a force-directed algorithm, to the circular layout. The algorithm pulls nodes with similar cytokine expression patterns closer together, while pushing apart those with dissimilar expression patterns. The layout of the network suggested the existence of disjoint patient and cytokine clusters, and revealed intercluster relationships such as how the patient clusters express particular cytokine clusters. However, quantitative methods must be used to identify cluster boundaries

expression to be pulled together, and those that are not similar to be pushed apart. The resulting layout suggests that there exist distinct clusters of patients and cytokines. Furthermore, the layout also reveals the intercluster relationships such as which patient clusters are most closely related with which cytokine clusters.

While the network layout suggests the existence of distinct clusters, it is not designed to reveal the members of each cluster. We therefore need to use quantitative methods that are explicitly designed to identify the boundaries of clusters based on a multivariate analysis of the data.

18.4.2 Quantitative Verification and Validation

There exist a wide range of quantitative methods to verify and validate patterns discovered through network visualization methods. While in principle any statistical method can be used to quantitatively analyze a pattern observed in a network, many patterns are often analyzed using graph-based methods (Newman 2010) that

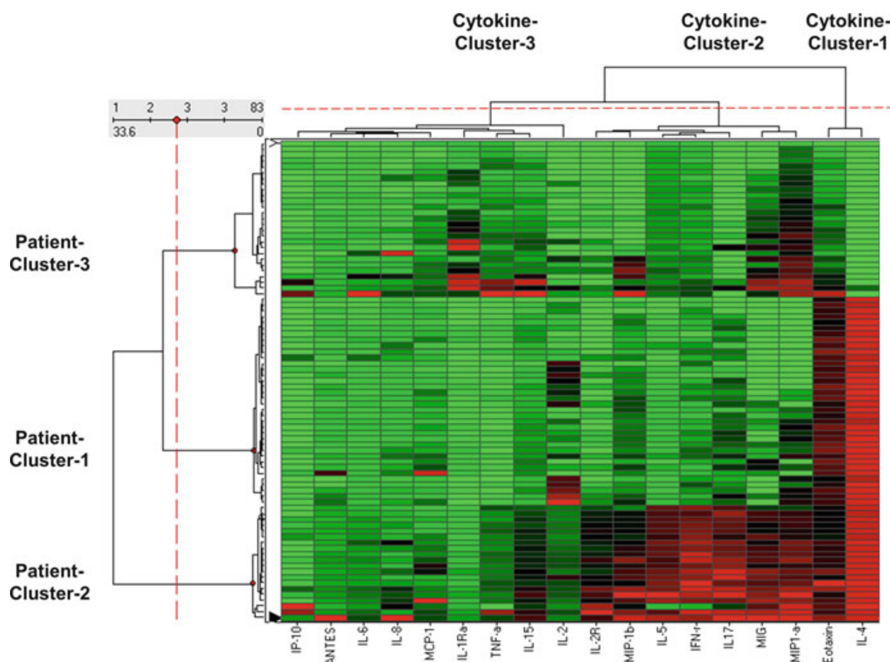

Fig. 18.5 A heat map where the *rows* represent patients, the *columns* represent cytokines, and the *colors* represent normalized cytokine values (*green* = 0, *red* = 1). The *rows* and *columns* are ordered based on the results of agglomerative hierarchical clustering. The patient and cytokine dendrograms are shown on the *vertical* and *horizontal axes*, respectively. Each dendrogram shows a natural break at three clusters indicated by the *red lines*

specialize in analyzing complex relationships. For example, *degree assortativity* measures whether one type of nodes in a network which have high weighted degree (e.g., patients who have large nodes in Fig. 18.3) are preferentially connected to another type of nodes that have high degree (e.g., cytokines that have large nodes in Fig. 18.1), or vice versa.

Another approach that can be used to verify patterns in a network is hierarchical clustering (Johnson and Wichern 1998). This unsupervised learning method attempts to identify the number and boundary of clusters in the data. For example, hierarchical clustering can be used to identify clusters of patients based on their relationship to cytokines, or clusters of cytokines based on their relationship to patients. The method begins by putting each node in a separate cluster, and then progressively joins nodes that are most similar based on their relationship to connected nodes. This progressive grouping generates a tree structure called a *dendrogram*, where distances between subsequent layers of the tree represent the strength of dissimilarity between the respective clusters; the larger the distance between two subsequent layers, the stronger the clustering. Analysts therefore determine the

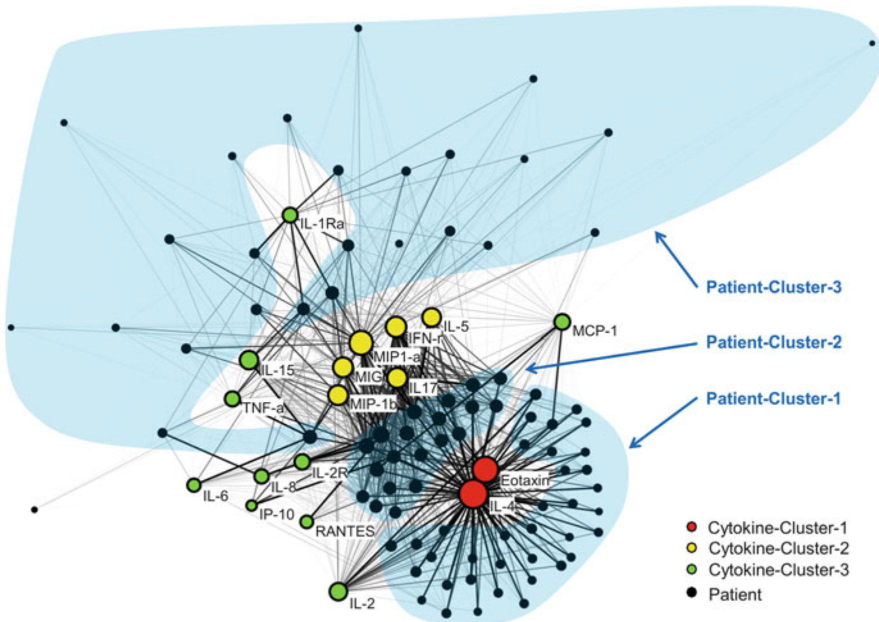

Fig. 18.6 Results of the agglomerative hierarchical clustering from Fig. 18.5 superimposed onto the *network* in Fig. 18.4 using *colors* to denote the three cytokine clusters and translucent *shapes* to denote the three patient clusters. The *network* shows that Patient-Cluster-1 highly expresses Cytokine-Cluster-1, while Patient-Cluster-3 highly expresses Cytokine-Cluster-3. The *network* also shows that Patient-Cluster-2 primarily expresses Cytokine-Cluster-2 and Cytokine-Cluster-1

number and membership of the clusters by identifying relatively large breaks between the layers in the dendrogram.

Given the wide range of quantitative methods available, the patterns in the network are used to guide the selection of the appropriate method. For example, if distinct clusters do not exist in a network, then it is not appropriate to apply a clustering algorithm to the network. This approach of selecting methods based on the inspection of the data is similar to how statisticians determine whether to use parametric or non-parametric inferential methods based on the underlying distribution of the data.

Because the network in Fig. 18.4 suggested the existence of disjoint clusters, hierarchical clustering was used to identify the boundary and members of the clusters. As shown in Fig. 18.5, the horizontal dendrogram represents the cytokine clusters, the vertical dendrogram represents the patient clusters, and the colored cells represent normalized cytokine expression ranging from green (0) to red (1). Each dendrogram shows a clear break at three clusters for cytokines, and for patients (as shown by the corresponding red dotted lines across each dendrogram).

While there may be clear breaks in the dendrograms, the overall pattern could have occurred by random chance. Patterns discovered in networks, and subsequently

the dendrograms, are therefore validated by determining their significance. One approach to do this is to compare the patterns in the data to random permutations of the network.

To test whether there were significant breaks in the dendrogram (denoting the existence of disjoint clusters), the variance, skewness, and kurtosis of the dissimilarities (generated by the hierarchical clustering algorithm) in the asthma network were compared to 1,000 permutations of the asthma network. For each network permutation, the number of nodes and the number of edges connected to each node, in addition to the edge weight distribution of patients were preserved when analyzing the cytokine dendrogram, and vice versa. Significant breaks in the asthma patient or cytokine dendrograms would result in a significantly larger variance, skewness, and kurtosis of the dissimilarity measures, compared to the same measures generated from the random networks.

As reported in (Bhavnani et al. 2011a) the results showed the clusteredness of the patients in the asthma network was significant as measured by the variance of the dissimilarities (Asthma=64.95, Random Mean=20.08, $p<0.001$ two-tailed test), skewness of the distribution of dissimilarities (Asthma=4.9, Random Mean=2.81, $p<0.001$ two-tailed test), and kurtosis of the distribution of dissimilarities (Asthma=30.24, Random Mean=14.78, $p<0.001$ two-tailed test). Furthermore, the results also showed that the clusteredness of the cytokine clusters was significant as measured by the variance of the dissimilarities (Asthma=837.62, Random Mean=46.69, $p<0.001$ two-tailed test), the skewness of the distribution of dissimilarities (Asthma=2.18, Random mean=0.49, $p<0.001$ two-tailed test), and kurtosis of the distribution of dissimilarities (Asthma=7.25, Random mean=2.49, $p<0.001$ two-tailed test).

To understand why the patients or cytokines were clustered, and how they related to each other, the cluster memberships were superimposed onto the network. As shown in Fig. 18.6, the cytokine nodes were colored to denote their membership in three separate clusters. In contrast, the patient clusters were denoted by closed translucent shapes to enable visual discrimination between patient and cytokine[2] clusters. As shown, Patient-Cluster-1 and Patient-Cluster-3 are enriched with Cytokine-Cluster-1 and Cytokine-Cluster-3, respectively. However, Patient-Cluster-2 is enriched with Cytokine-Cluster-1 and Cytokine-Cluster-2. The results of the quantitative analysis superimposed over the network visualization therefore helped to identify the intercluster relationships in the data.

[2] Such visual design decisions are currently loosely based on graphic design heuristics (Johnson and Wichern 1998) such as limiting the number of colors in the visualization to reduce visual overload. However, successful visualizations are often based on the graphic design expertise of the analyst who explores many variations of a visualization, and uses judgment to determine which one is most effective for the data, task, and mental representations of the domain experts who will be interpreting the results.

Table 18.1 Comparison of six independent pulmonary functions across the three patient clusters identified by the network analysis

Pulmonary function	*p* value with FDR correction
Max FVCpp/MPVLung	0.006*
Max FEV_1pp/MPVLung	0.0375*
Baseline FEV_1pp	0.0375*
Baseline FEV_1/FVC	0.1944
Max FEV_1 reversal	0.583
PC_{20} methacholine	0.0375*

Significant differences between the groups are indicated by asterisks based on a one-way, two-tailed Kruskal–Wallis test with an FDR correction. (*FVC* forced vital capacity, *FEV_1* forced expiratory volume in 1 s, *PC_{20} methacholine* dose of methacholine that produces 20 % fall in FEV_1, *FEV_1 albuterol reversal* percent change in FEV_1 in response to albuterol inhalation, *MPV* maximal postbronchodilator value, *pp* percent predicted). Permission pending

18.4.3 Inference of Biological Mechanisms and Asthma Phenotypes

While the visual and quantitative analysis helped to reveal patterns in the data, the ultimate goal of the network analysis is to infer the biological mechanisms involved, and the emergent sub-phenotypes in the data. This inferential step requires an integrated understanding of the molecular and clinical variables. One approach is to analyze how the patients in each emergent cluster (based on molecular profiles) differ in their clinical variables. This can be done with well-known statistical tests such as Kruskal–Wallis, a nonparametric test used to determine if the median of a variable is significantly different across many groups such as the clusters.

The Kruskal–Wallis test revealed patterns of pulmonary function across the three patient clusters (Bhavnani et al. 2011a). As shown in Table 18.1, four out of six pulmonary function measures were significantly different across the three clusters. In addition, we conducted a pairwise intercluster analysis, which revealed that Patient-Cluster-3 had three lung functions (Max FEV1pp/MPVLung, Baseline FEV1pp, and PC_{20} Methacholine) that were significantly higher than Patient-Cluster-1, and one lung function (Max FVCpp/MPVLung) that was significantly higher than Patient-Cluster-2. In contrast, Patient-Cluster-1, had only one lung function (Max FVCpp/MPVLung) that was significantly higher than Patient-Cluster-2. Patient-Cluster-3 therefore had less baseline airway obstruction (both FEV1 values were significantly higher), less hyper-reactivity to methacholine challenge (significantly higher PC20 Methacholine), and preserved pulmonary capacity (significantly higher FVC values) compared to the other two patient clusters.

The molecular and clinical profiles of the patients therefore helped to identify hypotheses for the mechanisms involved in asthma. As discussed in (Bhavnani et al.

2011a) the co-occurrence of Eotaxin and IL-4 (Cytokine-Cluster-1) is well aligned with a known sequence of molecular changes in asthma patients who often have a T-helper-2 (Th_2) lymphocyte-skewed immune response. This response results in the secretion of IL-4, which in turn triggers Eotaxin production by bronchial epithelial cells (Fujisawa et al. 2001). The resulting downstream actions include the activation and recruitment of tissue-resident eosinophils, a hallmark of early-stage asthma. The presence of Eotaxin and IL-4 in lung fluids therefore appears to indicate key substages of a complex molecular pathway in asthma, which explains their high co-occurrence in the network.

To comprehend the biological significance of cytokines in Cytokine-Cluster-2 (IL-5, IFN-γ, MIP1a, MIG, IL-17, and MIP-1β), they were entered into the Ingenuity Pathway Analysis (IPA) application. The results suggest that the frequent co-occurrence of these cytokines is regulated by the innate inflammatory nuclear factor-κB pathway (NF-κB). NF-κB is a potent pro-inflammatory transcription factor that activates expression of cytokine networks. In addition, persistent NF-κB activation has been linked to uncontrolled/acute exacerbations of asthma (Gagliardo et al. 2003). The frequent co-occurrence of this set of cytokines therefore implies the presence of a distinctly different pro-inflammatory state, when compared to the IL-4–Eotaxin process discussed above.

The above cytokine clusters combined with the pulmonary functions of the patients, provide a biological explanation for the patient clusters. The strong relationship of Patient-Cluster-1 to Cytokine-Cluster-1 suggests that patients in this cluster have disease driven primarily by Th_2 inflammation. In contrast, Patient-Cluster-2 has a strong relationship to both Cytokine-Clusters-1 and -2. This result implies that patients in Patient-Cluster-2 have a component of activated innate inflammatory pathways. Additional evidence for this inference of state-based clusters is evidenced by differences in pulmonary function across the clusters discussed earlier. Patient-Cluster-3 which has the lowest cytokine values for both of the above cytokine clusters also has the largest number of significant differences in obstructive airway disease parameters in pulmonary function testing, and lowest airway reactivity response to methacholine compared to Patient-Clusters-1 and -2. This result implies that Patient-Cluster-3 represents a subgroup of asthmatics with preserved pulmonary function and greatest response to albuterol without active inflammation.

Informed by these underlying molecular processes, the network analysis of patients and cytokines therefore implies a state-based classification of asthma patients. The results also provide evidence for the growing consensus (Bousquet et al. 2010) that asthma is a dynamic disease where the same patient could enter different asthmatic states based on environmental and/or other triggers. Future studies that include such information could lead to a better understanding of the relationship between triggers and resulting asthmatic states, which could translate into more effective personalized treatment and prevention approaches for each patient.

18.5 Strengths and Limitations of Network Analysis

Network analysis has several strengths and limitations, whose understanding can lead to informed uses of the method, appropriate interpretation of the results, and insights for future enhancements and complementary methods.

18.5.1 Strengths

Network visualization and analysis provide four distinct strengths for enabling rapid discovery of patterns in complex biomedical data.

1. Networks (that are based on graph theory) provide a tight integration between visual and quantitative analysis. For example, as shown in the Fig. 18.6, networks enable the simultaneous visualization of multiple raw values (e.g., patient–cytokine associations, cytokine values, patient attributes), aggregated values (e.g., sum of cytokine values), and emergent global patterns (e.g., clusters) in a uniform representation. This uniform visual representation leverages the parallel processing power of the visual cortex enabling the comprehension of complex multivariate, quantitative relationships.
2. Networks do not require a priori assumptions about the relationship of nodes within the data, in contrast to hierarchical clustering or k-means which assume the data is hierarchically organized or contain disjoint clusters, respectively. Instead, by using a simple pairwise representation of nodes and edges, network layouts enable the identification of multiple structures (e.g., hierarchical, disjoint, overlapping, nested) in a single representation (Nooy et al. 2005). Therefore, while layout algorithms such as Kamada–Kawai depend on the force-directed assumption and its implementation, such algorithms are viewed as less biased for data exploration because they do not impose a particular cluster structure on the data, often leading to the identification of more complex structures in the data (Bhavnani et al. 2010a). The overall approach therefore enables a more informed selection of quantitative methods to verify the patterns in the data.
3. Networks preserve highly correlated variables (such as cytokines) and display them through clustering. Furthermore, the bipartite network representation enables the comprehension of intercluster relationships such as between variable (e.g., cytokines) clusters and patient clusters. These features provide important clues to domain experts about the pathways that involve those variables. This is in contrast to many supervised learning methods which drop highly correlated variables in an attempt to identify a small number of variables that together can explain the maximum amount of variance in the data. While this approach is powerful for developing predictive models, the reduction in variables could limit the inference of biological pathways involved in the disease.
4. Networks enable high interactivity enabling the rapid modification of the visual representation to match the changing task and representation needs of analysts during the analysis process. For example, nodes that represent patients in a network can be

interactively colored or reshaped to represent different variables such as gender and race, enabling the discovery of how they relate to the rest of the network.

18.5.2 Limitations

Networks have three important limitations that need to be understood for their proper use.

1. While node shape, color, and size can represent different variables, there is a limit on the number of variables that can be simultaneously represented. Furthermore, a visual representation can get overloaded with too many colors and shapes, which can mask rather than reveal important patterns in the data. Therefore, while networks can reveal complex multivariate patterns in the data based on a few variables, they often require complimentary visual analytical representations such as Circos ideograms (Krzywinski et al. 2009; Bhavnani et al. 2011b) to explore data that is high dimensional (e.g., large number of attributes related to entities such as patients in the network).
2. While networks provide a rich vocabulary of graphical elements to represent data, their design and use requires iterative refinement based on an understanding of the domain, knowledge of graphic design and cognitive heuristics, and the use of complex interfaces that are designed for those facile in computation. This combination of knowledge required to conduct network analyses makes domain experts dependent on network analysts to generate and refine the representations, which can limit the rapid exploration and interpretation of complex data.
3. While network layout algorithms are designed to reveal complex and unbiased patterns in multivariate data, they often fail to show any patterns in the data resulting in what is colloquially called a "hairball." In such cases, the nodes appear to be randomly laid out providing little guidance for how to proceed with the analysis. While network applications offer many interactive methods to filter data such as by dropping edges and nodes based on different thresholds, many of these methods are arbitrary and therefore unjustifiable to use when searching for patterns especially in important domains such as biomedicine. There is therefore a need to develop more systematic and defensible methods to find hidden patterns in network hairballs.

18.6 Future Directions in Network Analysis Related to the Analysis of Biomedical Data

The limitations of networks discussed above motivate three important future research directions to make network analysis more effective for the analysis of biomedical data such as those related to asthma: (1) As nodes can only represent a

limited number of variables simultaneously, there is a need to use complementary visual analytical representations. This motivates the development of a framework designed to guide the selection and use of multiple visual analytical representations based on the nature of the tasks and of data. (2) Because network analyses currently require many iterations to design the representation through the use of complex interfaces, there is a need for systems that are streamlined for specific tasks such as biomarker discovery. (3) Given that many network layouts show no structure, future algorithms should attempt to integrate methods from supervised learning to enable the discovery of hidden patterns. These research directions could enable the rapid discovery of patterns in the age of big data and translational medicine.

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Part V
Biobehavioral Determinants in Asthma

Robert M. Rose

Overview of Chaps. 19 and 20

Part V of the volume "Heterogeneity in Asthma: Translational Profiling and Phenotyping" concludes with two chapters that provide insight into the larger contextual issues of how psychological and cultural forces impact the experience of asthma. Chapter 19 explores how studies of placebo responding and experimental induction of a mild asthmatic episode utilizing concurrent neuroimaging of the brain provide some clues as to how mind–brain interactions may impact the experience of asthma symptoms. Chapter 20 introduces the reader to current conceptualizations about culture and cultural analysis in the diagnosis and management of asthma.

Both these chapters complement the broad, multidisciplinary research that currently is employed in investigations that provide insight into asthma and can be considered part of the larger conceptualizing of systems biology.

Chapter 19: "Central Nervous System Influences in Asthma"

This chapter summarizes studies that attempt to understand how the brain influences asthma. They focus on a study that demonstrates significant physiological changes in lung function, using methacholine challenge, among individuals who show a placebo response compared to salmeterol. Research utilizing functional magnetic resonance imaging has demonstrated that neural circuitry when activated by exposure

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to asthma-laden words can influence allergen-induced asthmatic symptoms as well as circulating cytokines. These studies emphasize the role of the brain in mediating psychological influences in asthma and provide mechanistic insight into the role of stress, placebo, and other factors that alter the predisposition to and the nature of asthma.

Chapter 20: "Asthma, Culture, and Cultural Analysis: Continuing Challenges"

Studies of asthma in different countries and in different populations have demonstrated that asthma is not only complex clinically and scientifically, it also is an exemplar of the need for researchers to incorporate insights from systemic and holistic understanding of the role of culture. They argue, "Cultural analysis can document and enhance understanding of how established health institutions and research programs work" and "how asthma can be understood as a cultural phenomenon and problem." Because of stigma, the true prevalence of asthma is underestimated in various cultures and asthma goes untreated and the burden of the disease is underestimated. Cultural perspectives thus have the potential to integrate relevant, interlinked phenomena of biological, ecological, social, and economic factors that are relevant to a multidisciplinary understanding.

Chapter 19
Central Nervous System Influences in Asthma

Joel N. Kline and Robert M. Rose

Abstract Asthma is a biomedical disorder whose presentation can be markedly influenced by neurological and psychological factors. This chapter describes several approaches that provide insight into the role of psychological factors and brain function in asthma. These include the study of placebo responses and recent explorations using functional neuroimaging during the onset of asthma symptoms. Although the specific mechanisms involved remain uncertain, we are gaining an appreciation for some of the neurocircuitry that is involved. The insula and ACC may modulate inflammatory processes by their influence on neuroendocrine responses to stress, including highly studied effects on the HPA axis and its physiologic responses. However much we have recently learned, it is clear that further study of this topic is critical to fully explicate the role of the brain in asthma.

Keywords Asthma • Placebo response • Brain responses • Neuroimaging • Neurocircuitry • Insula • Asthma stroop test

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19.1 Introduction

It has long been recognized, if only implicitly, that the brain plays a role in the development and/or presentation of asthma. This ranges from the now archaic belief that asthma was purely psychogenic, perhaps induced by excessive maternal attention, to the more recent linkage between "stress" and asthma attacks. Nevertheless, how the central nervous system influences asthma and the identity and role of neurochemical linkages and mechanisms remain obscure. Until recently, the tools to explore this issue did not exist. Now, the development of functional neuroimaging has provided the opportunity to begin to understand the role of the brain in asthma.

Historically, asthma was believed to be, to a great extent, a psychogenic illness. Examples abound in the older literature of emotionally induced wheezing, and the derogatory phrase "smother mother" captured the idea that asthma resulted from a coddled but controlled childhood.

More recently, two major threads of evidence can be found that firmly establish that the brain can influence the lung. These can be summarized as the brain making asthma better (placebo response and power of positive thinking) or making it worse (stressful situations and environments leading to asthma exacerbations).

19.2 Placebo Response

The term *placebo* was coined in the nineteenth century to describe a medicine "more to please than benefit the patient" (Hooper 1843). Falsely assumed by many to have no significant impact on health when used to control clinical studies of new therapeutics, placebo administration clearly can improve measured outcomes in clinical trials for many indications.

Recent experimental studies have identified active brain responses to placebo that reflect how the brain influences peripheral processes as one example of the mind, brain, and body homeostatic regulatory system. Basically, external stimuli (e.g., therapeutic milieu and medication) are perceived, and their "meaning" centrally processed, which leads to changes in peripheral biology.

Unraveling the brain mechanisms through which these effects occur has been made possible by neuroimaging advances, which allow the linking of mental processes with underlying central nervous system (CNS) activity. The CNS continuously processes information (afferent signals) about the social environment to evaluate risk. In response to perceived threat, CNS-mediated activation of neural and endocrine signaling pathways (e.g., the sympathetic nervous system and the hypothalamic–pituitary–adrenal axis) can alter peripheral physiologic processes (Sapolsky 1986, 1990, 2000; Jacobson and Sapolsky 1991; Sapolsky et al. 1990; Sapolsky and Meaney 1986; Sapolsky and McEwen 1985), including the inflammatory biology of the lung. In the placebo response, the brain may downregulate such "danger" responses as well as activating alternate, beneficial pathways.

19.3 Placebo Mechanisms: Expectancy and Threat Appraisal

Before reviewing placebo responses in asthma, it is useful to present an overview of current understanding of how the brain is central to understanding the placebo. The current understanding of placebo is an active brain process with a substantial cognitive and evaluative component (Lieberman et al. 2004; Petrovic et al. 2002). Psychological constructs that have been invoked to explain the placebo response include enhanced expectancy, conditioning, reduced appraisal of threat, and relief of anxiety (Vallance 2006). Expectancies are predictions based on context and appraisal and involve translating cues (external stimuli that signal, in a specific context, the likelihood of a future outcome) into expectancies. Threat appraisals are very powerful stimuli of expectancies, likely due to the survival advantage associated with effective threat recognition.

Threat appraisal leads to complex and interactive behavioral and physiological responses that have been oversimplified as (but are not limited to) the "fight or flight" reaction. Appraisals arise in explaining past events as well as predicting future outcomes. In general, expectancies are formed on the basis of incomplete information, and behavior thus involves a process of forming expectancies based on available cues and revising them as more information becomes available. In this schema placebo responses (1) are initiated by cues; (2) induce a favorable appraisal; (3) promote positive expectancy; and (4) belief that a treatment will be effective in relieving symptoms. Placebo may also incorporate relief resulting from a reduction in appraisal of threat.

Expectancies often have both behavioral and physiological consequences. A substantial literature supports that individuals' expectations in improvement can markedly alter placebo response (Thomas 1987). Such experiments have been conducted in pain (Hrobjartsson and Gotzsche 2004), nausea (Wolf 1950), and respiratory symptoms (Leigh et al. 2003) along with other illness-related phenomena. Physiologic responses can be altered by expectancy when they are associated with subjective sensations, such as the airflow obstruction associated with respiratory symptoms (Leigh et al. 2003) and somatic responses associated with sympathomimetic or parasympathomimetic agents (Schachter and Singer 1962). Expectancy of a positive outcome may be enhanced openly (by informing subjects what to expect) or surreptitiously [e.g., demonstrating a treatment's efficacy by experimentally reducing the expected outcome (Wager et al. 2004)]. The relevant neural circuitry that coordinates behavioral and physiological responses relevant to placebo is described below, in Sect. 19.6.

In contrast to the learning that promotes expectancy, conditioning is the process of associating responses with stimuli. Although repeated experiences can both lead to learning and alter expectancy, conditioning generally refers to unconscious processes, usually outside conscious awareness. Classical conditioning requires the repeated pairing of a conditional stimulus (cue) with an "active" unconditional stimulus (e.g., feeding), with the result that a conditioned response follows the cue in the absence of the unconditional stimulus. Conditioning can also affect systemic

responses that are not accessible to conscious control, such as immunosuppression (Ader and Cohen 1975).

It is likely that both conditioning and expectancy play a role in placebo: "placebo responses are mediated by conditioning when unconscious physiological functions such as hormonal secretion are involved, whereas they are mediated by expectation when conscious physiological processes such as pain and motor performance come into play" (NCCAM 2004). Although these may operate via separable mechanisms, there is substantial overlap between them. Finally, a reduction in anxiety (either general or symptom/disorder related) has been proposed as a feature of placebo effects. In support of this theory, placebo may suppress affective but not sensory ratings of experimental pain (Gracely et al. 1979) [although others have found that placebo inhibits both sensory and the affective aspects (Zubieta et al. 2001)]. Placebo analgesia correlates well with expectation of anxiety relief (Petrovic et al. 2005); anxiety as a target symptom responds very well to placebo (Hrobjartsson and Gotzsche 2001); and many of the same attributes predict response to placebo as to *verum* therapy (Woodman et al. 1994). Anxiety reduction may be a primary response to placebo or may represent reduced threat appraisal.

In summary, several psychological constructs may mediate placebo responses:

- Placebo-induced changes in perceived threat of a stimulus or condition may modify or interact with the brain regions that participate in context-based learning aspects of threat appraisal.
- Placebo-induced changes in positive expectancies may be reflected by activity in brain regions that initiate or modify affective appraisals and threat/reward responses.
- Placebo-induced changes in physiologic responses may be reflected by modulation of activity in brain regions that participate in homeostatic regulation of the autonomic nervous and endocrine systems.
- Placebo-induced reduction in anxiety may be associated with any of the above response patterns or induction of alternate neural circuitry.

There is a gap in knowledge regarding the role and contribution of these overlapping mechanisms for placebo response. Functional neuroimaging (see below) allows us to specifically probe each of these psychological constructs.

19.4 Placebo Response in Asthma

With a rising interest in links between the brain and health across a spectrum of health-related conditions, the role of placebo response in asthma has received increasing attention. In an oft-quoted, but flawed, meta-analysis, Hrobjartsson and Gotzsche found that placebo administration induced beneficial changes in subjectively assessed outcomes, such as pain and depression, but not in objectively defined medical outcomes. Partially based on such assertions, we decided to study if there is a placebo response in objective measures of lung function in the context of asthma,

and, if so, to estimate the magnitude of that effect relative to natural variation in lung function and response to active drug (Kemeny et al. 2007).

In order to evaluate the role of placebo in asthma, we recruited a population with mild asthma and subjected them to a series of methacholine challenges. First, we carried out two studies in untreated patients to validate that their airway hyperreactivity (as assessed by methacholine PC20) was stable. Next we repeated the challenge following open-label administration of salmeterol (50 mcg, dry powder inhaler) to ensure that all subjects demonstrated a bronchoprotective response to this bronchodilator. Finally, we carried out (on different days) a pair of double-blinded methacholine challenges that followed, in a random test order, inhalation of salmeterol or placebo. We also further divided the test population to receive "positive" or "neutral" messages regarding the efficacy of the administered agents.

We found that there was an overall placebo effect in this group of asthma subjects, with methacholine PC20 nearly doubling following administration of placebo. Although this was much weaker an effect than seen following salmeterol administration (approximately 1/3 the benefit), nearly a fifth of the subjects had far greater effects, with an improvement in methacholine PC20 of at least two doses. Surprisingly, half of these individuals normalized their airway reactivity, with a methacholine PC20 > 25 mg/ml. In this study, we identified neither psychological predictors of placebo responsivity nor an overall effect of the message (positive/neutral) with which we attempted to manipulate these responses. Although not all agree that placebo can modulate objective measures of asthma control (Hrobjartsson and Gotzsche 2001), our results strongly suggest that further study of placebo phenomena in asthma is warranted.

19.5 Stress and Asthma

As with placebo responses, there is substantial interest as well as confusion regarding the role of stress in asthma (Leigh et al. 2003; Akiyama et al. 2005; Bienenstock 1991, 1998, 2002; Wright et al. 2002, 2004a, 2005), although the responsible mechanisms remain unclear (Busse et al. 1995). Current research suggests that stress may modulate inflammation, which then can influence airflow, symptoms, chronicity, treatment response, and exacerbations (Lehrer et al. 1993a, b; Maes et al. 2001; Dickerson et al. 2004; Dickerson and Kemeny 2004).

Chronic stress is a stimulus for asthma; it is a significant risk factor for disease (Chen et al. 2002a; Apter et al. 1999; Litonjua et al. 1999) and increased severity (Wright et al. 1998, 2002, 2004b, 2005; Wright 2004, 2005, 2006; Wright and Steinbach 2001; Kubzansky et al. 2006; Gold and Wright 2005; Chen et al. 2002b). Prevalence and severity of asthma have also been shown to relate to socioeconomic status (SES).

One potential peripheral mechanistic link between SES and asthma may include altered immune system homeostasis (McDade et al. 2006; Taylor et al. 2006; Loucks et al. 2006; Rathmann et al. 2006; Alley et al. 2006; Lubbock et al. 2005; Owen et al. 2003; Frieri 2003; Buske-Kirschbaum et al. 2003; Kang and Fox 2001;

Marshall and Agarwal 2000; Sternberg 2001), including increased expression of Th2 cytokine expression (Chen et al. 2003a, b, 2006) and reduced glucocorticoid and β-receptor expression (Miller and Chen 2006). Central stress pathways likely include the HPA axis and the sympathetic nervous system, which serve as "effector" arms of the threat appraisal system. Recent studies linking adverse socio-environmental conditions to increased leukocyte expression of pro-inflammatory genes suggest potential immunobiological mechanisms for such relationships (Miller and Chen 2006; Chen et al. 2009; Cole et al. 2007; Miller et al. 2009).

Acute stress may also exacerbate asthma, and asthma treatment is less effective in "stressed" individuals (Rietveld and Creer 2003). Stressful negative emotion can increase respiratory resistance in asthma (Ritz et al. 2000). Likewise, in work led by William Busse, asthmatic students had greater airway inflammation and allergen-induced airflow obstruction during final examination week—a period of significantly heightened stress—compared to their baseline responses (Liu et al. 2002). This stress also leads to enhanced Th-2 cytokines (Kang et al. 1997). Similar dynamics are seen in animal models of airway inflammation in which both antigen exposure and behavioral stress are experimentally manipulated (Joachim et al. 2003, 2006). With few exceptions, this literature lacks objective measurement of the effects of psychological factors on airway inflammation, despite the strong reciprocal relationship between emotion and inflammation.

19.6 Asthma and the Brain: Neurocircuitry

Observations that psychological factors can influence asthma symptoms underscore the critical role of the brain, since only the brain can transduce stimuli to exert downstream changes on peripheral biological systems important to asthma. Yet, research directed toward understanding asthma's pathophysiology has largely ignored the brain's role. Rosenkranz and colleagues at the University of Wisconsin have identified neural circuitry involved in this interaction within the insula and anterior cingulate cortex (ACC). In a seminal study, this group has evaluated neural circuitry in atopic asthmatics during the development of "late-phase" responses to allergen inhalation (Rosenkranz et al. 2005). This work required development of an asthma-specific "Stroop" task to use as a stimulus during fMRI scanning. The Stroop task is a psychological test for interference with attention in the setting of two conflicting mental signals. The original task asked the subjects to name the color of a displayed word; when the word and its color are discordant (e.g. the word "brown" is written in green), the subject's reaction time (e.g. in naming the color) is increased. The relative slowing of reaction time when stimulus features capture the subject's attention is referred to as interference, which increases with the "emotional salience" or perceived threat of the stimulus and can be general (e.g., "murder") or specific. Words that are specific to an individual's situation or condition (e.g., disease) promote interference effects (Whalen et al. 2006). The Asthma Stroop compares the response to asthma-related words (e.g., wheeze, cough, and choke) and neutral words.

In Rosenkranz's study, atopic asthmatics underwent fMRI scanning at baseline and then early and late after a double-blind methacholine or allergen challenge (crossover design). At the late timepoint after allergen challenge (when subjects were asymptomatic with normal airflow but had increasing airway inflammation and were primed to develop late-phase bronchospasm), the "asthma" words elicited significant changes in neural activity, including activation in the anterior cingulate cortex and in the insula. These regions process information related to physiological responses [e.g., dyspnea (Banzett et al. 2000; Liotti et al. 2001)] but are also closely linked with emotional processing including threat responses (Devinsky et al. 1995; Yasui et al. 1991).

The anatomical projections to and from the insula and ACC implicate these structures in monitoring changes in physiological status, integrating this information with external sensory, cognitive, and emotional information and directing the appropriate behavioral and peripheral physiological (i.e., homeostatic) responses (Rosenkranz and Davidson 2009). They also are involved in threat appraisal and response, both prominent in stressful settings. These findings strongly support the concept that these specific "brain regions may be hyperresponsive to disease-specific emotional and afferent physiological signals, which may contribute to the dysregulation of peripheral processes, such as inflammation." They also suggest the possibility of bidirectional links, a potential avenue for mind–brain modulation of peripheral physiology.

In addition to the modulated neural activity, study of peripheral blood leukocytes demonstrated altered sensitivity to glucocorticoids (reduced ability of dexamethasone to suppress LPS-stimulated induction of cytokines). Thus these neural circuits are in close communication with peripheral immune processes and likely exert mutual modulatory action. Therefore the consequences of threat perception may include activation of the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic nervous system via hormone and neurotransmitter receptors on inflammatory cells (Bhatia and Tandon 2005; Collins 2001)

19.7 Conclusions

It is now clear that the brain modulates the asthma phenotype; there are sufficient data from epidemiologic studies of acute and chronic stress, clinical trials invoking the placebo response, and recent explorations using functional neuroimaging to make this case confidently. Although the specific mechanisms involved (and they are likely to differ, for example, between placebo and stress-invoked responses) remain uncertain, we are gaining an appreciation for some of the neurocircuitry that is involved. The insula and ACC may modulate inflammatory processes by their influence on neuroendocrine responses to stress, including highly studied effects on the HPA axis and its physiologic responses. However much we have recently learned, it is clear that further study of this topic is critical to fully explicate the role of the brain in asthma.

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Chapter 20
Asthma, Culture, and Cultural Analysis: Continuing Challenges

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Abstract Recent research indicates that asthma is more complicated than already recognized, requiring a multilateral approach of study in order to better understand its many facets. Apart from being a health problem, asthma is seen as a knowledge problem, and as we argue here, a cultural problem. Employing cultural analysis we outline ways to challenge conventional ideas and practices about asthma by considering how culture shapes asthma experience, diagnosis, management, research, and politics. Finally, we discuss the value of viewing asthma through multiple lenses, and how such "explanatory pluralism" advances transdisciplinary approaches to asthma.

Keywords Environmental health • Transdisciplinarity • Explanatory pluralism • Anthropology of science • Cultural frames • Qualitative methods • Compliance and cultural competency

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20.1 Introduction

Asthma is at once (1) a heterogeneous chronic disease experienced by hundreds of millions of people around the world, living in very different settings; (2) a standardized (although variable in practice) diagnostic category deployed by health care professionals to direct disease management and enable disease surveillance; (3) a research focus of scientists employing a wide array of methods in fields as varied as pulmonology, genetics, epidemiology, immunology, air chemistry, and cultural anthropology; and (4) a sentinel condition that motivates environmental research, activism, and regulation. In short, asthma is a complex condition, with multiple dimensions, prompting diverse efforts to understand and manage it. All of these efforts need further development and refinement, even after decades of research and concerted effort. Asthma has resisted our most dedicated efforts to understand and care for it.

Furthermore, recent research developments call attention to the possibility that asthma is even more complicated than has already been recognized. An editorial in *The Lancet* in 2008, for example, noted that the "notion of asthma as one unifying disease concept is disappearing further into the realm of historical over simplification" (Lancet editorial 2008). A 2011 article by veteran asthma researcher Stephen Holgate and coauthors (Holmes et al. 2011) also points to over simplification in asthma research to date: "No single method is sufficient to model a syndrome as complex as asthma accurately," they write (Holmes et al. 2011). Their critical review was directed primarily at the limitations of animal models of asthma, which "use the concept that allergen-driven Th2-type inflammation is the underlying abnormality in asthma" (Holmes et al. 2011). In the view of Holgate and coauthors, animal models "have failed to recapitulate important features of the disease," especially those more closely connected to "infection (viral and bacterial), air pollution, diet, environmental tobacco smoke, drugs and other chemicals, and their interplay with genetic factors." What is called for, in their view, is the development of a "tool-kit" of multiple approaches and methods that "when combined, build a holistic picture of the disease."

Statements such as those made by Holgate and coauthors indicate that asthma is not only a health problem but also a knowledge problem, calling for new knowledge practices and forms. Asthma is also, we argue here, a cultural problem. The complex multidimensionality of asthma makes it difficult to deal with clinically and scientifically, and also culturally—because it challenges us to create new modes of thought and practice that differ from those behind conventional ways of thinking about health, research, and politics.

Modern health care and research aim to identify the cause and mechanism of disease, progressively ruling out what is not determinative. The logic is binary: Is it X, or not? If not X, is it then Y, and not Z? Modern health care and research are also organized into disciplinary specializations, allowing for impressive depth of knowledge and analysis, but making it difficult to see "the whole (bodily, much less environmental) system." And modern health care and research privilege "in-body"

constructs of disease; air chemists and exposure scientists are usually not housed within Schools of Medicine; few medical training programs cover environmental health stressors; the U.S. National Institute of Environmental Health Sciences enjoys only 2 % of the overall budget for the National Institutes of Health; social scientists—especially the qualitative kind—are not seen as deeply relevant to understanding and care of disease.

Intense focus on bodily mechanisms and singular causation has enabled amazing advances in medicine over the last century. It has also fueled what anthropologist Mary Jo Good (2007) has termed a "medical imaginary" that fosters belief that modern medicine can provide both explanation and cures for any range of ills. Even in the most difficult cancer cases, Good argues, there is often great expectation that biomedicine will come through—with clear explanations and solutions to the problem of illness. This drives people in varied positions—patients, family members, doctors, researchers, the media—to conceive, represent, and deal with disease in a particular manner: one which is straightforward and clearly actionable. We all want to know what, *exactly*, to do when someone is sick. And we count on health care experts, institutions, and research to tell us this.

But asthma is much more complex. And the acknowledgment of asthma's complexity by researchers like Holgate and his coauthors indicates a potential convergence, we also argue, between biomedical researchers' recognized need for more holistic perspectives and anthropologists' long-standing goal of systemic, holistic understanding of culture. That convergence in turn creates new opportunities for genuine "transdisciplinary" work involving not only diverse biomedical researchers but also cultural analysts as well.

Complex conditions such as asthma cannot be adequately explained or cared for within current cultural frames. Cultural innovation is therefore required. Cultural analysis can document and enhance understanding of how established health institutions and research programs work, drawing out the assumptions that undergird them. Cultural analysis can also draw out the explanatory limits of biomedicine—what it cannot address because of habitual, often discipline-specific modes of thought and practice that efforts at "transdisciplinarity" are intended to overcome.

As Harvard physician–anthropologist Paul Farmer (renowned for his work in Haiti and Africa with Partners for Health) has emphasized: a critical epistemology of disease is needed that addresses the multifactorial nature of disease emergence. This kind of innovation starts, Farmer argues, through examination of existing conceptual frameworks, asking: "What is obscured in this way of conceptualizing disease? What is brought into relief?" (Farmer 1996). Cultural analysis, carried out in dialog with biomedical researchers, physicians, and all other involved in asthma care and knowledge production, can advance this kind of conceptual examination. Cultural analysis can highlight cultural innovation, the development of new modes of thought and practice in all areas of the "asthma complex system"—from researchers like Holgate, to clinicians, to asthma sufferers themselves.

Below we describe how asthma can be understood as a cultural phenomena and problem. In the first section, we describe how the theories, methods, and techniques of contemporary cultural analysis have been updated for study of complex societies,

dealing with complex problems. We then describe how cultural analysts have examined asthma experiences, diagnosis and management, research, and politics—collectively drawing out the many ways that asthma is a cultural phenomena. The final section discusses the cultural challenge and value of multifactorial, pluralistic ways of understanding asthma systemically, "holistically," or "transdisciplinarily."

20.2 Theories, Methods, and Techniques of Cultural Analysis

Cultural analysis not only strives to document all that is going on in a "system" but also seeks to explain how and why things occur. It maps the many influences that shape human perceptions and actions, and the forms and patterns of language, thought, behavior, and social interaction that characterize a particular context. In cultural anthropology, the ambition is holistic. Cultural anthropologists strive to explain the parts, relationships, and dynamics of whole sociocultural systems—their structure, functioning, and context, and how they change (or resist change). They do this through observation of systems at work, through research that draws out historical and external forces on a system and through sustained dialogue with human actors within the system.

In early anthropological research, the system studied was often a village or particular language or ethnic community, seeking to explain how religious and economic practices, family and political structures, and aesthetic and narrative forms synchronized to produce the particular system at hand. An array of ways to study whole systems emerged. Researchers learned, for example that focusing on women's roles, practices, and perceptions was a reliable way to understand how a system was held together (Weiner 1976).

In the last few decades, cultural anthropologists have studied increasingly complex societies and phenomena, where the "system" studied could be a residential or scientific community, a particular hospital or urban space, an organization like Doctors Without Borders that works across geographic locations, and large-scale phenomena such as globalization and neoliberalism (see e.g., Fischer 2003). This has entailed a need to "study up" (Nader 1972), focusing on the cultures and practices of elites and professionals, including health professionals. The anthropology of science has also developed as a cutting-edge subfield, with work focused on how scientists think, work, and are organized and on ways science is understood by citizens, patients, lawmakers, and other stakeholders (see Fischer 2007 for a summary). A pulmonologist with supplementary training in allergies and immunology, anxiously trying to meet three grant application deadlines to keep his laboratory at a major university medical center active and productive—driven by a complex mixture of curiosity, socioeconomic need, and desire to heal—is thus treated as much as a cultural actor as a Mexican-American mother of three, who is an evangelical Catholic, with

limited English, and resides in Los Angeles County, California, while managing both her own relatively mild asthma and the more severe asthma of two of her children. Organizations are also analyzed as cultural actors. Bureaucracies formed in different historical and geographic contexts, for example, delineate and address complex problems like asthma in different ways and thus need to be analyzed and understood as cultural actors and producers.

"Culture," in this way of doing cultural analysis, has a dynamic character. Culture is not so much a stable and consistent "worldview," conceived largely as a set of beliefs, as it is habitual modes of perception, action, and articulation—patterned ways of seeing and conceptualizing problems, and figuring out how to respond. Culture provides terms and techniques for making sense of things (Fischer 2003) and is thus both a resource and a limitation.

20.3 Culture and the Experience of Asthma

How asthma is experienced differently according to historical, geographical, and cultural context has been studied by cultural analysts using a range of approaches and methods, including interviews, ethnographic fieldwork, videography, and participant observation—although much work remains to be done (Adams et al. 1997; Chalfen and Rich 2004, 2007; Gabe et al. 2002; Rudestam et al. 2004; Williams 2000; Wind et al. 2004). Some cultural anthropologists also analyze literary texts to illuminate how asthmatic experience is shaped by culture; as an example of this approach, we consider here journalist Tim Brookes' (1994) autobiographical account *Catching My Breath: An Asthmatic Explores His Illness.*

As an adult, Brookes was nearly killed by an asthma attack, which set in motion a remarkable process of exploration to figure out *the* cause. Brookes consulted specialists in numerous medical fields, experimented (unsuccessfully) with homeopathy, and learned about the wide array of alternative treatments asthmatics have tried, including yoga, acupuncture, hypnotism, and cockroach tea. He wrangled with insurance companies and rode with a mobile health unit in New York City serving poor communities—all aiming to understand the spectrum of factors that shape asthma experience and outcomes. At the end of his book, Brookes decides that the "culprit" behind his life-threatening asthma attack was an antacid tablet containing yellow dye. To test his theory, he contacted his pulmonologist and again ingested a yellow-dyed antacid tablet in the controlled environment of the Pulmonology Function Lab. But there was no emergency. In this instance at least, the yellow-dyed antacid did not trigger a life-threatening asthma attack.

Brookes did not thus conclude that he was safe with yellow dye. Instead, he recognized that his own response was shaped by what we have referred to here as the "modern" culture of explanatory simplicity: a habit of working to identity *the* cause behind any effect—be it an asthma attack, another disease outcome, or some other complex problem. "Even after all this time," Brookes reflected, and all his effort to explore the multiple complexities of asthma, "I was still looking for the single

causes, simple cures." Brookes desperately wanted to know *the* cause of his asthma attacks and felt that this was what was needed to care for, if not cure, himself. Brookes thus provides a powerful example of how a "medical imaginary" can powerfully shape asthma experience.

And that cultural effect is not limited to asthma sufferers but is a systemic effect that works on multiple levels of the asthmatic condition including on biomedical researchers. Reflecting on the long history of asthma research in which he has played a prominent role, Fernando Martinez likens research on asthma to kindred research on rheumatoid arthritis, for which scientists "yearn[ed] for a theory that would fit it all together, under one formula, one idea, one mechanism" (Martinez 2007, quoting Weyand and Goronzy 2006). Such ideas would have to be relinquished, Martinez argues, and researchers must, "with apologies to William of Ockham," learn to tolerate "less parsimonious" explanations that do not rely on any simple cause or mechanism, biological or environmental, but grapple instead with "weak linkages" and "indirect, undemanding, and low-information regulatory connections" that are "highly flexible" and thoroughly heterogeneous. We return to these cultural analyses and their implications for future asthma research in the conclusion.

20.4 Culture in the Diagnosis and Management of Asthma

Culture also shapes asthma diagnoses and management by health professionals. As medical anthropologist David Van Sickle (2005, 2009) has documented, for example, biomedical professionals in Chennai, India do not see and diagnose asthma in the way prescribed by the ISAAC (International Study of Allergy and Asthma in Children) protocol for the study of asthma incidence across nations. In part, this is because of the stigma attached to asthma, and resulting hesitation to confirm an asthma diagnosis. Van Sickle argues that because patients and their families do not want to be diagnosed with asthma, doctors (who are trying to make their patients happy in a very competitive medical market place) tend not to diagnose asthma in these individuals. This means that asthma goes untreated, is not controlled, and comes to adversely shape life trajectories in significant ways. A culture which frames asthma as stigma thus unleashes a cascade of effects on patients and shapes what physicians see and do.

Van Sickle concludes that "many practicing clinicians in India differ in the perception and interpretation of common asthma symptoms depicted in the ISAAC video," and that the cumulative effect of these many cultural differences "may account for the low rates of reported asthma observed in epidemiological studies conducted in Chennai and other parts of India, and suggests that rates of diagnosed asthma among these populations underestimate the true burden of disease" (Van Sickle 2005). The ISAAC protocols and research effort have been crucial to our understanding of asthma internationally. But Van Sickle's research also suggests that Chennai physicians have ways of understanding respiratory illness outside

expected frames. A key challenge for cultural analysis and global public health is to leverage the difference in perspectives—not seeing the perspective of Chennai physicians as a better one than that embedded in the ISAAC study, but as a different one, and thus a means to critically reflect on different ways of conceptualizing and managing asthma. Again following Paul Farmer, such differing perspectives shaped by culture become an opportunity to ask, of ISAAC protocols and Indian physicians alike: “What is obscured in this way of conceptualizing disease? What is brought into relief?” (Farmer 1996).

20.5 Culture in Asthma Science

In his history of asthma and allergy in the USA, historian of medicine Greg Mitman tells the story of Oren Durham, a photographer and amateur “pollen collector” of the early 1920s who supplied his uncle—one of the first physicians in the USA to practice immunotherapy on his hay fever patients—with pollen samples for his immunization treatments. He quickly turned professional and developed the first detailed pollen maps of Kansas City and Chicago, linking pollen levels to urban neglect in the process. Durham went on to build a collaboration with the US Weather Bureau, using its networks to coordinate and standardize a system of pollen collection and analysis that led to the first national pollen maps. Durham was later hired as chief botanist by Abbot Laboratories to develop a market for pollen extracts (1930), foreshadowing the huge role the pharmaceutical industry would come to play in asthma knowledge and care. Mitman goes on to describe intricate links between the pharmaceutical industry and professional societies, questioning how commercial interests have shaped how we approach asthma. Part of the story is about increasing standardization in the way asthma has been conceptualized, regardless of context. Mitman’s overarching message is that we should “widen our focus on the causes and prevention of [asthma] and invest more in research that takes into account the ecological relationships between illness and place” (Mitman 2007).

Mitman lays empirical and analytic ground for understanding the wide array of sciences that contribute to asthma knowledge. There is an intriguing array of actors just in the story he tells, and there are even more in the contemporary asthma picture. Cultural analysts need to map their connections, as Mitman did for Durham, questioning how these connections have staged and influenced their work on asthma. Cultural analysts can also work to understand how the disciplinary frames that scientists and health professionals from different fields bring to their work. These disciplinary frames provide important analytic purchase, yet can also make it difficult for people from different fields to work together.

The atomistic way that both medical fields and scientific disciplines are configured in modern societies illustrates a cultural tendency to deal with problems by breaking them apart to explore different possible mechanisms—assumed to be isolatable. This way of organizing knowledge production and translation both embodies and reinforces assumptions that causality is singular, usually linear, and, once

delineated, can be "fixed." Cross-systems cascades and cumulative effects are hard to get at within such a regime. And it is hard for individual researchers to rise above it. This is not because individual researchers do not recognize the need for systems approaches and interdisciplinarity. Many researchers do. But a researcher trained in a particular discipline is deeply encultured to see in a particular way and to have a particular "thought style." Indeed, this is what it means to be disciplined.

Working across disciplines—creating what historian of science Peter Galison has called "trading zones" can thus be enabled by cultural analysis that draws out the thought styles of different fields, increasing the visibility of their assumptions and logics. Cross-disciplinary understanding is a form of cross-cultural understanding; it does not solve the problem of cultural differences within the sciences and among medical fields, but it can animate methodological reflexivity and the kind of creativity crucial in transdisciplinary effort.

20.6 Culture in Asthma Politics

Numerous cultural analyses of biomedicine have documented its tendency to "individualize" disease (Good 1994, 2007; Frankenberg 1980). The difficult paradox involved becomes all too clear in the case of asthma; clinicians and researchers alike must attend to the heterogeneous specifics of an individual's respiratory distress, yet doing so can occlude the social, cultural, economic, and political determinants of illness. Cultural analysts can help reach the goal of a more systemic understanding of asthma, bringing its political dimensions back into view.

Sociologist Phil Brown and colleagues, for example, have studied "not how the illness shapes the individual experience," but how community organizations can "create a collective identity around the experience of asthma" that "links social and physical realities" that can "transform the personal experience" into collective effort and environmental advocacy (Brown et al. 2003). The Brown group analyzed how Alternatives for Community and Environment (ACE), in the Boston neighborhood of Roxbury, built new scientific collaborations with researchers at the Schools of Public Health at Harvard and at Boston University, developing the AirBeat project to install neighborhood air monitors, collect air quality data, and analyze the relationship between air quality data and doctor and hospital visits. A similar group, West Harlem Environmental Action (WE ACT), partnered with researchers at Columbia School of Public Health to develop an even more extensive research program. These collective efforts resulted in a way of thinking about asthma that was overtly political. As one community leader put it, "it's the underlying conditions of poverty and social injustice that are contributing to all these things…[Y]ou just can't get rid of cockroaches and expect asthma to go away. For that matter, you can't just put in better buses and expect asthma to go away. It's all got to be approached in a social justice framework" (Brown et al. 2003). Foregrounding the social and political dimensions of asthma in these cases led to more, and better, biomedical research.

In other cases, biomedical researchers more focused on the systemic forces shaping asthma lead to recognition of its political dimensions through the lens of health disparities research. Pulmonologist Rosalind Wright and Diane Gold have been active in the Asthma Coalition on Community, Environment, and Social Stress (ACCESS), a long-term prospective cohort study noteworthy for its multilevel approaches including gene–environment interaction studies, molecular profiling, socioeconomic analysis, indoor and outdoor exposure monitoring, behavioral factors, and numerous other measures of differential social support or violence, all modeled in an effort to better understand how psychosocial stress may shape the health disparities visible in asthma. They conclude that "in the United States, effective reduction in disparities in asthma morbidity will be dependent only in part on specific measures like establishment of smoking cessation programs, home allergen reduction in sensitized asthmatic children, physician feedback, and/or health education. The long-term success of any of these specific measures is likely to depend, in great part, on more general improvements in living conditions and life opportunities" (Gold and Wright 2005).

Health disparities in asthma incidence and severity exist beyond US borders, of course. Cultural analysis is important at the global level as well, again bringing out the more systemic political and cultural forces that shape asthma. The Global Alliance for Clean Cookstoves (http://www.cleancookstoves.org), for example, is a private–public coalition of academic organizations like Columbia University, multilateral organizations like the World Health Organization, and numerous corporations working collectively to produce the cultural, technological, and political changes necessary to reduce the global burden of respiratory diseases, including asthma, resulting from traditional cookstove use that disproportionately affects low-income women and children.

20.7 Toward a Culture of Explanatory Pluralism

Today's global asthma epidemic exemplifies the kind of complex problems that involve interlinked systems of different kinds—biological, ecological, social, economic, and technical. Responding to complex problems requires extraordinary levels of coordination in research and practice, a capacity to work beyond established paradigms, and the drive for "holistic," systemic understandings shared by both cultural analysts and asthma researchers. The complex dynamics of asthma thus present an opportunity where conversation and collaboration can occur between cultural anthropologists and biomedical researchers and clinicians focused on asthma.

Section 20.2 analyzed biomedicine's tendencies toward simplification and unification, and their effects on asthma sufferers and researchers alike. Anthropologist Ian Whitmarsh sees this tendency at work in the long history of efforts to deal with asthma. Since the end of the nineteenth century, Whitmarsh (2008) points out, "asthma has been viewed as neurosis or physiological predisposition; caused by dust, pollution,

heredity, parental emotions, the unclean modern home (carpets harbouring dust mites), or the continually cleaned modern home (underexposure to infections); and treated with stimulants and depressants, dieting, steroids, and various tonics." Asthma researcher Fernando Martinez (2007) reinforces the cultural anthropologist's analysis, noting how understanding of asthma has constantly shifted from one "unifying explanation" to another, while never successfully unifying. Whitmarsh notes that what is most remarkable in this history, however, "is not the plurality of definitions, causes, and diagnostic techniques" in its own right, "but rather the attempt to reduce this plurality". The plurality of definitions, practices, and etiologies has been framed as something to be resolved rather than something to be actively leveraged.

We emphasize again: this is a cultural phenomena and problem. Modern biomedical culture expects pluralities to be reduced, "mysteries" to be cleared up, with straightforward and preferably singular explanations, after sufficient rational effort has been expended.

Here is where cultural analysis can suggest a different frame. Rather than be frustrated or mystified by asthma's resistance to singular explanation, we can cultivate what historian of science Evelyn Keller calls a culture of "explanatory pluralism"—the idea that science, rather than seeking or needing one definitive answer, in fact thrives best on multiple interpretations that do not try to reduce or eliminate each other. Keller argues that throughout history, the culture of the life sciences has been one of "de facto multiplicity of explanatory styles in scientific practice, reflecting the manifest diversity of epistemological goals which researchers bring to their task. Explanatory pluralism, I suggest, is now not simply a reflection of differences in epistemological cultures but a positive virtue in itself, representing our best chance of coming to terms with the world around us…[T]he investigation of processes as inherently complex as biological development may in fact require such diversity" (Keller 2002).

Valuing and cultivating explanatory pluralism in asthma research and care will be a cultural challenge. But here is where the reframing that cultural analysis can offer joins with biomedical researchers' own efforts to reframe scientific practice, thinking, and organization to better understand and cope with complexity. Attempts to create "transdisciplinary" approaches to complex conditions like cancer and asthma are recognition, in our view, that biomedical research faces the challenge of transforming its own culture from one geared toward unifying explanations to one that values explanatory pluralism. But as Colditz et al. (2012) discuss in the context of cancer research, although the "benefits of transdisciplinary teams…have been touted for decades…few such teams have been successfully assembled." They argue that "operating in this collaborative way is not intuitive," so "investigators…regress toward the mean of siloed research, which is familiar and routine." The mutual recognition by researchers and anthropologists alike that biomedical research culture needs to change if we are to effectively deal with asthma presents an opportunity for more dialog. Perhaps cultural analysis, with its ability to comprehend and reframe "familiar and routine" understandings and elicit alternatives, can help biomedical researchers meet the challenges of transdisciplinarity and, in turn, the global challenges of asthma.

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Part VI
Conclusions and Future Directions

Chapter 21
Conclusions and Future Directions

William J. Calhoun and Allan R. Brasier

21.1 Introduction

21.1.1 Heterogeneity of Manifestations of Asthma

Considerable variation exists in the clinical expression of asthma. As detailed in the corresponding section of this monograph, the heterogeneity of asthma is expressed clinically, by commonly observable characteristics, but these clinical phenotypes overlap and have indistinct borders. By a variety of assessment technologies and approaches (epidemiologic, physiologic, and clinical), this syndromic disease defies clear and unambiguous classification. Collectively, clinical phenotyping has not led to reliable categories of asthma that inform treatment, predict progression of lung function abnormalities, or define risk for exacerbations and death. It is for this reason that—omics approaches have been explored, and by which new stratagems for molecular phenotyping have been developed.

21.1.2 Genetics, Epigenetics, and Gene Expression Profiling

The last half of the twentieth century was marked by exponential growth in the understanding of human genetics and its application to human diseases.

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The advances in technology that have allowed these advances to occur span several orders of magnitude in throughput. Studies of structural genetics have informed understanding of risk factors, severity, and characteristic features of asthma but have not been as helpful in understanding temporal variation in this disease. The advent of gene expression profiling (genomics), and proteomics, in which quantities of gene product are measured and analyzed, offers the potential to understand the occurrence of exacerbations, time-dependent variation in lung function and other manifestations of asthma, and the critically important interaction between host (structural genetics) and the environment (allergens, viruses, etc.). Finally, the emerging field of epigenetics is allowing us to understand how environmental exposures may modify the noncoding structure of DNA in ways that are stable, affect gene transcription, and are heritable within cellular generations. Collectively, these fields offer great promise to understand and explain the heterogeneity of disease expression in asthma.

21.1.3 Proteomics, Metabolomics, and Systems Biology

As discussed in this edition and in the supporting literature, asthma is a heterogeneous disease with a complex phenotype that resists clear and absolute classification. To decipher the pattern of symptoms and derive a molecular description of the disease requires multidisciplinary approaches, with an unbiased focus. That is, at a basic level, a combination of global analyses spanning genomics, proteomics, and metabolomics may lead to a description of the molecular events that lead to the complex phenotypes we collectively define as "asthma." The integration of the "omics" observations into a coherent "system" would be described as the "systems biology" of asthma and likely lead to astonishing insights into its etiology.

This edition illustrates the major sensors and signaling molecules constituting the airway innate immune response (IIR), its coupling to adaptive immunity, and how these molecular events may lead to acute decompensation/exacerbations. Recent advances in quantitative proteomics technology that can be used to monitor the status of the IIR. In patients with severe asthma, the mechanisms for glucocorticoid (GC) resistance have remained enigmatic. Applications of functional proteomics can be used to characterize GC signaling, estimate GC receptor function, and characterization GC resistance through posttranslational modifications and differential abundance measurements. These new exciting methodologies and approaches for measurement of dysregulation of the metabolism, inflammatory subtypes, including the innate immune pathway, and insights into normal and dysfunctional signaling of the GC receptor will be informative in translational research. As these technologies evolve, integrating metabolomics with functional proteomics will reveal novel insights into disease and response to treatments.

21.1.4 New Analytical Approaches

Analysis of complex datasets involves two philosophically distinct approaches: one, the application of supervised learning to develop a predictive model of disease outcome based on some mathematical combination of measured variables and two, unbiased analysis in which no preconceived hypothesis regarding the relationships among variables is assumed. This is an ordered process where statistical methods are used to rank the variables based on the degree to which they are significantly different between the classes and to remove uninformative or correlated data. An illustration of supervised classification using multivariate cytokines has been informative to show that protein patterns in BAL can be meaningfully combined into predictive models of airway reactivity. The emerging field of visual analytics tightly integrates these methods with powerful interactive visualizations designed to enable comprehension of complex patterns using one of the most developed methods called network analysis. Network analysis enables both a visual and a quantitative analysis of the data using a unified representation and has led to the identification of a previously unrecognized innate endotype in BAL proteins. These kinds of approaches hold great promise for deconvoluting the complexities of biologic systems and their perturbations in disease.

21.1.5 Neural and Behavioral Contributions to Asthma Heterogeneity

The questions of how psychological and cultural forces impact the experience of asthma, and thereby contribute to the heterogeneity of its expression are critically important to a full understanding of this disorder. These chapters complement the broad, multidisciplinary research that currently is employed in investigations that provide insight into asthma and can be considered part of the larger conceptualizing of systems biology, using two different perspectives: how the central nervous system, and its cognitive processes influence the expression of asthma via neurochemical mechanisms, and how cultural and sociologic factors influence the expression of disease. Although the latter seems at first to be unrelated to biological heterogeneity, in fact the true prevalence of asthma in underestimated in various cultures and asthma goes untreated and the burden of the disease is underestimated. Cultural perspectives thus have the potential to integrate relevant, interlinked phenomena of biological, ecological, social, and economic factors that are relevant to a multidisciplinary understanding.

21.2 Challenges and Opportunities in Translational Research

Simply put, translational research seeks to improve human health. To accomplish meaningful health impact of a new drug, devices, or practice modification, a broad range of expertise must be effectively engaged. Scholars who have considered the translational spectrum have broken the process into at least two domains, the first concerned with proving an intervention that modifies a biological process has efficacy in humans, a process known as "bench-to-bedside" translation (T1 phase), and the second proving that intervention applied to real world populations has measurable benefit, informing evidence-based practice and health policy, a process referred to as "bedside-to-curbside" translation (T2-4 phase, Khoury et al. 2007; Woolf 2008). The complexity and regulatory issues surrounding this broad concept of translational research continuum poses major challenges to its efficient conduct (Sung et al. 2003).

As a result of increasing emphasis on bringing basic biomedical discoveries into improved human health, the NIH Roadmap initiative created is the Clinical and Translational Sciences Award (CTSA), whose primary goal is to stimulate the speed and effectiveness of translational research (Zerhouni 2007). Currently, 61 CTSAs and the NIH Hatfield Clinical Research Center have been funded across academic health centers in the USA that are providing strategies and promoting institutional transformation to overcome the inherent bottlenecks in the translational research continuum.

One important strategy taken by CTSA has been to place increasing emphasis on team science approaches. The justification for team science has begun to be studied by the emerging field of the "Science of Team Science" (SciTS, Borner et al. 2010; Falk-Krzesinski et al. 2010). In science and engineering research, the approach of teams has dramatically accelerated over the last quarter century, making multiuniversity collaborations the fastest growing authorship structure (Jones et al. 2008). Research and intellectual property developed by highly functioning multidisciplinary research teams has greater impact in peer recognition through citations and patent uses than research products from siloed investigators (Wuchty et al. 2007). This transition has been accelerated by the recognition that increasingly specialized scientific fields must develop collaborations to enhance creativity and accelerate the pace of discovery to address major societal health problems (Disis and Slattery 2010).

A local implementation taken by the UTMB CTSA is the development of multidisciplinary translational teams (MTTs, Calhoun et al. 2013). We consider an MTT to be a hybrid academic–business construct embracing academic missions of generating new knowledge, educating trainees, and building capacity, yet focused on development of product-like translational goals. These goals would be to develop or apply a device, therapeutic, or intervention to improve human health (Calhoun et al. 2013). Of relevance here, the content of this book has been developed by the severe asthma MTT in collaboration with other leading investigators. The severe asthma

MTT is composed of physicians, cellular biologists, proteomics, psychiatrists, bioethicists, biomedical informaticians, and involves health profession trainees as a vehicle for learning about team roles and processes. As a result, the physicians leading the severe asthma MTT has published work in molecular classification, endotyping and network analysis, for example, that would not have been produced without the team construct (Bhavnani et al. 2011; Brasier et al. 2008, 2010; Pillai et al. 2012).

As an example, translating discovery science in asthma, and using this information to inform classification of distinct molecular phenotypes could be a first step towards an ultimate goal of personalized medicine. In asthma, the expression of "disease" is a common phenotype (wheezing and bronchoconstriction) that may be the result of distinct pathological processes. This translational model is conceptually based on several concepts (1) subtypes of disease are difficult to identify with conventional clinical criteria and (2) application of multidimensional profiling—mRNA, proteins, and metabolites can improve risk assessment and treatment over clinical assessment alone. In this book, we have provided a number of findings that indicate that these translational approaches to medicine are in fact applicable to diagnosis and management of asthma.

The application of molecular profiling to inform personalized medicine shifts emphasis of health care to early interventions using the most efficacious therapies. For example, accurate identification of the small subgroup of patients with severe asthma, a subgroup that represents the highest rate of morbidity may lead to selection of optimal dosing and alternative therapies (Moore et al. 2007; Wenzel and Busse 2007). We contend that molecular identification of the severe asthmatic subgroup will reduce trial and error prescription of steroids, reducing side effects and morbidity from trial and error therapy. The application of evidence-based decisions for selection of therapy will mean that drugs are safer—less adverse reactions and reduce cost of health care. From a clinical investigation perspective, molecular profiling will also improve clinical trial design and reduce time and cost for drug approval.

Challenges in translational research remain. The pathway through T2 to T4 (1,2) is arduous, expensive, and usually time consuming. Even a compelling and validated finding in T1 may not have the performance characteristics necessary for diagnostic or predictive utility in T2 and T3 populations. Hence, development pathways may stall for reasons unforeseen at the beginning of a promising project. As an approach advances through the translational timeline, increasing numbers of human subjects are generally required, with proportionately increasing fiscal costs. Whether these costs are borne by federal, industrial, or academic resources, the constraints on research resources will have a constraining influence on T2–T4 translation. Finally, the uptake of new approaches and technologies necessary for T4 translation by the broader array of professional organizations, federal advisory boards, guideline committees, and other stakeholders is subject to considerations well beyond the scope of scientific merit alone.

21.3 Promises and Pitfalls of Personalized Medicine

Personalized medicine is an articulated vision in modern medicine. It is a medical model that customizes medical decisions, practices, and/or products being tailored to unique molecular features of a specific patient, derived from T1–T3 translational research. Many academic health care organizations include such concepts in their statement of purpose, or their marketing tag lines, or other institutional documents. The concept of delivering the correct molecule, at the correct dose, with the correct timing in order to maximize therapeutic efficacy and to reduce the occurrence or severity of adverse effects is attractive to physicians, patients, and ultimately to third-party payers as well. This highly customized approach is distinct from traditional practice of medicine where clinical diagnosis and management relies on expression of clinical signs and symptoms, which are imprecise and overlapping. It is true however that personalization of therapy has always been part of medical practice, whether incorporated as clinical judgment, or as empiric use of therapeutic agents for individual patients and monitoring clinical responses, or by considering patient preferences in the selection of medical or surgical therapies.

How personalization occurs has been driven by technology, from group response approaches, to clinical phenotyping, to simple single parameter biochemical tests of blood, urine, or other biologic fluids. The concept of personalized medicine, however, gained most traction from the application of genetics to patients with disease. Polymorphisms in genes (both single nucleotide polymorphisms, SNPs, and other more substantive coding variations) are associated with therapeutic responses to warfarin, short-acting beta2 agonists, leukotriene inhibitors, corticosteroids, cancer therapeutics, and several others; other polymorphisms confer risk of developing diseases: asthma, atopy, bronchial hyperresponsiveness, etc. Although in some cases the clinical effect size is moderate, in most of the polymorphisms so far described, the clinical effect size is small, suggesting that other factors must also be important in the clinical expression of disease.

As developed in this book, a number of other biochemical (gene expression profiling/genomics, epigenetics, proteomics, and metabolomics) and data analysis approaches also have potential to discriminate mechanistically similar groups of patients, and by so doing, reduce therapeutic variability. Asthma is a disease of temporal variability, marked by times of minimal symptoms and signs, interrupted by periods of exacerbations. This temporal variability makes the conceptual linkage between structural (unchanging) genetics and temporal variability in asthma difficult. In contrast, genomics, epigenetics, proteomics, and metabolomics involve the study of regulated expression of genes (including splice variants), their protein expression, and the consequences of protein expression and activity on biologic molecules. Accordingly, these platforms may provide better understanding of the temporal variability in the expression of clinical asthma. Finally, because of the relatively small effect size for most genetic variations (a limitation that may also apply to other -omic technologies), it is likely that combinations of analytes will be more predictive as a panel than any one of the markers in isolation. Advanced

analytic techniques, as describe herein, have the potential to advance the field significantly.

Personalization has potential application in many areas related to asthma (1) in the clinic to predict therapeutic response, adverse events, and the course of disease; (2) in clinical research, stratifying subjects by predictive factors that reduce variability of response can improve the signal-to-noise ratio of a study, leading to a smaller, or more cost-effective study, or a more definitive result; and (3) in the basic or translational research enterprise, phenotyping strategies that isolate specific pathways of disease allow for definitive understanding of new mechanisms of disease pathogenesis. This field has been recently reviewed (Bhakta and Woodruff 2011).

Personalization of therapy does confer some downside as well. From a drug development standpoint, each aspect of personalization of asthma therapy will likely result in a smaller potential market; for example, if a particular approach identifies a mechanistically homogeneous group of patients that is 10 % of those patients with asthma, then therapeutic approaches developed for that group will be potentially marketable only to those 10 %. This consideration makes personalization in the drug-development arena a two-edged sword: more effectiveness or less toxicity, in a smaller market. In addition, predictive biomarker development must precede Phase II and III drug development, which may impact on the usable patent life. Ultimately, personalized medicine may increase the costs of bringing medications to patients.

There are conceptual concerns with the concept of personalized medicine as well. It is possible that the number of different measurements required for a new biomarker to be determinative is so high as to be impractical or prohibitively expensive. Other unmeasured factors, such as diet, supplements, socioeconomic status, living conditions, and a myriad of other disease modifiers, could individually or collectively have a greater effect on the expression of asthma than the determinants in a predictive biomarker. Finally, particularly in asthma, chance encounters with viruses and other infectious causes of exacerbation are largely unpredictable, and encounters with allergens, although predictable may well be unavoidable. These known triggers of asthma exacerbations could reduce the utility of predictive phenotyping.

For personalized medicine to become a reality, a number of obstacles must be overcome (Hamburg and Collins 2010). The success of personalized medicine will rely on using accurate diagnostic tests in the clinic, which may be laboratory-based, multivariate tests whose interpretation relies on complex machine-learning algorithms. For this to occur, development of coherent regulatory policy and a pathway for personalized medical tests approval will need to be done by regulatory agencies including the Federal Drug Administration (FDA)'s Critical Path Initiative (Hamburg and Collins 2010). Practicing physicians will need to adopt and use clinical information support systems. The adoption of electronic medical record provided by the health information technology act will be an important driver for acceptance of information systems in clinical practice. Another obstacle is payer resistance for tests that do not show cost–benefit, such as CMS medicare rules that do not reimburse for screening tests in absence of signs or symptoms. This policy limits the

application of personalized medicine, because the molecular profiles used as diagnostics extend clinical assessment and are not specifically indicated by them. Another challenge is that big pharmaceutical industries are reluctant to develop drugs for small patient populations, which reduces their potential market.

Finally, the development of predictive biomarkers for personalized medicine in asthma is in its infancy. Biomarker development requires a large capital investment, and many years, to navigate the processes of identification, validation, measurement of performance characteristics in relevant populations, and ultimately incorporation of such tests into clinical care.

Predictive phenotyping as a basis for implementing personalized medicine has great potential, employing novel biochemical and statistical analyses for the purpose of improving care of patients with the complex syndromic disease called asthma. Despite these and other significant challenges, the promise of personalized medicine is powerful and compelling.

21.4 Future Directions in Molecular Phenotyping in Asthma

A key obstacle in this field is a lack of “gold standard” phenotypes against which a novel biomarker, genetic profile, or molecular phenotype can be assessed. The overlap in phenotypes within the US SARP experience (Moore et al. 2007) is not a manifestation of lack of rigor in the evaluation of these patients, but rather a reflection of the considerable variation in clinical presentation of asthma. A novel conceptual framework to bring clarity to this field would be to establish an empiric molecular phenotype as the “gold standard” and interpret clinical subgroups of asthma (mild, moderate, and severe persistent disease) in that context (Bhavnani et al. 2011). The experience of Woodruff et al. is instructive in that regard, as a cytokine expression pattern reliably predicted responsiveness to corticosteroids (Woodruff et al. 2009).

Although none currently exist, a predictive biomarker for the important clinical outcomes of exacerbations, and accelerated decline in lung function would have important implications for drug development, improved management strategies, and prognosis. Both of these outcomes are best assessed over the long term, which adds both complexity and expense to their development.

Proteins, which mediate the interaction between a host (patient) and his or her environment, are attractive candidates for molecular phenotyping, because their level and activity are both important considerations that can be measured. This situation contrasts with structural genetics, which are largely invariant over life; hence, a compelling case for genetics as a proximate cause of the temporal variation of expression of asthma is difficult to make. Accordingly, measurement of factors, which vary in relationship to the clinical expression of disease, including gene expression profiling, epigenetics, and proteomics, appears to hold the most promise for developing truly predictive biomarkers that will usher in the era of personalized medicine.

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