Exome array analysis of pulmonary function in smokers with and without chronic obstructive pulmonary disease (COPD)

By Margaret M. Parker, MHS

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Abstract:
Chronic obstructive pulmonary disease (COPD) is a progressive respiratory disease characterized by airflow restriction and decreased lung function. It is the 3rd leading cause of death worldwide, accounting for approximately 3 million deaths in 2010. It is diagnosed using spirometric measurements, including the forced expiratory volume in one second (FEV₁) and the forced vital capacity (FVC). These measures reflect the severity of airway obstruction and predict population morbidity and mortality. The primary environmental cause of COPD is cigarette smoking, but genetics also play a role in individual susceptibility and disease progression. Genome-wide association studies (GWAS) have identified over 30 loci associated with lung, but together the identified variants can only explain a small proportion of the variation in spirometric measures and a small proportion of the estimated heritability. We hypothesized: 1) rare functional variation also affects lung function; and 2) genetic variation (both common and rare) affects longitudinal changes in lung function. This dissertation tests these hypotheses using data from the COPDgene study, a large multicenter study of current and former smokers.
Thesis committee

Terri Beaty, Ph.D. (advisor)
Professor of Epidemiology

Ingo Ruczinski, Ph.D., M.S.
Professor of Biostatistics

Rasika Mathias, Sc.D.
Associate Professor of Medicine

Thesis readers

Terri Beaty, Ph.D. (advisor)
Professor of Epidemiology

Priya Duggal, Ph.D.
Associate Professor of Epidemiology

Rasika Mathias, Sc.D.
Associate Professor of Medicine

Margaret Taub, Ph.D.
Assistant Scientist of Biostatistics
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Chapter 1. Introduction and literature review
Chapter 1. Introduction and literature review

Chronic obstructive pulmonary disease (COPD) is a progressive respiratory disease characterized by airflow restriction and decreased lung function. COPD results from both small airway disease (obstructive bronchitis) and parenchymal destruction (emphysema) and typical symptoms include dyspnea, chronic cough, and chronic sputum production. These symptoms tend to worsen over time, as and the pathological changes associated with COPD are not fully reversible. Individuals with COPD can vary greatly in symptom severity, but disease progression typically leads to a decreased capacity for exercise, an increased risk of serious comorbidities, and an overall increased mortality rate\textsuperscript{1,2}.

COPD burden

COPD is a highly prevalent disease resulting in a substantial economic burden that is increasing\textsuperscript{3}. According to the WHO’s Global Burden of Disease, Injuries, and Risk Factors Study, COPD is the 3\textsuperscript{rd} leading cause of death worldwide, accounting for approximately 3 million deaths in 2010\textsuperscript{4}. It is estimated 4.2\% of working adults in the United States (or 15 million individuals) have COPD, and this number is projected to increase as the population ages\textsuperscript{5}. This not only poses a large medical burden, but also an economic one. The National Heart, Lung and Blood Institute (NHLBI) estimates that along with asthma, COPD costs exceeded $60 billion USD in 2008, of which $53.7 billion USD were direct costs\textsuperscript{6}. The high prevalence and massive economic burden of COPD highlight the need for greater understanding of disease etiology to better predict individual risk, prevent future disease and treat diagnosed cases.

Measurement of COPD

The airflow restriction characteristic of COPD is most often quantified via spirometry, including forced expiratory volume in one second (FEV\textsubscript{1}), forced vital capacity (FVC),
and the ratio of FEV₁/FVC. These measurements quantify the volume and speed at which an individual can exhale air, providing a useful indication of lung function. To obtain spirometric measurements, patients are asked to take a deep breath and exhale into a sensor as hard as possible, for as long as possible. From this maneuver, one can measure: 1) the volume of air exhaled in one second (FEV₁); 2) the total volume of air exhaled (FVC); and 3) FEV₁ as a percent of the predicted value based on height, age, sex, and race (FEV₁ percent predicted).

Individuals without obstructive airway disease can expire all (or nearly all) of their vital capacity in one second, resulting in an FEV₁/FVC ratio about equal to one. Alternatively, an FEV₁/FVC ratio below 0.7 defines airflow obstruction and is used to diagnose COPD (Figure 1.1).

Spirometric measurements are easily obtained, inexpensive and provide useful benchmarks for COPD progression. Moreover, they reliably predict population mortality. In a longitudinal study of 15,759 individuals followed over 11 years, those with the most severe lung function impairment (FEV₁/FVC < 0.7 and FEV₁ % predicted < 50%) were at a 5.7 times higher risk of death (95% CI = 4.4 - 7.3) than those with normal lung function, highlighting the importance of these measurements as indicators of population health.

However, spirometry is not without limitations. The measurement procedure is highly dependent on patient cooperation and effort, and therefore can vary substantially among individuals. Additionally, spirometry does not fully describe all aspects of disease pathogenesis, including changes in airway thickness, emphysema and severity of symptoms. Thus, while these measurements can confirm the presence of airflow obstruction, they do not provide any specific etiologic diagnosis.
Etiology of COPD

COPD is a multifactorial disease with the primary cause being cigarette smoking\textsuperscript{10}. However, only 25\% of cigarette smokers ever develop COPD\textsuperscript{11} and even among those that do, the rate of lung function decline varies considerably among smokers with similar exposure levels\textsuperscript{12,13}. This suggests additional risk factors, including genetics, may play some role in individual susceptibility to COPD development and disease progression.

Environmental risk factors

Cigarette smoking

The primary environmental cause of COPD is cigarette smoking. In a 25-year prospective cohort study of 8,045 individuals, current smokers were 6.3 times more likely to develop clinically significant COPD than never smokers (95\% CI = 4.2-9.5)\textsuperscript{4}. Approximately 80\% of COPD deaths are caused by smoking, and smokers are 12-13 times more likely to die from COPD than never smokers\textsuperscript{14}.

Cigarette smoking leads to airflow obstruction and COPD symptoms through a complex physiological process. Repeated exposure to the irritants in cigarette smoke causes chronic inflammation of the airways, leading to an influx of inflammatory mediator molecules (e.g. neutrophils, B cells, and T-lymphocytes)\textsuperscript{15}. Over time, this chronic inflammation causes irreversible structural and physiological changes in the lung including airway constriction, excess mucus production and dysfunctional cilia\textsuperscript{16}. These changes result in expiratory airflow limitation, especially in the smaller (< 2mm) airways, and hallmark COPD symptoms, such a wheezing, coughing and dyspnea\textsuperscript{17}.

Sex

There are distinct sex differences in COPD prevalence. Overall, men are more likely to have COPD, but over the past 20 years COPD prevalence and mortality have increased
more rapidly among women\textsuperscript{18}. Sex differences in COPD are frequently attributed to
different exposure rates (e.g. smoking) between men and women, but there is increasing
evidence that biological factors may also play a role. Female participants of the National
Emphysema Treatment Trial (NETT) had fewer pack-years of smoking history than men
but had similarly severe COPD\textsuperscript{19}. Additionally, a meta-analysis by Gan et al. found that
females had a faster annual rate of decline in lung function (measured as FEV\textsubscript{1}) than
males of similar smoking levels\textsuperscript{20}. Overall, research suggests women develop more
severe COPD at younger ages with lower levels of exposure to smoking\textsuperscript{19–21}. Proposed
explanations for this disparity include: 1) differential susceptibility to the effects of
tobacco, 2) anatomical differences (e.g. smaller lungs and airways), and 3) differential
responses to treatments\textsuperscript{22}. The effect of sex on COPD development and progression is
likely due to a combination of environmental and biological factors, however the exact
mechanism resulting in this disease disparity remains unresolved.

Race

Differences in lung function between European Americans (EAs) and African Americans
(AAs) are well documented. Prevalence of any impairment in lung function is higher
among EAs than AAs\textsuperscript{23}. According to the CDC, the age adjusted mortality rate from
COPD is 46.0 per 1000 in EA and 27.2 per 1000 in AA individuals\textsuperscript{24}.

However, mortality from COPD is increasing more rapidly among AAs\textsuperscript{25}, and there is
evidence that AAs may be more susceptible to the negative effects of tobacco
smoke\textsuperscript{26,27}. In 2004, Chatalia et al. reported that among 80 EA and 80 AA patients with
advanced COPD, AAs were younger with less cumulative smoking history despite
comparable lung function\textsuperscript{26}. More recently, differences in between EA and AAs have
been studied in the COPDgene study, an observational case-control study of 10,000
smokers with and without COPD\textsuperscript{29}. Investigators found AAs were much more likely to
have severe-early onset COPD than EAs (42% vs. 14%) and had different patterns of emphysema distribution over the lung region\textsuperscript{30}. The potential impact of racial differences in COPD development has important implications on disease is screening, diagnosis and treatment, and more research is needed to understand why racial disparities may exist.

**Age**

Aging is associated with a progressive decline in lung function even among non-smoking adults\textsuperscript{31}. It remains unclear if this change results from the normal aging process (i.e. age-related changes in pulmonary mechanics, respiratory muscle strength, gas exchange and ventilatory control)\textsuperscript{32} or if age reflects the sum of cumulative exposures throughout life leading to lung function decline\textsuperscript{3}.

**Other environmental risk factors**

A number of additional risk factors are associated with an increased risk of COPD including occupational dust exposure\textsuperscript{21,34}, air pollutants\textsuperscript{35}, history of respiratory infection\textsuperscript{36}, asthma\textsuperscript{37–39}, nutrition\textsuperscript{40,41}, second-hand smoke exposure\textsuperscript{42}, and socio-economic status\textsuperscript{43}. The relative contribution of these diverse risk factors to COPD development and progression remains unresolved, and many may be especially important contributors to disease in non-smokers\textsuperscript{3}.

**Genetic risk factors**

There is substantial evidence that genetic factors can influence COPD susceptibility\textsuperscript{44–51}. Early familial aggregation studies found severe, early onset COPD probands were significantly more likely to have first-degree relatives with decreased FEV\textsubscript{1} than controls\textsuperscript{51}. More recently, a large population-based study of 821 cases and 776 smoking controls showed individuals with a parental family history of COPD were 1.73 times more
likely to have disease than those without a family history, indicating COPD aggregates in families which could reflect either shared genetics or shared environment.

The estimated heritability, or proportion of variance in lung function attributable to the additive effects of genes, is 52%, 54% and 45% for FEV₁, FVC and FEV₁/FVC (respectively). This means a considerable proportion of the variation in these spirometric measurements is due to genetic factors.

**Alpha-1 antitrypsin deficiency**

The best understood genetic risk factor of COPD is a mutation on chromosome 14 in the gene coding the serine protease inhibitor alpha-1 antitrypsin. The role of alpha-1 antitrypsin deficiency in lung disease was first identified in 1963 by Carl-Bertil Laurell when he noted the absence of alpha-1 antitrypsin protein in the plasma of many early onset emphysema cases. This absence was eventually traced back to a missense mutation in the alpha-1 antitrypsin gene that occurs when a glutamic acid is substituted for a lysine at amino acid position 342 leading to a non-functional protease inhibitor. Individuals homozygous for this null mutation display markedly decreased alpha-1 antitrypsin levels, typically leading to lung tissue degradation and early-onset, severe COPD. Although well-characterized, this mutation is relatively rare in the population, and accounts for only 1-2% of all COPD cases, suggesting other genetic factors control disease susceptibility.

**Genome-wide association studies**

A number of investigators have attempted to characterize common genetic variation influencing lung function using genome-wide association studies (GWAS). In 2009, Pillai et al. identified an association between variants in the *CHRNA3/CHRNA5* gene cluster on chromosome 15q25 and COPD case-control status in 823 cases and 810 smoking
controls of European descent\textsuperscript{55}. Subsequent work has replicated this association and implicated a nearby gene in tight linkage disequilibrium, \textit{IREB2}, as the likely candidate gene underlying this association\textsuperscript{56}. Concurrent to the Pillai et al. GWAS, Wilk et al. conducted an independent GWAS of lung function in 7,691 Framingham Heart Study participants, using FEV\textsubscript{1}/FVC ratio as a quantitative outcome\textsuperscript{57}. They identified 4 single nucleotide polymorphisms (SNPs) near the hedgehog interacting protein (\textit{HHIP}) gene as genome-wide significant (p < 5x10\textsuperscript{-8}). \textit{HHIP} is a regulatory protein in the hedgehog signaling pathway and may influence fetal lung development, although its role in lung disease remains unclear\textsuperscript{57}. Finally, Cho et al. identified an association between \textit{FAM13A} loci and case-control status using in 2,940 cases and 1,380 controls from 3 independent study populations\textsuperscript{58} (ECLIPSE\textsuperscript{59}, NETT\textsuperscript{60}, and Norway\textsuperscript{61}). In summary, early GWASs with modest sample sizes identified three genes (\textit{IREB2}, \textit{HHIP}, and \textit{FAM13A}) associated with COPD and its related qualitative outcomes.

\textbf{Large scale meta-analyses}

More recently, investigators have leveraged the large sample sizes afforded by ongoing cohort studies to test for association between common markers and lung function outcomes. Three large meta-analyses of GWAS data have identified 25 additional loci associated with lung function outcomes. In 2010, Hancock et al. identified 4 novel loci associated with FEV\textsubscript{1}/FVC in 20,890 participants of European descent in the Cohorts for Heart and Aging Research in Genetic Epidemiology (CHARGE) studies. Associated SNPs were located in or near the genes: \textit{GPR126}, \textit{ADAM19}, \textit{PTCH1} and \textit{PID1}\textsuperscript{62}. Concurrently, Repapi et al. identified 5 novel loci in 20,288 individuals who were part of the SpiraMeta Consortium studies. Associated markers were located in the following genes: \textit{TNS1}, \textit{GSTCD}, \textit{HTR4}, \textit{AGER}, \textit{THSD4}\textsuperscript{63}. Finally, in the largest meta-analyses of lung function to date, Soler-Artigas et al. combined these two large meta-analyses
(CHARGE and SpiraMeta) to evaluate 2.5 million SNPs in 48,201 individuals of European ancestry. This analysis identified 16 novel genetic loci associated with FEV1/FVC including variants in and near the genes: MFAP2, TGFB2, HDAC4, RARB, MECOM, SPATA9, ARMC2, NCR3, ZKSCAN3, CCDC38, C10orf11, LRP1, CCDC38, MMP15, CFDP1 and KCNE2. Previously identified associations between genetic markers and lung function in European derived populations are summarized in Figure 1.2.

**Lung function decline**

COPD development is likely influenced by both: 1) impaired attainment of maximal lung size and function before adulthood; and 2) accelerated lung function decline. Cross-sectional studies cannot differentiate between these pathways, and it is probable different risk factors (both genetic and environmental) separately influence each pathway. However, few studies have assessed the genetics of lung function decline, as this requires longitudinal data.

To date, there have been three published GWASs of lung function decline, all in populations of European ancestry. A summary of these studies is provided in Table 1.1. Overall, 6 genes have been identified as associated with lung function decline, none of which overlap the reported associations from studies of cross-sectional lung function. However, there is little agreement between the 3 published studies regarding which genes contribute to this decline phenotype, suggesting additional replication and further research is necessary.

**Genetic risk factors in African Americans**

Despite increasing evidence that African Americans may be especially susceptible to the negative effects of tobacco smoke, there has been little analysis of the genetic
determinants of lung function in this sub-population. GWAS analysis in 3,260 AA participants of the COPDgene study identified one locus significantly associated with FEV₁/FVC (near BC011998 on chromosome 5, p-value=1.31x10⁻⁵) and one marginally significant locus associated with FEV₁ (near MGAT3 on chromosome 22, p-value = 9.19x10⁻⁸). Additionally, admixture mapping of this population identified an intronic variant in FAM19A2 as being associated with FEV₁/FVC. However, compared to European-derived populations, few studies have been conducted in African Americans, and little is known about the genes controlling lung function in this sub-population.

Motivation and specific aims for this study

Substantial advances have been made in our understanding of the genetic etiology of COPD through GWAS and candidate gene studies. However, despite large sample sizes, together all known associated markers account for only 3.2% of the estimated heritability of FEV₁/FVC and 1.5% of the estimated heritability of FEV₁. Moreover, little is known about the genetic determinants of these measurements in African Americans. Together, this suggests much of the genetic variation controlling reduced pulmonary function has yet to be discovered. We seek to address this issue through the following specific aims:

1. To identify functional genetic variants associated with lung function in European and African American participants of the COPDgene study using exome array data.

2. To identify genetic variants associated with longitudinal changes in lung function in European American and African American participants of the COPDgene study using GWAS and exome array data.
Study description

COPDgene is an observational study conceived in 2008 to investigate the genetic and environmental etiology of COPD\textsuperscript{29}. Participants included 10,280 self-identified AAs or EAs between the ages of 45 and 80 years with a minimum of 10 pack-years of smoking history recruited from 21 study centers across the United States. Study exclusion criteria included those with a history of lung disease (other than asthma), previous surgical removal of at least one lung lobe, active cancer treatment, suspected lung cancer, metal in the chest, exacerbated COPD treated with antibiotics/steroids, known alpha-1 antitrypsin deficiency, recent eye surgery, and inability to use albuterol, self-identified as multiple racial categories, having a first or second degree relative in the study, and pregnant women. Subjects completed detailed questionnaires, pre- and post-bronchodilator spirometry, volumetric computed tomography (CT) of the chest, and provided a DNA sample for genotyping.

Genotyping platforms

Available genotyping platforms included: 1) The Illumina Omni Express BeadChip GWAS array containing approximately 733,000 common single nucleotide polymorphic (SNPs) variants; and 2) Illumina HumanExome chip 12v1-1 (or 12v1-2) exome array containing approximately 233,000 (mostly) functional genetic variants located in gene exons.

Longitudinal follow-up

In 2013, eligible COPDgene participants returned for a 5-year follow-up assessment, affording investigators the opportunity to assess determinates (both genetic and environmental) of decline in lung function. As of November 2015, longitudinal data from 2,000 participants was available for analysis.
Summary

Many recent advances have been made in our understanding of COPD risk factors. However, important determinates (both genetic and environmental) remain unidentified. This dissertation aims to identify functional genetic risk factors associated with reduced lung function and longitudinal lung function decline, using exome and genome-wide array data. Ultimately, improved understanding of COPD risk factors can aid in tailoring treatment, discovering novel therapeutic interventions and in developing effective prevention strategies, and this is especially timely given the large and increasing global burden of COPD.

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Figure 1.1
Volume to time curve for a normal versus obstructed airflow from spirometry. FEV₁ is the forced expiratory volume in one second. FVC is the forced vital capacity. A FEV₁/FVC ratio of less than 0.7 defines COPD (Figure adapted from the Global Initiative for COPD report³).
Figure 1.2
Genes associated with lung function (measured as FEV₁ or FEV₁/FVC) in European-derived populations (Figure adapted from Wain et al., 2012⁷¹).

Definition of abbreviations: ¹CHARGE= Cohorts for Heart and Aging Research in Genetic Epidemiology, ²FHS = Framingham Heart Study
Table 1.1 Summary of published GWAS studies of lung function decline.

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<tr>
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<td>2014</td>
<td>CHARGE or Spriometa Consortium Studies</td>
<td>change in FEV₁</td>
<td>IL16/STARD5/TMC3 (15), ME3 (11)</td>
</tr>
</tbody>
</table>

Definition of abbreviations: ¹EGEA = Epidemiological Study of the Genetics and Environment of Asthma, ²SAPALDIA = Swiss Cohort Study on Air Pollution and Lung and Heart Disease in Adults, ³ECRHS = European Community Respiratory Health Survey, ⁴LHS = Lung Health Study, ⁵CHARGE = Cohorts for Heart and Aging Research in Genetic Epidemiology
Chapter 2. Exome Array Quality Control
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Introduction

Genome-wide association studies (GWAS) have successfully identified common variation related to many complex phenotypes, but typically associated variants account for only a small fraction of the estimated heritability. Rare variants (defined here as variants with a minor allele frequency less that 5%) are hypothesized to play an important role in complex traits but are poorly characterized by GWAS arrays. Sequencing, either with whole exome or genome sequencing, can directly assay these rare variants but it is expensive and data interpretation is challenging given the large amount of data sequencing produces. Therefore, the Illumina HumanExome genotyping array (or “the exome array”) was developed to capture known protein coding variation at a relatively low cost.

The exome array contains over 240,000 markers chosen from approximately 12,000 sequenced exomes based on the following criteria: 1) non-synonymous variants if observed at least three times in two or more studies; 2) stop-altering variants if observed at least two times in two or more studies; and 3) splice site variants if observed at least two times in two or more studies\(^1\). The array also contains 3,241 ancestry informative markers, 4,761 GWAS tag markers, 3,369 identity-by-descent markers, and 4,651 randomly selected synonymous markers to aid in quality control\(^1\). Previous research comparing the exome array to exome sequencing indicates that the coverage of rare, functional variants on the array is around 70\(\%\)^2–5 and is comparable between European-derived and African-derived populations\(^4\).
Although the exome array affords an exciting opportunity to assess rare coding variation, it also introduces novel data processing challenges. Unlike sequencing, genotyping platforms depend on automated clustering algorithms (e.g. Illumina’s GenCall) to detect and assign genotype calls. These algorithms, originally designed for common variant detection, have difficulty accurately making genotype calls for rare variants because few observations exist in the heterozygote and homozygote minor allele clusters\(^6,7\). This can result in misclassified data (i.e. the incorrect assignment of a genotype to a cluster) or missing data (i.e. the inability to assign a genotype to a cluster). Additional sources of variability in exome array genotype calling include: 1) the sample size available for clustering (larger sample sizes improve genotype calling accuracy\(^5\)); and 2) the manual re-clustering steps required by many exome array protocols, which can be subjective\(^6\).

This chapter will describe the quality control (QC) procedures completed in exome array data from the COPDgene study, an observation case-control study of smokers with and without COPD\(^8\). A total of 9,858 Non-Hispanic White (NHW) and African American (AA) participants were genotyped on Illumina’s HumanExome platform in addition to completing detailed questionnaires, pre- and post- bronchodilator spirometry, and volumetric computed tomography (CT) scans. This rich phenotyping, paired with the exome array data genotyping, provide a unique opportunity to extend the allelic and functional spectrum of genetic variation underlying COPD and COPD-related phenotypes. However, adding to the data processing challenges of the exome array, the 9,858 COPDgene subjects were genotyped in two batches, introducing the possibility of batch effects to contend with during the QC process (2,306 subjects on HumanExome array version 1.1 at University of Washington and 7,552 subjects on array version 1.2 at the Center for Inherited Disease Research). With this in mind, we undertook data
cleaning with the goal of producing a set of high quality functional variants for use by COPDgene investigators.

This chapter aims to: 1) provide a comprehensive description of the COPDgene exome array quality control procedures and; 2) to summarize variants available for analysis with regard to frequency and functional annotation.

**Methods**

**Sample selection**

All eligible participants of the COPDgene study with available DNA (n=9,858) were genotyped on Illumina’s HumanExome Beadchip in two batches. Samples in batch one were selected based on extreme phenotypes and included NHW subjects with severe COPD and NHW disease-resistant smoking controls (n=2,306). Samples in batch two included the remaining NHW subjects (not genotyped in batch one) and all AA subjects (n=7,552). Therefore, in NHW subjects, chip membership is phenotypically determined (Table 1).

**Exome array genotyping**

Batch one was genotyped using version 1.1 of the HumanExome Beadchip (Illumina) at the University of Washington. Batch two was genotyped using version 1.2 of the HumanExome Beadchip (Illumina) at the Center for Inherited Disease Research at Johns Hopkins University. Each batch contained replicate samples (n= 128 in batch 1, n=38 in batch 2) and 121 samples were genotyped on both exome arrays to assess batch effects. In addition, batch two contained 166 control samples from 8 HAPMAP populations. Because of differences between array version 1.1 and 1.2, we were unable to pool the two batches for joint genotyping calling. Thus, genotypes were called separately by
batch using Illumina’s GenTrain clustering algorithm (version 1.0) in GenomeStudio (version 2011.1).

**Exome array QC overview**

An overview of the COPDgene exome array QC is provided in Table 2. Briefly, we performed: 1) initial single nucleotide variant (SNV) QC in order to combine the two exome array datasets 2) sample QC 3) SNV QC; and lastly 4) separated NHW and AA subjects for data analysis. All exome array QC was performed in using PLINK version 2.0⁹ or R¹⁰.

**Initial SNV QC**

Because marker identifiers on different exome array versions are inconsistent, we mapped all available SNVs to dbSNP version 141. In this process, we removed indels (n=276), markers with probable strand issues (n=135), markers that mapped to the wrong variant type (e.g. mapped to indel but was a SNV) (n=2), multi-allelic markers (n=2,680), and markers that did not map to dbSNP141 (n=617). Additionally, the Illumina exome array contains duplicate markers (n=833 on version 1.1, n=815 on version 1.2). For each duplicate marker, we checked concordance, dropped any discordant markers (n=107), and dropped the SNV with more missingness for concordant markers (n=1,541). Together, this initial SNV QC enabled us to merge the two exome array datasets for sample QC. Table 2.1 summarizes the initial SNV QC process.

**Sample QC**

A summary of sample QC is provided in Table 2.2.

**GWAS concordance**

All but 5 samples had been previously genotyped with Illumina’s OmniExpress GWAS array and thus we could rely on previously performed QC for many of the sample checks.
as long as concordance between samples was established. Therefore, we calculated genotype concordance between exome array samples and GWAS samples on SNVs that overlapped between the two platforms (n= >17,000 SNVs). This was performed separately for the first and second chips, as some samples were known to be GWAS discordant on the first chip and were re-genotyped on the second chip for that reason. Subjects with greater than 500 discordant sites between the two arrays were removed from analysis, excluding a total of 13 subjects.

**Call rate**
A low call rate, or fraction of called SNVs per sample, can indicate poor sample quality. We calculated call rate per sample using PLINK. This was performed by chip, as some markers were unique to either the first chip (n=3,219) or the second chip (n=406). Using a cutoff of 95%, no samples were dropped for low call rate.

**Sex check**
We relied on previously performed GWAS QC to exclude subjects with sex discrepancies. This excluded one subject. Additionally, there were 7 subjects with sex chromosome abnormalities identified in GWAS analysis that were genotyped on the exome chips (3 XO individuals, 3 XXY individuals, and 1 XY individual that self-identified as a female). These subjects were used in GWAS analysis and are therefore included in the exome chip dataset.

**Heterozygosity**
Given a homogenous sample, the heterozygosity rate, or fraction of non-missing genotype calls that are heterozygous, can help identify problematic samples (low heterozygosity may indicate inbreeding, while high heterozygosity may sample contamination). We calculated the heterozygosity using PLINK, separately in NHW and
Using a cutoff of greater or less than 6 standard deviations from the mean, 8 NHW and 4 AA outliers were identified and excluded from analysis.

**Relatedness**

To identify cryptic relatedness, we estimated kinship coefficients between all pairwise samples using the Kinship-based Inference for GWAS (KING) software\textsuperscript{11} (using the option: "-kinship -related -degree 2"). All samples were estimated together (excluding known duplicates), using all available SNVs. A total of 152 second degree relationships were identified by KING, of which 122 had been previously identified and were removed (114 removed during GWAS QC process and 8 removed during GWAS concordance checks). Additionally, 23 relationships (representing 7 subjects), were removed for suspected contamination. Seven additional relationships identified as possible second degree relationships by KING (kinship coefficient 0.88-0.95), but not flagged during the GWAS QC process, were retained.

**Population outliers**

To identify population outliers, we ran principal components analysis (PCA) separately in NHWs and AAs using unlinked, autosomal polymorphisms (n=14,961 SNPs in NHW and 19,884 SNPs in AAs). These results (using markers from the exome chip) were nearly identical to the CPA results using markers from the GWAS chip, and therefore we excluded subjects identified as population outliers in the GWAS analysis (n=52) (Figure 2.3).

**Replicate and control samples**

Replicate samples were genotyped on the first chip (n=128), the second chip (n=38), and across the 2 chips (n=121). For all replicates, we calculated genotype concordance
between the samples and dropped the one with more missingness. Additionally, we excluded 166 HAPMAP genotyping control samples included on chip # 2.

**Fraction of singleton/doubletons**

An excess or depletion of very rare variants can indicate sample quality issues including sample contamination, inbreeding or population outliers. Thus, we looked at the number of singletons and doubletons per person (separate in NHW and AAs), but did not exclude any subjects based on this metric (Figure 2.4).

**Additional GWAS exclusions**

Seven subjects were excluded from analysis for having confirmed alpha-1 antitrypsin deficiency. One subject was flagged as mislabeled during the GWAS QC process, and therefore was also excluded from the exome array analysis.

**Single Nucleotide Variant (SNV) QC**

A summary of SNV QC is provided in Table 2.3.

**Call rate**

We assessed SNV call rate using PLINK. Because there are some markers unique to only one chip, this was performed separately by chip. Using a 95% call rate cutoff, a total of 1,394 SNVs were dropped from the first chip, and a total of 4,874 SNVs were dropped from the second chip.

**Frequency differences between chips**

To test genotype quality, we compared the allele frequencies of overlapping markers on chip v1.1 to allele frequencies on chip v1.2 in NHW subjects. We used two frequency mismatch cutoffs to exclude markers: 1) SNVs with absolute frequency differences greater than 0.1; and 2) SNVs that were significantly different on a Fisher's exact test of variant frequency between controls on each chip (cutoff = p-value < 10^{-4}). In total, this
excluded 180 SNVs. Additionally, we flagged (but did not exclude) markers with p-values between $10^{-3}$ and $10^{-4}$ on the Fisher’s exact test of frequency differences.

**Hardy-Weinberg equilibrium**

We calculated Hardy-Weinberg equilibrium (HWE) by race in unaffected (FEV$_1$/FVC $> 0.70$) subjects using PLINK. Markers with HWE p-values less than $10^{-8}$ were excluded (n=150 in NHW, n=143 in AAs). Markers with HWE p-values less than $10^{-4}$ were flagged but not excluded from the final dataset. Manhattan plots of HWE p-values by race are provided in Figure 2.5.

**Minor allele concordance**

Using the duplicate samples within each chip (n=166) and between the two chips (n=121), we calculated minor allele concordance (MAC) and dropped SNVs with MAC $< 95\%$ (n=1,677).

**Non-autosomal markers**

A total of 5,540 markers on the X, Y, XY, and mitochondrial chromosomes were removed from analysis. These SNVs were not included in the QC process and require additional QC if used in future analyses.

**Chip-specific markers**

There are markers unique to only chip one (n=3,219) or chip two (n=406). Markers genotyped on only 1 of the 2 chips are included in the analyzed dataset, but we created a flag for consideration during analysis as these SNVs will have substantial missingness which may be non-random (chip membership is phenotype-dependent in NHW subjects).

**Annotation**

SNVs were annotated using ANNOVAR$^{11}$ with the RefSeq reference genome$^{12}$. 
Results

A total of 6,581 subjects and 233,263 SNVs passed QC in NHWs, and 3,221 subjects and 233,255 SNVs passed QC in AAs. The overall call rate on chip one was 99.8% (sample range: 98.1% - 100.0%). The overall call rate on chip two was 98.0% (sample range: 95.4% – 98.0%).

Minor allele frequencies and functional annotation

The minor allele frequency (MAF) distributions for successfully genotyped SNVs are described (by race) in Table 2.4. There were 82,832 monomorphic SNVs in NHWs (35.5%) and 86,605 monomorphic SNVs in AAs (37.1%). The majority (> 87%) of variants in both NHWs and AAs have a MAF < 5% and many (> 37%) have a MAF < 0.005%. NHW subjects have more variants with a MAF less than 0.001 (90,946 in NHW vs. 55,132 in AAs), likely attributable to the marker contents of the chip (chosen from primarily NHW exomes) and to the number of AAs genotypes (about half the number of NHWs).

Greater than 92% of genotyped SNVs in both NHWs and AAs are functional (annotated as nonsynonomous, stopgain, stoploss, or splicing). The most common function category is nonsynonomous, as it accounts for 90% of all genotyped SNVs. A summary of the functional categories of observed SNVs by MAF is provided in Table 2.5.

Genes with multiple SNVs

Given that the vast majority of observed variants are rare, traditional approaches testing for the association of a single variant and outcome are underpowered. To address this, many have suggested different collapsing methods where sets of rare variants in a given genomic unit (e.g. gene) are “collapsed” and collectively tested for an association with the outcome. This test is more powerful than a single variant test if the collapsed
variants are causal. To assess our ability to perform “collapsing” tests by gene, we summarized the number of variants in each gene by MAF category (Table 2.6).

There are 16,142 genes in NHWs and 15,932 genes in AAs with more than one observed variant. The median number of rare (MAF < 5%) variants per gene was 5 in both NHW and AAs. Three genes had an usually high number (>100) of observed variants $TTN$, $MUC16$ and $OBSCN$, but these genes are large and known to contain a high number of variants. Together, these analyses indicate that the majority of genes observed in the COPDgene exome array data have multiple rare variants, and are therefore eligible to be used in “collapsing” tests.

**Coverage of COPD-associated genes**

Previous research has been successful in identifying common variation associated with COPD through genome-wide association studies (GWAS)$^{13}$. While GWAS-associated SNPs may identify important genetic regions, they are likely not causal and only tag functional mutations. Because the exome array directly queries functional variants, we have the opportunity to potentially elucidate causal mutations explaining previously identified GWAS signals. Thus, we assessed the number of observed variants by MAF category in 26 genes associated with lung function in a recent large meta-analysis of 48,201 NHW subjects$^{14}$ (Figure 2.6).

The number of variants per lung function-associated gene ranged from (0-50). One gene ($CDC123$) was not observed in our data. As expected, the majority of SNVs in lung function-associated genes were rare and functional.
**Batch Effects**

The COPDgene NHW subjects were genotyped at two time points, with different versions of the exome array. However, 121 subjects were genotyped on both chips. We used these duplicated samples to assess batch effects.

Figure 2.7 shows variant counts in different frequency categories (e.g. singleton, doubleton) in the 121 duplicates samples. If no batch effect were present, we would expect counts in each category to be equal. However, we see that there are slightly fewer variants called and slightly more monomorphic markers in the first chip than the second chip, suggesting there may be some under calling of rare variants in chip one. Potential reasons for this include: 1) differences in genotype clustering and calling between the 2 arrays; and 2) the larger sample size used for calling chip two.

We also calculated minor allele concordance (the number of concordant minor alleles over the total number of minor alleles) between duplicated samples. Overall the minor allele concordance between samples genotyped on both chips was high (> 99%) for all frequencies (Table 2.7). Together, this suggests there are subtle but present batch effect in the NHW exome array data.

**Discussion**

This chapter describes the quality control procedures completed in the COPDgene exome array data. Through multiple subject and variant QC steps, we produced a high quality dataset to be tested for associations with lung function and the wide array of COPD-related outcomes available in the COPDgene study. We have shown that this data consists of mostly rare and potentially protein altering variants, including many genes with multiple rare variants and genes previously associated with lung function. This data provides researchers with an opportunity to extend the allelic and functional
spectrum of genetic variation underlying COPD, and this chapter serves as a resource for those using it.
References


Figure 2.1
Distribution of FEV₁/FVC by chip in NHW COPDgene subjects. Chip one samples were selected based on extreme phenotypes (severe COPD cases and disease-resistant smoking controls). Chip two samples included all other COPDgene NHW subjects.
Figure 2.2
Overview of COPDgene exome array quality control process, including the number of subjects and SNVs used for each QC step. We performed: 1) initial SNV QC; 2) Sample QC 3) SNV QC; and 4) separated NHW and AA subjects for data distribution.
Table 2.1
Summary of initial SNV quality control. We mapped all SNVs to dbSNP 141 to create consistent marker names. During this process, we dropped indels, SNVs with probable strand issues, markers that were the wrong type (SNV versus indel), multi-allelic markers, duplicate markers, and SNVs that were unable to be mapped to dbSNP141.

<table>
<thead>
<tr>
<th>Flag</th>
<th>N SNVs dropped Chip #1</th>
<th>N SNVs dropped Chip #2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indel</td>
<td>140</td>
<td>136</td>
</tr>
<tr>
<td>Strand Problem</td>
<td>50</td>
<td>85</td>
</tr>
<tr>
<td>Wrong marker type</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Multi-allelic</td>
<td>1340</td>
<td>1340</td>
</tr>
<tr>
<td>Duplicate Marker</td>
<td>865</td>
<td>890</td>
</tr>
<tr>
<td>Not mapped to dbSNP</td>
<td>473</td>
<td>144</td>
</tr>
</tbody>
</table>
Table 2.2
Summary of subject QC in the COPDgene exome array data. A total of 662 subjects were flagged (including 27 subjects flagged more than once) resulting in 9,802 subjects available for analysis.

<table>
<thead>
<tr>
<th>Flag</th>
<th>Cutoff</th>
<th>Number of Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>GWAS discordant</td>
<td>&gt; 500 discordant sites</td>
<td>13</td>
</tr>
<tr>
<td>Call Rate by Subject</td>
<td>&lt; 95%</td>
<td>0</td>
</tr>
<tr>
<td>Sex Check</td>
<td>GWAS exclusions</td>
<td>1</td>
</tr>
<tr>
<td>Heterozygosity</td>
<td>&gt;</td>
<td>6 SD</td>
</tr>
</tbody>
</table>
| Relatedness              | GWAS exclusions and samples related to  
2 ≥ 3 people            | 111                |
| Population outliers      | GWAS exclusions                                      | 42                 |
| GWAS excluded            | Alpha 1 Anti-trypsin or mislabeled                   | 8                  |
| Known Duplicates         | Sample with less missingness                         | 287                |
| Known Duplicate          | Failed one of above flags                           | 22                 |
| Sample flagged           |                                                      |                    |
| HAPMAP controls           |                                                      | 166                |
Figure 2.3
Comparison of principal components generated from NHW exome array data and NHW GWAS data. The x-axis is principal component 2. The y-axis is principal component 3. A total of 52 subjects were excluded as population outliers (colored red).
Figure 2.4
Singleton count versus total minor allele count in A) NHW COPDgene subjects (n=6,581) and B) AA COPDgene subjects (n=3,321). Red lines indicate mean counts, gray line indicate standard deviations. As expected, NHW subjects have fewer total minor alleles than AA subjects (mean NHW = 15,270, mean AAs = 17,000). Outliers in counts of singletons tend to match up with principal component outliers.
Table 2.3
Summary of SNV QC in the COPDgene exome array data.

<table>
<thead>
<tr>
<th>Flag</th>
<th>Cutoff</th>
<th>Number of SNVs dropped</th>
</tr>
</thead>
<tbody>
<tr>
<td>Call rate</td>
<td>&lt; 95% by chip</td>
<td>Chip #1: 1,394</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chip #2: 4,874</td>
</tr>
<tr>
<td>Frequency differences between chips</td>
<td>MAF difference &gt; 0.1</td>
<td>180</td>
</tr>
<tr>
<td></td>
<td>Fisher exact test P &lt; 10^{-4}</td>
<td></td>
</tr>
<tr>
<td>Hardy-Weinberg Equilibrium</td>
<td>Race specific P &lt; 10^{-8} in control samples</td>
<td>NHW: 150</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA: 143</td>
</tr>
<tr>
<td>Minor allele concordance</td>
<td>&lt; 95% in replicate samples</td>
<td>1,677</td>
</tr>
<tr>
<td>Non-autosomal markers</td>
<td>X, Y, XY, MT</td>
<td>5,540</td>
</tr>
</tbody>
</table>
Figure 2.5
Manhattan plots of Hardy Weinberg p-values for A) NHW controls (FEV₁/FVC > 0.70) and B) AA controls (FEV₁/FVC > 0.70). The dashed red line denotes the p-value cutoff for exclusion (p < 10⁻⁸).
<table>
<thead>
<tr>
<th>MAF Interval</th>
<th>Non-Hispanic Whites (n=6,581) (%)</th>
<th>African Americans (n=3,221) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>82,832 (35.5)</td>
<td>86,605 (37.1)</td>
</tr>
<tr>
<td>(0, 0.001]</td>
<td>90,946 (39.0)</td>
<td>55,132 (23.6)</td>
</tr>
<tr>
<td>(0.001, 0.005]</td>
<td>19,654 (8.4)</td>
<td>32,933 (14.1)</td>
</tr>
<tr>
<td>(0.005, 0.01]</td>
<td>5,305 (2.3)</td>
<td>10,224 (4.4)</td>
</tr>
<tr>
<td>(0.01, 0.05]</td>
<td>9,167 (3.9)</td>
<td>18,715 (8.0)</td>
</tr>
<tr>
<td>(0.05, 0.1]</td>
<td>4,143 (1.8)</td>
<td>6,426 (2.8)</td>
</tr>
<tr>
<td>(0.1, 0.2]</td>
<td>5,800 (2.5)</td>
<td>7,035 (3.0)</td>
</tr>
<tr>
<td>(0.2, 0.5]</td>
<td>15,630 (6.8)</td>
<td>16,351 (7.0)</td>
</tr>
</tbody>
</table>
Table 2.5
Counts of observed variants by functional type in COPDgene NHW and AAs. SNVs were annotated using ANNOVAR with RefSeq reference genome.

<table>
<thead>
<tr>
<th>Variant Type</th>
<th>All Sites</th>
<th>MAF &lt; 1%</th>
<th>MAF 1-5%</th>
<th>MAF &gt; 5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsynonymous</td>
<td>122,656</td>
<td>106,344</td>
<td>7,554</td>
<td>8,758</td>
</tr>
<tr>
<td>Stopgain/Stoploss</td>
<td>2,538</td>
<td>2,395</td>
<td>73</td>
<td>70</td>
</tr>
<tr>
<td>Splicing</td>
<td>1,074</td>
<td>983</td>
<td>36</td>
<td>55</td>
</tr>
<tr>
<td>Synonymous</td>
<td>8,521</td>
<td>4,507</td>
<td>562</td>
<td>3,452</td>
</tr>
<tr>
<td>Other</td>
<td>1,318</td>
<td>982</td>
<td>98</td>
<td>238</td>
</tr>
</tbody>
</table>

Non-Hispanic White (n= 6,581)

<table>
<thead>
<tr>
<th>Variant Type</th>
<th>All Sites</th>
<th>MAF &lt; 1%</th>
<th>MAF 1-5%</th>
<th>MAF &gt; 5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsynonymous</td>
<td>119,407</td>
<td>90,893</td>
<td>16,463</td>
<td>12,051</td>
</tr>
<tr>
<td>Stopgain/Stoploss</td>
<td>2,003</td>
<td>1,760</td>
<td>150</td>
<td>93</td>
</tr>
<tr>
<td>Splicing</td>
<td>873</td>
<td>718</td>
<td>75</td>
<td>80</td>
</tr>
<tr>
<td>Synonymous</td>
<td>8,761</td>
<td>3,735</td>
<td>1,126</td>
<td>3,900</td>
</tr>
<tr>
<td>Other</td>
<td>1,320</td>
<td>846</td>
<td>191</td>
<td>283</td>
</tr>
</tbody>
</table>

African American (n= 3,221)
Table 2.6
Counts of observed variants by gene. The majority of genes have more than one variant and are therefore eligible to be included in a collapsing test.

<table>
<thead>
<tr>
<th></th>
<th>Non-Hispanic Whites</th>
<th>African Americans</th>
</tr>
</thead>
<tbody>
<tr>
<td>N genes with observed variant(s)</td>
<td>18,053</td>
<td>17,924</td>
</tr>
<tr>
<td>N genes with 1 observed variant</td>
<td>1,911</td>
<td>1,992</td>
</tr>
<tr>
<td>Median N variants per gene (range)</td>
<td>6 (1-572)</td>
<td>6 (1-558)</td>
</tr>
<tr>
<td>Median N variants MAF &lt; 5% per gene (range)</td>
<td>5 (1-541)</td>
<td>5 (1-496)</td>
</tr>
<tr>
<td>Median N variants MAF &lt; 1% per gene (range)</td>
<td>5 (1-483)</td>
<td>4 (1-434)</td>
</tr>
</tbody>
</table>
Figure 2.6
Counts of observed variants in known COPD-associated genes in NHW COPDgene exome array. COPD-related genes (n=26) from a large meta-analysis of 48,201 NHW subjects\textsuperscript{14}. Dark gray bars indicate counts of variants with MAF > 5\%, gray bars indicate counts of variants with a MAF between 1\% and 5\%, and light gray bars counts of variants with MAF < 1\%.
Figure 2.7
Counts of variants by frequency category in 121 duplicated samples. Singletons are variants observed once in the dataset, doubletons are variants observed twice in the dataset. Overall, there are fewer variants called on the first chip than the second chip in these duplicated samples.
Table 2.7
Minor allele concordance in 121 subjects genotyped on both exome array #1 and exome array #2. Overall minor allele concordance is high (>99%) in all frequency categories.

<table>
<thead>
<tr>
<th></th>
<th>N markers</th>
<th>Concordance %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Singletons</td>
<td>15,734</td>
<td>99.39</td>
</tr>
<tr>
<td>Doubletons</td>
<td>4,702</td>
<td>99.36</td>
</tr>
<tr>
<td>MAF 1-5%</td>
<td>9,898</td>
<td>99.79</td>
</tr>
<tr>
<td>MAF &gt;5%</td>
<td>26,262</td>
<td>99.86</td>
</tr>
<tr>
<td>Overall</td>
<td>56,596</td>
<td>99.64</td>
</tr>
</tbody>
</table>
Chapter 3. Exome Array Analysis of Lung Function in the COPDgene Study
Chapter 3. Exome Array Analysis of Lung Function in the COPDgene Study

Introduction

Chronic obstructive pulmonary disease (COPD) is a progressive respiratory disease characterized by airflow obstruction and decreased lung function. It is the 3rd leading cause of death worldwide, accounting for approximately 3 million deaths in 2010. COPD is diagnosed based on spirometric measures of lung volume and flow, specifically the ratio of forced expiratory volume in one second (FEV₁) to forced vital capacity (FVC). The FEV₁/FVC ratio reflects the severity of airway obstruction and can predict morbidity and mortality in individuals.

The primary risk factor for COPD is cigarette smoking, but genetics also play a role in individual susceptibility and disease progression. Over the past several years, substantial advances have been made in our understanding of the genetics underlying COPD using genome wide association studies (GWAS) and large-scale meta analyses. However, associated genetic risk factors account for only a small fraction of the estimated heritability of lung function, suggesting much of the genetic variation in spirometric measures has yet to be discovered.

Some of this “missing heritability” may reflect the effects of rare, protein coding variation, which cannot be easily tested using the GWAS approach. Nearly every gene contains functionally important rare variants and recently developed exome array technology, which directly types these variants, has been successful in identifying additional loci associated with many complex traits.

To determine if rare coding variation plays a role in lung function, we genotyped 9,858 participants from the COPDgene study with Illumina’s HumanExome Beadchip. We
performed both: 1) traditional single variant analysis, which tests for statistical
association between one variant and an outcome; and 2) gene-based analysis, whereby
all variants in a gene are “collapsed” and tested together for association with an
outcome. These gene-based tests are more powerful than single variant tests whenever
multiple variants in a gene are causal, and can be particularly useful for rare variants
(minor allele frequency < 5%), where single variant tests offer little statistical power.

Methods

Study participants
We genotyped 9,858 participants of the COPDgene study, an observational study
conceived in 2008 to investigate the genetic and environmental etiology of COPD. A
complete study protocol for COPDgene had been described elsewhere\textsuperscript{15}. Briefly, self-
identified Non-Hispanic Whites (NHWs) and African Americans (AAs) between the ages
of 45 and 80 years with a minimum of 10 pack-years smoking history were enrolled at 21
study centers across the United States. In addition to completing detailed
questionnaires, pre- and post- bronchodilator spirometry, and volumetric computed
tomography (CT) of the chest, participants provided whole blood for DNA extraction and
genotyping.

Genotyping and quality control (QC)
Genotyping was performed with the Illumina HumanExome BeadChip v1.1 at the
University of Washington or with v1.2 at the Center for Inherited Disease Research
(CIDR). Genotype calling was performed using GenTrain v1.0 in GenomeStudio v2011.1
(Illumina). The quality control (QC) process resulted in several samples being dropped
from the analysis. Sample exclusions included sex discordances (n=1), sample
duplications (n=309), discordance with previous GWAS typing (n=13), population outliers
(n=42), unexpected related subjects (n=111) and samples with high overall heterozygosity (n=12). This resulted in a final sample size of 6,581 NHW and 3,221 AA subjects.

A total of 247,870 single nucleotide variants (SNVs) were genotyped on Illumina HumanExome BeadChip array v1.1, and a total of 244,770 SNVs were genotyped on v1.2. SNVs exclusions were based on call rate < 95% (n=6,268), Hardy-Weinberg p < $10^{-8}$ (n=293), minor allele concordance between duplicate samples < 95% (n=1,677), non-autosomal SNVs (n=5,540) and significant frequency differences between the two genotyped arrays (n=180). This resulted in 150,299 SNVs in NHWs and 146,654 SNVs in AAs available for analysis. A complete description of all quality control procedures is provided in chapter 2 of this dissertation.

**Phenotypes**

We analyzed: 1) the ratio of forced expiratory volume in one second to forced vital capacity (FEV$_1$/FVC), and 2) the ratio of forced expiratory volume in one second as a percent of the predicted value (FEV$_1$ % predicted). After taking a maximal deep breath, FEV$_1$ is the volume of air exhaled in one second, while FVC is the total volume of air exhaled. The ratio of these two measurements (FEV$_1$/FVC) defines COPD when values are < 0.7. Forced expiratory volume in one second as a percent of the predicted value (FEV$_1$ percent predicted) is calculated using regression models incorporating race, gender, age and height, and is indicator of the severity of airflow obstruction$^{16}$. Spirometry measurements were obtained using a standardized spirometer (EasyOne by ndd Medical Technologies) after administering two puffs (180 mcg) of albuterol.

**Statistical analyses**

*Single variant analysis*
We tested SNP-trait associations for FEV₁/FVC and FEV₁ percent predicted separately in NHWs and AAs assuming an additive genetic model using linear regression. All analyses were adjusted for age, gender, pack-years smoked, exome array version, and principal components (PCs) to control for population stratification within racial groups. PCs were calculated using unlinked, autosomal polymorphisms (n= 14,975 in NHW and n=19,498 AAs) in Eigensoft¹⁷. Single variant tests included only SNVs with a MAF > 0.5% annotated as being functional (nonsynononomous, stop-gain, stop-loss, splicing or ncRNA). Statistical significance for each quantitative phenotype was determined by Bonferroni correction (0.05 ÷ 21,394 tests = 2.34 x 10⁻⁶ in NHWs and 0.05 ÷ 38,519 tests = 1.30 x 10⁻⁶ in AAs).

**Gene-based analysis**

We performed gene-based tests using the sequence kernel association test (SKAT)¹⁸. SKAT has been shown to perform well under various scenarios, including when protective, deleterious and null variants are present within a single gene. We tested putatively functional, rare (minor allele frequency [MAF] < 5%) variants in genes with ≥ 5 observed variants. Statistical significance was determined by Bonferroni correction for the number of genes tested in each population (α = 5.83 x10⁻⁶ for NHW analysis, α = 5.49 x 10⁻⁶ for AA analysis).

For statistically significant genes, we tested the relative contribution of each variant by removing one SNV at a time, and re-calculating the evidence for association across the gene using the SKAT statistic. All analyses were adjusted for age, gender, pack-years smoked, exome array version and principal components to control for population stratification.
Variant annotation and *in-silico* functional prediction

SNVs were annotated using ANNOVAR\(^\text{19}\) with the RefSeq\(^\text{20}\) reference genome. In an effort to assess the potential functional consequences of variants identified as significant, we used the combined annotation dependent depletion (CADD) algorithm \(^\text{21}\) to predict deleterious effect on the gene product and genomic evolutionary rate profiling (GERP) scores \(^\text{22}\) to indicate the degree of evolutionary conservation of variants.

**Results**

**Study participants**

Clinical characteristics of the NHW (n=6,562) and AA (n=3,182) subjects with available spirometry data are provided in Table 1 and Figures S3.1-S3.2. On average, AA subjects were younger with fewer pack-years exposure and had higher mean FEV\(_1\)/FVC and FEV\(_1\) percent predicted.

**Genotypes**

A total of 150,299 SNVs in NHWs and 146,654 SNVs in AAs passed QC and were not monomorphic. Greater than 87% of these were rare (MAF < 5%) and greater than 92% were putatively functional (annotated as nonsynonomous, stop-gain, stop-loss, ncRNA or splicing). The most common functional category was nonsynonomous, representing over 90% of all genotyped SNVs.

**Single variant analysis**

We identified four significant single variant associations with spirometry, all of which were nonsynonymous mutations (Table 3.2 and Figure 3.1). Three of these four associations had been previously identified in GWAS as genetic risk loci for lung function: rs16969968 in CHRNA5 (Asp398Asn, MAF = 36.6%) and rs2070600 in AGER (Gly38Ser, MAF = 4.2%). The identified nonsynonomous variant in CHRNA5 had a
phred-scaled CADD score of 10.5, indicating it was in the top 10% of deleterious predictions\textsuperscript{21}, and a GERP score of 3.88, indicating a high degree of evolutionary constraint (a cutoff of 2.0 is commonly used to indicate “constraint” \textsuperscript{22}). Similarly, \textit{in-silico} functional predictions using CADD and GERP suggest the identified nonsynonomous variant in \textit{AGER} is likely deleterious (CADD score = 18.0, GERP score of 5.82).

We identified one novel association between a nonsynonomous SNV and FEV\textsubscript{1} percent predicted in AA COPDgene subjects (rs34664882, \(p = 2.42 \times 10^{-7}\), MAF= 1.7\%). The associated SNV is in the gene encoding the ankyrin 1 (\textit{ANK1}) protein and the minor allele (A) is associated with significantly lower FEV\textsubscript{1} percent predicted (\(\beta = -11.59\), Figure 3.2). This mutation causes the substitution of a valine for alanine in amino acid position 1503, however CADD and GERP did not predict this change to be deleterious (CADD score = 2.1, GERP score = -2.53). This variant (rs34664882) was observed in NHW subjects at a comparable frequency (MAF = 2.9\%), but no association between genotype and FEV\textsubscript{1} percent predicted was observed in this sub-population (\(p=0.93\)). Manhattan and QQ plots for all single variant association tests are shown in Figures S3.3-S3.5.

**Gene-based analysis**

We identified three significant gene associations with FEV\textsubscript{1}/FVC or FEV\textsubscript{1} percent predicted using SKAT (Table 3.3 and Figure S3.6). Associated genes include: \textit{AGER} on chromosome 6 (associated with FEV\textsubscript{1}/FVC in NHWs, \(p = 2.01 \times 10^{-9}\)); \textit{ProSAPiP1} on chromosome 20 (associated with FEV\textsubscript{1} percent predicted in NHWs, \(p = 2.63 \times 10^{-6}\)); and \textit{ANK1} on chromosome 8 (associated with FEV\textsubscript{1} percent predicted in AAs, \(p = 5.27 \times 10^{-6}\)). All gene-based associations were population-specific, and we observed no evidence of signal overlap between NHWs and AAs (SKAT p-value for \textit{AGER} in AAs = 0.52, SKAT p-value for \textit{ProSAPiP1} in AAs = 0.19, SKAT p-value for \textit{ANK1} in NHWs = 0.93).
To quantify the relative contribution of each SNV to the gene-based signals, we removed one SNV at a time and re-calculated the evidence for association across the gene using SKAT (Figure 3.3). In each of the three associated genes, this identified a single, rare SNV that accounted for the gene-based signal (i.e. every time that variant was included in the re-calculated SKAT, gene-based results were significant, but when it was excluded, SKAT results were not significant). The identified SNV in *AGER* (rs2070600) and *ANK1* (rs34664882) was the same SNV previously identified in the single variant analysis. The identified SNV in *ProSAPiP1*, rs140282982, was observed in 10 subjects (MAF = 0.00076), and a single copy of the minor allele (C) reduced FEV1 percent predicted by an average of -31.7 (Figure S3.7).

**Discussion**

We performed single variant and gene-based exome array analysis in NHW and AA participants from the COPDgene study to identify functional variation associated with quantitative spirometric phenotypes. We replicated previously known GWAS associations (rs2070600 in *AGER* and rs16969968 in *CHRNA5*), and we identified two novel associations in the *ANK1* and *ProSAPiP1* genes. Association signals in these genes were largely driven by a single rare, nonsynonomous variant with a large effect (rs34664882 in *ANK1* and rs140282982 in *ProSAPiP1*).

*ANK1* is part of the ankyrin family of proteins that act to attach integral membrane proteins to the spectin-actin based cytoskeleton. It plays a key role in cell motility, cell activation, cell proliferation, and the maintenance of specific cellular membrane domains\(^\text{20}\). *ANK1* is highly expressed in the brain and muscle, and moderately expressed in the lung\(^\text{23}\). Mutations in *ANK1* are responsible for hereditary spherocytosis\(^\text{24}\), and this gene (and others in the ankyrin family of proteins) have been associated with lung function in previous research: In 2012, Imboden et al. identified a
variant in \textit{ANK1} (rs7006290) as associated with \textit{FEV}_1 decline in asthmatics \textit{p}=5.19 \times 10^{-6} \textit{)} \textsuperscript{25}. Additionally, Hansel et al. identified an association in the \textit{ANK3} gene with \textit{FEV}_1 decline in 4,048 Lung Health Study participants \textit{26}. 

\textit{ProSAPiP1}, or the proline-rich synapse-associated protein-interacting protein 1 (also known as \textit{LZTS3}), is thought to play a role in regulating cellular growth, although its biological function remains largely unknown\textsuperscript{27}. This gene is primarily expressed in the brain and kidney, but shows moderate expression in multiple other tissues including the lung\textsuperscript{23}. There are no known associations between this gene and clinically defined COPD or COPD-related phenotypes, and its role in lung disease remains unclear.

Our results indicate low frequency, coding variants may account for some known GWAS signals, and association signals in newly identified genes may explain a small proportion of the population variation in lung function. However, overall we found no widespread impact of low-frequency coding variation in lung function. One limitation of our study is that the exome array does not cover all functional variants. Specifically, very rare variants (such as uncharacterized private mutations) and regulatory variation outside of the exome are not genotyped on this platform, and whole genome sequencing may be needed for a comprehensive assessment of al variation. We have identified interesting results warranting further research to understand the mechanism underlying their role in COPD etiology.
References


Table 3.1
Characteristics of the COPDgene study population with available spirometry data.

<table>
<thead>
<tr>
<th></th>
<th>Non-Hispanic Whites</th>
<th>African Americans</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>6562</td>
<td>3182</td>
</tr>
<tr>
<td>Mean Age</td>
<td>62.1 (8.8)</td>
<td>54.7 (7.2)</td>
</tr>
<tr>
<td>Gender (% Male)</td>
<td>52.3 %</td>
<td>55.9 %</td>
</tr>
<tr>
<td>Mean pack-years smoked</td>
<td>47.4 (26.0)</td>
<td>38.3 (21.6)</td>
</tr>
<tr>
<td>Mean FEV$_1$/FVC</td>
<td>0.64 (0.2)</td>
<td>0.72 (0.1)</td>
</tr>
<tr>
<td>Mean FEV$_1$ % Predicted</td>
<td>0.74 (0.3)</td>
<td>0.82 (0.2)</td>
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</table>
Table 3.2
Single variant associations with spirometry. Putatively functional SNVs with MAF > 0.5% were tested for association with FEV₁/FVC and FEV₁ percent predicted in 6,051 NHW and 3,321 AA COPDgene subjects.

### Non-Hispanic Whites

<table>
<thead>
<tr>
<th>SNP</th>
<th>Chr</th>
<th>Hg19 Position</th>
<th>Outcome</th>
<th>Beta</th>
<th>P-value</th>
<th>Gene</th>
<th>Function</th>
<th>MAF</th>
<th>P-value AA</th>
<th>MAF AA</th>
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<tr>
<td>rs2070960</td>
<td>6</td>
<td>32151443</td>
<td>FEV₁/FVC</td>
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<td>1.67E-08</td>
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<td>78882925</td>
<td>FEV₁ % P</td>
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<td>2.26E-12</td>
<td>CHRNA5</td>
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<td>0.366</td>
<td>0.064</td>
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</table>

### African Americans

<table>
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<th>Chr</th>
<th>Hg19 Position</th>
<th>Outcome</th>
<th>Beta</th>
<th>P-value</th>
<th>Gene</th>
<th>Function</th>
<th>MAF</th>
<th>P-value NHW</th>
<th>MAF NHW</th>
</tr>
</thead>
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<tr>
<td>rs34604882</td>
<td>8</td>
<td>41543673</td>
<td>FEV₁ % P</td>
<td>-11.59</td>
<td>2.42E-07</td>
<td>ANK1</td>
<td>nonsynonymous</td>
<td>0.017</td>
<td>0.529</td>
<td>0.029</td>
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Figure 3.1
Boxplot of spirometry by A) rs2070600 genotype in the AGER gene; B) rs16969968 genotype in the CHRNA5 gene; C) rs16969968 genotype in the CHRNA5 gene; and D) rs34664882 genotype in the ANK1 gene.
Figure 3.2
Regional association results (- log₁₀ p-value) for SNVs in the ANK1 gene for FEV₁ percent predicted in AA COPDgene subjects. Linkage disequilibrium (r²) values from the 1000G African population.
Table 3.3
Genes significantly associated with spirometry. Gene-based analyses were conducted using SKAT. Only rare (MAF < 5%), nonsynonymous, stop-gain, stop-loss, splicing, and non-coding RNA variants in genes with ≥ 5 variants were considered. Genes presented in the table are significant after Bonferroni correction for the number of genes tested (α = 5.83 x 10^-6 in NHWs, α = 5.49 x 10^-6 in AAs).

<table>
<thead>
<tr>
<th>Population</th>
<th>Outcome</th>
<th>Gene</th>
<th>Chr</th>
<th>Range</th>
<th>Number of variants</th>
<th>SKAT P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHW</td>
<td>FEV1/FVC</td>
<td>AGER</td>
<td>6</td>
<td>32148744-32152099</td>
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<tr>
<td>NHW</td>
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<td>ProSAPIP1</td>
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<td>7</td>
<td>2.63 x 10^-4</td>
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<tr>
<td>AA</td>
<td>FEV1 % P</td>
<td>ANK1</td>
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<td>41510248-41754280</td>
<td>19</td>
<td>5.27 x 10^-4</td>
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</table>
Figure 3.3
Assessment of the relative contribution of each SNV to the gene-based SKAT test for A) SNVs in AGER; b) SNVs in ANK1; and C) SNVs in ProSAPiP1. We re-calculated SKAT p-values removing one SNV at a time, adjusting for age, gender, pack-years smoked, and principal components. Variant MAFs are presented in red.
Supplementary Figure 3.1
Histograms of A) FEV₁/FVC and B) FEV₁ percent predicted in NHW COPDgene participants (n=6,562).
Supplementary Figure 3.2
Histograms of A) FEV$_1$/FVC and B) FEV$_1$ percent predicted in AA COPDgene participants (n=3,182).
Supplementary Figure 3.3
Manhattan plot of $-\log_{10}$ p-values from a linear regression of outcome on SNV (coded additively) controlling for age, gender, pack-years smoked, and 5 principal components in NHW subjects (n=6,501). Panel A contains results from the FEV₁/FVC analysis. Panel B contains results from the FEV₁ percent predicted analysis. Only functional variants with a MAF > .5% were considered. The red dotted line indicates significance level, as determined by Bonferroni correction for the number of SNVs tested (n=21,394). Significant SNVs are labeled with their gene name.
Supplementary Figure 3.4
Manhattan plot of $-\log_{10}$ p-values from a linear regression of outcome on SNV (coded additively) controlling for age, gender, pack-years smoked, and 5 principal components in AA subjects (n=3,221). Panel A contains results from the FEV₁/FVC analysis. Panel B contains results from the FEV₁ percent predicted analysis. Only functional variants with a MAF > .5% were considered. The red dotted line indicates significance level, as determined by Bonferroni correction for the number of SNVs tested (n=38,519). Significant SNVs are labeled with their gene name.
Supplementary Figure 3.5
Quantile-quantile (QQ) plots for single variant analyses in PLINK by minor allele frequency category.
Supplementary Figure 3.6
Boxplot of spirometry by rs140282982 genotype in the ProSAPIP1 gene. A copy of the minor allele at this SNV reduces FEV1 percent predicted by -31.7 in NHWs (calculated by linear regression of outcome on genotype, coded additively, controlling for age, gender, pack-years smoked, and the first 5 principal components, p-value = 2.63 x10^{-5}).
Supplementary Figure 3.7
Quantile-quantile (QQ) plots for gene-based analysis using SKAT.
Chapter 4. Genetic determinants of decline in lung function in the COPDgene study
Chapter 4. Genetic determinants of decline in lung function in the COPDgene study

Introduction

Spirometric measurements, including the forced expiratory volume in one second (FEV₁) and the forced vital capacity (FVC), reliably reflect the physiological state of the airways, and are used to diagnose chronic obstructive pulmonary disease (COPD)¹². These measures are heritable³⁴ and a multitude of previous research has assessed genetic risk factors for cross-sectional lung function⁵⁻⁹, including a large scale meta-analysis of 48,201 individuals that identified a total of 26 genetic loci associated with this outcome⁹. However, reduced lung function is likely influenced by both: 1) a failure to attain maximal lung size and function before adulthood; and 2) accelerated lung function decline after maturity. Cross-sectional studies cannot differentiate between these two pathways, and it is probable different risk factors (both genetic and environmental) separately influence each one. However, few studies have assessed risk factors for lung function decline, as this requires longitudinal data.

Fletcher and Peto first established cigarette smoking as a key risk factor for accelerated lung function decline¹⁰, but genetic variation is also hypothesized to play an important role. Heritability estimates of longitudinal change in lung function using family and twin data range from 10% to 39%⁴,⁷,¹¹. To date, there have been three published genome-wide association studies (GWASs) of lung function decline, all in populations of European ancestry¹²⁻¹⁴. Overall, 6 genes have been identified as associated with lung function decline (DLEU7, TMEM26, FOXA1, ANK3, IL16/STARD5/TMC3, and ME3), none of which overlap with the reported associations from studies of cross-sectional lung function. However, there is little agreement between these 3 published studies regarding
which genes contribute to this decline phenotype, suggesting additional replication and further research is necessary.

To assess the role of genetic risk factors in longitudinal changes in lung function, we used genotyping array data from a large multicenter study of current and former smokers to determine genetic predictors of change in FEV₁ and change in the FEV₁/FVC ratio.

Methods

Study Participants
Subjects who have completed the 5-year follow-up phase of the COPDgene study were included in this preliminary analysis (n=2,000). A complete study protocol for COPDgene has been described elsewhere but briefly, Non-Hispanic Whites (NHWs) and African Americans (AAs) between the ages of 45 and 80 years with a minimum of 10 pack-years smoking history were enrolled at 21 study centers across the United States. In addition to completing detailed questionnaires, pre- and post- bronchodilator spirometry, and volumetric computed tomography (CT) of the chest, participants provided whole blood for DNA extraction and genotyping.

Genotyping and Quality Control (QC)
Samples were genotyped using two array platforms: 1) Illumina’s OmniExpress GWAS array, which contains ~730,000 common single nucleotide polymorphisms (SNPs); and 2) Illumina’s HumanExome Beadchip (v1.1 or v1.2), which contains ~250,000 functional, primarily low frequency variants in recognized exons of the human genome. Sample and variant quality control (QC) were performed separately for both the genome-wide and exome arrays. A summary of the quality control procedures for the GWAS and exome arrays is provided in Table 1. A complete description of the GWAS array QC is provided at: http://www.copdgene.org/sites/default/files/GWAS_QC_Methodology_20121115.pdf,
and a complete description of the QC for the exome array is provided in Chapter 2 of this dissertation.

After quality control, genotyping data from the genome-wide marker panel and exome arrays were combined (excluding exome array variants also on the genome-wide array), and only variants with a minor allele frequency (MAF) > 0.5% were considered for analysis. This resulted in a total of 1,394 subjects and 654,976 variants available for analysis in the NHW group and 606 subjects and 727,583 variants available for analysis in the AA group.

**Principal component analysis**

We performed principal component analysis (PCA) separately in NHW and AA subjects using unlinked, polymorphic (MAF > 5%) SNPs that were in Hardy Weinberg equilibrium and not in regions known to have long range linkage disequilibrium (LD)\(^{16}\). These inferred principal components summarize genetic ancestry and were applied as covariates in subsequent statistical analyses to control for population stratification within racial group.

**Phenotype and covariate assessment**

We analyzed: 1) the change in FEV\(_1\) percent predicted (calculated as FEV\(_1\) percent predicted at visit 2 minus FEV\(_1\) percent predicted at visit 1); and 2) the change in FEV\(_1\)/FVC ratio (calculated as FEV\(_1\)/FVC at visit 2 minus FEV\(_1\)/FVC at visit 1). At both baseline and the 5 year follow-up, spirometry measurements were obtained using a standardized spirometer (EasyOne by ndd Medical Technologies) after administering two puffs (180 mcg) of albuterol. FEV\(_1\) percent predicted was calculated using race and gender-specific regression models based on age, age\(^2\), and height\(^2\). We used
demographic data (including age, gender and smoking habits) from the baseline visit for our analysis.

**Statistical Analysis**

*Genome-wide association studies*

We performed linear regressions of 5-year change in spirometry on SNP genotype coded additively using PLINK\(^\text{17}\) separately in NHWs and AAs. All association models included baseline age, gender, pack-years smoked, baseline spirometry, height, and the first 5 (NHWs) or 6 (AAs) principal components to control for population stratification. SNP-trait analyses using genotype data from the exome array were also adjusted for chip version (v1.1 or v1.2). We declared a variant-trait association to be genome-wide significant if the p-value was less than 5 \(\times 10^{-8}\), and a variant-trait association nominally significant of the p-value was less than \(<1\times10^{-5}\).

*Candidate lung function decline genes*

We examined genes previously reported in the literature to be associated with longitudinal changes in lung function to determine if SNPs in or near these genes were associated with change in FEV\(_1\) percent predicted or FEV\(_1\)/FVC in the COPDgene cohort. In total, we tested 13 regions, including nine genes/gene clusters with a p-value \(< 1 \times 10^{-5}\) in the Tang et al. paper (ST3GAL3, NFIA, ESRRG/GPATCH2, BAZ2B, TMCO3, IL16/STARD5/TMC3, SV2B, MYH11, CACNG4)\(^\text{14}\), one gene from the Imboden et al. paper (DLEU7)\(^\text{13}\), and three genes from the Hansel et al. paper (TMEM26, FOXA1, ANK3)\(^\text{12}\). Because not all of the sentinel variants in previous studies were available in our data, we annotated all SNPs using ANNOVAR\(^\text{18}\) and the RefSeq\(^\text{19}\) reference and those annotated to one of the 13 gene regions were assessed for association. This totaled 2,023 variants in the NHW group and 2,193 variants in AA group.
Results

Subject characteristics

Clinical characteristics of subjects with follow-up data are presented in Table 2. This represents 20.3% of the total NHW cohort and 18.4% of the total AA cohort expected to complete 5-year follow-up as part of COPDgene. At visit two, 270 subjects (13.5%) had changed smoking status, with 219 subjects (11.0%) changing from current to former smokers and 51 subjects (2.6%) changing from former to current smokers.

GWAS of change in FEV₁ percent predicted

The mean 5-year change in FEV₁ percent predicted among NHWs was -1.77 ± 9.63, and the mean 5-year change in FEV₁ percent predicted among AAs was -2.97 ± 12.03 (Supplementary Figure 1). There was considerable variability in change in FEV₁ percent predicted (range in NHW: -46.7 to 36.2, range in AA: -39.1 to 48.6) with 820 subjects (41.2%) increasing FEV₁ percent predicted between visit 1 and visit 2.

Our genome-wide association analyses of change in FEV₁ percent predicted yielded no statistically significant associations (Figure 1). Quantile-quantile (QQ) plots presented in Figure S5 show that there was no evidence of type 1 error inflation (genomic inflation factor NHW analysis = 0.994, genomic inflation factor AA analysis = 0.997). There were 6 SNPs in the NHW analysis and 8 SNPs in the AA analysis that were nominally associated (p < 1x10⁻⁵) with change in FEV₁ percent predicted (Supplementary Table 1). None of the associated variants overlapped between the NHW and AA analyses.

The most statistically significant SNP among NHWs was rs2867387 (β = 2.28, p = 2.7 x 10⁻⁶) located just downstream of the FGA gene. Interestingly, another variant in the 3’ UTR of this gene, rs2070022, was also nominally associated with change in FEV₁ percent predicted (β = 2.19, p = 6.8 x 10⁻⁶). The most statistically significant association
in the AA analysis was an exonic variant in the *NUP153* gene (rs16879902, $\beta = -10.9$, $p = 9.06 \times 10^{-7}$). This variant has a low minor allele frequency (MAF = 0.03) and results in a missense substitution of an aspartic acid to an asparagine at amino acid position 90.

**GWAS of change in FEV$_1$/FVC ratio**

Mean 5-year change in FEV$_1$/FVC ratio among NHWs was $-0.014 \pm 0.06$, and mean 5-year change among FEV$_1$/FVC ratio in AAs was $-0.021 \pm 0.07$ (Supplementary Figure 2). Change in FEV$_1$/FVC ranged from -0.34 to 0.29 in NHWs and from -0.29 to 0.31 in AAs with 684 subjects (37.0 %) increasing their FEV$_1$/FVC ratio between visit 1 and visit 2.

Our genome-wide association analysis of change in FEV$_1$/FVC yielded no statistically significant associations (Figure 2). QQ plots presented in Figure S5 show there was no evidence of type 1 error inflation (genomic inflation factor NHW analysis = 1.010, genomic inflation factor AA analysis = 0.999). A total of 11 SNPs in NHWs and 6 SNPs in AAs were nominally associated with change in FEV$_1$/FVC ratio ($p < 1 \times 10^{-5}$) (Supplementary Table 2). Nominally significant associations did not overlap the FEV$_1$ percent predicted analysis nor did they overlap between the NHW and AA analyses.

The most statistically significant associations in the NHW analysis were two SNPs in high LD ($r^2=0.9$) located in the intergenic region between the *LOC10050720* and *HNRNPKP3* genes. (rs12574104, $\beta = -0.027$, $p = 6.25 \times 10^{-7}$ and rs16937161, $\beta = -0.027$, $p = 9.54 \times 10^{-7}$). The most statistically significant association in the AA analysis was a variant, rs1998292, in the *RASSF2* gene ($\beta = -0.028$, $p = 2.24 \times 10^{-6}$). This variant is located in the intronic regions of the *RASSF2* gene and has a minor allele frequency of 0.13 in AAs.
Candidate lung function decline genes

We sought corroborative evidence of association for genes previously associated with lung function decline (n=13). The most statistically significant SNP association from each of the 13 genes is presented in Table 4 (for change in FEV$_1$ % predicted) and Table 5 (for change in FEV$_1$/FVC). Considering p < 0.001 as suggestive evidence, none of these SNPs were associated with change in FEV$_1$/FVC, and three SNPs were associated with change in FEV$_1$ percent predicted. Interestingly, SNPs within the NFIA gene were associated with change in FEV$_1$ percent predicted in both NHW and AA groups (SNP=rs17377218, p=0.0006 in NHWs and SNP= rs1712138, p=0.0009 in AAs). Additionally, a SNP within the BAZ2B gene was also associated with change in FEV$_1$ percent predicted in NHWs (p=0.0002). This variant is located just downstream of the previously reported variant associated with change in lung function (genomic position = 160223047, genomic position of previously associated = 160250021). These two SNPs are in moderate linkage disequilibrium ($r^2 =0.8$) in the HAPMAP CEU population$^{20}$.

Discussion

Although genetic risk factors for cross-sectional lung function have been well-studied in large scale GWASs$^{5,9,21}$, comparatively less information is known about longitudinal changes in spirometry. We sought to assess the role of genetic risk factors in longitudinal changes in FEV$_1$ percent predicted and FEV$_1$/FVC using GWAS and exome array data from the COPDgene study, a large multicenter study of current and former smokers.

In our genome-wide analyses, no variants reached statistical significance, suggesting no single variant can explain a large portion of the phenotypic variance in lung function decline. The top SNP in the change in FEV$_1$ percent predicted analysis, rs17688693, is located in the nucleoporin 153 gene (NUP153) on chromosome 6p22. Nucleoporins are
responsible for regulating the movement of macromolecules between the nucleus and cytoplasm, but they have no known role in lung disease\textsuperscript{22}. The two top associations in the change in FEV\textsubscript{i}/FVC analysis, rs12574104 and rs16937161, were located in the intergenic region between two pseudo-genes (\textit{LOC100507205} and \textit{HNRNPKP3}). The function of these pseudo-genes remains unknown\textsuperscript{22}.

Additionally, we sought corroborative evidence of association for genes previously associated with lung function decline in three population-based GWASs\textsuperscript{12–14}. One gene, nuclear factor 1A (\textit{NFIA}), showed suggestive evidence of association with change in FEV\textsubscript{i} percent predicted in both the NHW and AA groups (\textit{p}=0.0006 in NHWs and \textit{p}=0.0009 in AAs). Genes in the nuclear factor 1 family are highly conserved and function as cellular transcription factors. These genes are known to be essential for lung maturation\textsuperscript{23} and variants in this gene have been previously implicated in the asthma plus allergic rhinitis phenotype\textsuperscript{24}. Our results support the involvement of this gene in change in lung function among adults.

There are a few limitations to our study. With 1,394 NHW subjects and 606 African American subjects, we have limited power to detect associations, especially those with modest effect sizes. Additionally, we modeled change in lung function by subtracting measures at visit 1 from measures at visit 2 (the “delta method”). Although easy to interpret, this may not fully capture differences in disease trajectory in this population. Specifically, there is evidence those with moderate disease at baseline have a more rapid decline than those with more advanced disease\textsuperscript{25–27} (Supplementary Figure 4.4 and Supplementary Figure 4.4). Future analyses may benefit from stratification by baseline disease severity. Lastly, changes in lung function are likely to be affected by environmental exposures, especially cigarette smoking. Our analyses controlled for cumulative pack-years smoked, but did not explicitly model changes in smoking status.
between visit 1 and visit 2. Additional work is needed to understand how environmental factors and gene by environment interactions affect the rate of lung function decline.

In summary, we performed genome-wide association studies of change in FEV₁ percent predicted and FEV₁/FVC in a large cohort of smokers. This study should be viewed as preliminary analysis, as a sizable portion of COPDgene study participants have yet to complete their 5-year follow-up visit. Additional analyses with this larger sample size are warranted and will provide improved statistical power to detect associations.
References


11. Finkel D, Pedersen NL, Reynolds CA, Berg S, de Faire U, Svartengren M. Genetic and environmental influences on decline in biobehavioral markers of


Table 4.1
Summary of the GWAS and exome array quality control procedures. Genotyping data from the GWAS and exome arrays were combined for analysis, resulting in a total of 1,394 subjects and 654,976 variants available for analysis in the NHWs and 606 subjects and 727,583 variants available for analysis in the AAs. A complete description of the GWAS array QC is provided at: http://www.copdgene.org/sites/default/files/GWAS_QC_Methodology_20121115.pdf, and a complete description of the exome array QC is provided in Chapter 2 of this dissertation.

Definition of abbreviations: HWE= Hardy Weinberg Equilibrium; MAF= minor allele frequency; NHW = Non-Hispanic White; AA = African American; GWAS= genome-wide association study.

GWAS array

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Table 4.2
Clinical characteristics of COPDgene subjects with follow up data. Change in FEV₁ percent predicted was calculated as FEV₁ percent predicted at visit 1 – FEV₁ percent predicted at visit 2. Change in FEV₁/FVC ratio was calculated as FEV₁/FVC at visit 1 – FEV₁/FVC at visit 2.

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<td>-2.97 (12.03)</td>
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Figure 4.1
Manhattan plot of $-\log_{10} p$-values from a linear regression of change in FEV$_1$ percent predicted on SNP (coded additively) controlling for age, gender, pack-years smoked, height, baseline spirometry and principal components. Panel A contains results from the NHW analysis. Panel B contains results from the AA analysis. Only variants with a MAF > .5% were considered. The red dotted line indicates genome-wide significance level ($p < 5 \times 10^{-8}$) and the blue line indicates the nominal significance level ($p < 1 \times 10^{-5}$).
Manhattan plot of $-\log_{10} p$-values from a linear regression of change in FEV1/FVC on SNP (coded additively) controlling for age, gender, pack-years smoked, height, baseline spirometry and principal components. Panel A contains results from the NHW analysis. Panel B contains results from the AA analysis. Only variants with a MAF > .5% were considered. The red dotted line indicates genome-wide significance level ($p < 5 \times 10^{-8}$) and the blue line indicates the nominal significance level ($p < 1 \times 10^{-5}$).
Table 4.4
Assessment of previously associated lung function decline genes (n=13) with change in FEV$_1$ percent predicted in COPDgene. All SNPs annotated to the 13 regions were tested for association. The most significant SNP for each region is presented. P-values less than 0.001 are highlighted.

**Definition of abbreviations:** SNP = single nucleotide polymorphism; P= P-value; NHW=Non-Hispanic White; AA= African American.

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Table 4.5
Assessment of previously associated lung function decline genes (n=13) with change in FEV₁/FVC in COPDgene. All SNPs annotated to the 13 regions were tested for association (n=2,023 variants in NHWs, n=2,193 variants in AAs). The most significant SNP for each region is presented. P-values less than 0.001 are highlighted.

Definition of abbreviations: N=number; SNP = single nucleotide polymorphism; P= P-value; NHW=Non-Hispanic White; AA= African American.

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<th>Gene</th>
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Supplementary Figure 4.1
Histograms of change in FEV₁ percent predicted in A) COPDgene NHW subjects completing follow-up (n=1,394) and B) COPDgene AA subjects completing follow-up (n=606). The red dashed line indicates mean change in FEV₁ percent predicted (NHW mean = -1.77, AA mean = -2.97).
Supplementary Figure 4.2
Histograms of change in FEV₁/FVC in A) COPDgene NHW subjects completing follow-up (n=1,394) and B) COPDgene AA subjects completing follow-up (n=606). The red dashed line indicates mean change in FEV₁, percent predicted (NHW mean = -1.77, AA mean = -2.97).
Supplementary Figure 4.3
Quantile-quantile (QQ) plots for single variant analyses of decline phenotypes in PLINK. Genomic inflation factors are presented in the upper left hand corner.
**Supplementary Table 4.1**

Associations of the most statistically significant SNPs ($P < 1 \times 10^{-5}$) with change in FEV$_1$ percent predicted in COPDgene NHW ($n=1,394$) and AA subjects ($n=606$). Reported results are from a linear regression of change in FEV$_1$ percent predicted on SNP (coded additively) controlling for age, gender, pack-years smoked, height, baseline spirometry, and principal components.

*Definition of abbreviations: CHR = chromosome; SNP = single nucleotide polymorphism; MAF = minor allele frequency.*

### Non-Hispanic Whites

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### African Americans

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Supplementary Table 2. Associations of the most statistically significant SNPs (P < 1 x 10^{-5}) with change in FEV1/FVC in COPDgene NHW (n=1,394) and AA subjects (n=606). Reported results are from a linear regression of change in FEV1/FVC on SNP (coded additively) controlling for age, gender, pack-years smoked, height, baseline spirometry, and principal components.

**Definition of abbreviations:** CHR = chromosome; SNP = single nucleotide polymorphism; MAF= minor allele frequency.

### Non-Hispanic Whites

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Supplementary Figure 4.4
Change in FEV₁ percent predicted by GOLD classification. GOLD 0 is defined as FEV₁/FVC > 0.7. GOLD 1 is defined as FEV₁/FVC < 0.7 and FEV₁ ≥ 80% of normal. GOLD 2 is defined as FEV₁/FVC < 0.7 and FEV₁ 50- 80% of normal. GOLD 3 is defined as FEV₁/FVC < 0.7 and FEV₁ 30- 50% of normal. GOLD 3 is defined as FEV₁/FVC < 0.7 and FEV₁ <30% of normal. GOLD 0 and GOLD 4 subjects have the smallest decline in FEV₁ percent predicted.
Supplementary Figure 4.5
Change in FEV$_1$/FVC by GOLD classification. GOLD 0 is defined as FEV$_1$/FVC > 0.7. GOLD 1 is defined as FEV$_1$/FVC < 0.7 and FEV$_1$ ≥ 80% of normal. GOLD 2 is defined as FEV$_1$/FVC < 0.7 and FEV$_1$ 50-80% of normal. GOLD 3 is defined as FEV$_1$/FVC < 0.7 and FEV$_1$ 30-50% of normal. GOLD 3 is defined as FEV$_1$/FVC < 0.7 and FEV$_1$ <30% of normal. GOLD 4 subjects have the smallest decline in FEV$_1$/FVC.
Chapter 5. Summary of key finding and conclusions.
Chapter 5. Summary of key finding and conclusions.

Chronic obstructive pulmonary disease (COPD) is the 3rd leading cause of death worldwide, accounting for approximately 3 million deaths in 2010\(^1\). Although cigarette smoking is the primary environmental risk factor, COPD susceptibility is related in part to genetic variation\(^2\). Most previous research has focused on identifying associations of common variants with cross-sectional lung function. We hypothesized: 1) rare functional variation also affects lung function; and 2) genetic variation (both common and rare) affects longitudinal changes in lung function. This dissertation addresses these two hypotheses with the following specific aims:

1. To identify functional genetic variants associated with lung function in Non-Hispanic White (NHW) and African American (AA) participants of the COPDgene study using exome array data.

2. To identify genetic variants associated with longitudinal changes in lung function in Non-Hispanic White and African American participants of the COPDgene study using GWAS and exome array data.

Summary of key findings

*Exome array analysis of lung function*

We aimed to identify rare coding variation associated with quantitative spirometric phenotypes through single variant and gene-based exome array analysis in the COPDgene study. We replicated previously known associations from genome-wide association studies (rs2070600 in *AGER* and rs16969968 in *CHRNA5*), illustrating the potential utility of studying the coding genome to clarify GWAS loci. In addition, we identified two novel associations in the *ANK1* and *ProSAPip1* genes. Association signals
in these genes were largely driven by a single rare, nonsynonomous variant with a large
effect (rs34664882 in ANK1 and rs140282982 in ProSAPiP1). Additional replication and
functional validation of these variants is necessary, but together these results suggest
searching the exome for additional loci influencing lung function loci may improve our
understanding of genetic risk factors for COPD.

Analysis of longitudinal changes in lung function

We assessed the role of genetic risk factors in longitudinal changes in spirometric
measures (FEV1 percent predicted and FEV1/FVC) using GWAS and exome array data
from the COPDgene study. This preliminary genome-wide analysis yielded no
statistically significant findings, suggesting no single variant can explain a large portion
of the phenotypic variance of lung function changes over a 5-year period. The top SNP
associated with change in FEV1 percent predicted analysis, rs17688693, is located in the
nucleoporin 153 (NUP153), gene and the top associations in the change in FEV1/FVC
analysis, rs12574104 and rs16937161, were located in the intergenic region between
two pseudo genes (LOC100507205 and HNRNPKP3). Replication and functional
validation of these associations is necessary. Additionally, we sought confirmatory
evidence of association for genes previously associated with decline in lung function6–8.
Our results support the involvement of one gene, nuclear factor 1A (NFIA), which
showed suggestive evidence of association with change in FEV1 percent predicted in
both the NHW and AA groups (p=0.0006 in NHWs and p=0.0009 in AAs).

Although the heritability of lung function decline is established9,10, genes controlling this
phenotype remain largely unknown. To date, large GWAS analyses, including those
presented in this dissertation, have failed to produce replicable results8. This negative
result is likely due to: 1) imprecise measurement of smoking behaviors (especially failure
to account for changes in smoking behavior over time); and 2) differential lung function decline by disease severity (i.e. lung function decline may decelerate in those with more severe disease). However, despite these challenges, identifying risk factors for accelerated disease progression is key to identifying those at highest risk and subsequently improving clinical outcomes. Future large epidemiological studies are warranted.

**Strengths and limitations**

**Strengths**

This dissertation has a few key strengths. The COPDgene study is a large, well-characterized study that included both Non-Hispanic White and African American participants. Spirometric measurements were carefully collected, post-bronchodilation, using a standardized protocol. Unlike many population-based cohorts, COPDgene is unique in that many subjects have severe or very severe COPD (FEV₁ percent predicted < 50%).

Additionally, this is one of the first studies to assess the role of rare genetic variation in influencing quantitative measures of lung function and lung function decline. While traditional candidate gene and GWAS analyses can identify indirect associations between tagging SNPs and a quantitative phenotype, the exome array affords the opportunity to directly observe functional variation, potentially aiding to elucidate the mechanisms underlying risk of COPD. Additionally, we queried common and functional variants across the genome in a manner that it is unbiased with respect to previous knowledge of lung function etiology. Therefore, our analyses had the potential reveal causal genes not previously suspected in disease etiology, allowing for hypothesis generation.
Limitations

This dissertation should be interpreted in light of its limitations. While we have detailed which smoking behaviors we analyzed, these common measures of exposure may not fully capture critical aspects of smoking, especially given these data are based on self-report. Specifically, participants may not have reported changes in smoking behaviors over their lifetime, potentially resulting in residual confounding that could bias reported results. Moreover, available measures of smoking behaviors (pack-years smoked and current smoking status) may not completely capture the toxic effects of tobacco.

Additionally, this dissertation used genotype data from Illumina’s HumanExome array (chapters 3 and 4) and Illumina’s OmniExpress GWAS array (chapter 4). Although these genotyping platforms cover common and coding variants relatively well\textsuperscript{11,12}, they do not cover all genetic variation. Specifically, rare variants in non-coding regions and very rare variation (e.g. private mutations) are not covered. It is possible that loci affecting lung function will not be represented on the tested platforms, and therefore could not have been identified in this study.

Lastly, our genome-wide analyses of lung function decline had limited sample size (n=1,394 NHWs and n=606 AAs), and therefore limited statistical power to detect genetic associations. Failure to identify statistically significant results likely reflects this limitation. As longitudinal follow-up continues in the COPDgene study, this additional data will greatly improve our ability to detect associations.

Future directions

Additional COPD-related phenotypes

Spirometric phenotypes are reliable indicators of lung function and predict population mortality\textsuperscript{13}. However, they do not fully describe the heterogeneous nature of COPD.
Specially, they do not differentiate between small airway disease and emphysema, the two main components of COPD pathogenesis. One unique advantage of the COPDgene study is that all participants underwent computed tomography (CT) imaging, allowing for more refined phenotyping to assess structural lung disease. Future analyses will focus on identifying genetic determinants of COPD-related phenotypes other than spirometry, including, percent emphysema, percent gas trapping, airway wall thickness and chronic bronchitis.

**Whole exome and whole genome sequencing**

In order to comprehensively study all genetic variation for its association with lung function, sequencing data is necessary. The COPDgene study is generating whole exome and whole genome sequencing on a subset of its 10,000 participants, and these data will allow for more complete evaluation of very rare and non-coding variation with COPD-related outcomes.

**Ongoing longitudinal follow-up**

The longitudinal analysis included in this dissertation represented approximately 20% of the COPDgene cohort expected to completed 5-year follow-up. The additional sample size afforded as more longitudinal data is collected will aid in the continued study of risk factors (both genetic and environmental) related to changes in lung function over time in this cohort of adults at high risk of COPD.

**Public health significance**

This study aims to elucidate genetic variants associated with lung function and longitudinal lung function decline. Reduced lung function defines COPD, a common disease with a high global burden\(^1\). This is one of the first studies to assess the role of rare genetic variants in disease pathogenesis, and we identified novel loci associated...
with obstructive pulmonary disease. With replication, identification of associated variants can broaden our understanding of the biological pathways related to this disease.

Ultimately, the goal of studying COPD genetics is to provide evidence for clinical practice and prevention. Improved understanding will aid in tailoring treatments to defined COPD subtypes, discovering novel therapeutic interventions and developing effective prevention strategies. This is especially timely given the large and growing burden of COPD1.

References


Curriculum Vitae
Margaret Parker
Curriculum Vitae

PERSONAL DATA:
Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health
615 North Wolfe Street W6517, Baltimore, MD 21205
Phone: 617-312-1191
megparker@jhu.edu

EDUCATION:
PhD in Genetic Epidemiology      Expected 2015
Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
Advisor: Dr. Terri Beaty
Thesis Committee: Dr. Ingo Ruczinski, Dr. Rasika Mathias

MHS in Genetic Epidemiology/Human Genetics    May 2011
Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
Advisor: Dr. Terri Beaty
Thesis Committee: Dr. Rasika Mathias, Dr. W.H. Linda Kao

BA in Biology, BA in Community Health    May 2007
Tufts University, Medford, MA

PROFESSIONAL EXPERIENCE:
Johns Hopkins Bloomberg School of Public Health, Baltimore, MD    Dec 2009-Present
Senior Research Assistant
Principal Investigator: Dr. Terri Beaty

Tufts Medical Center, Boston, MA        May 2007-July 2009
Clinical Research Coordinator (Feb 2008-July 2009)
Laboratory Technician (May 2007-Feb 2008)
Principal Investigator: Dr. Johanna Seddon

PUBLICATIONS:
Published Peer-Reviewed Articles:


Under Review:


In Preparation:

POSTERS:
Presenter:


Co-author:


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AWARDS:
Anna Huffstultler Stiles Award, JHSPH Epidemiology Department May 2011
Marilyn Menkes Award, JHSPH Epidemiology Department May 2013
2nd place poster, Johns Hopkins Genetics Research Day Feb. 2014
Honorable Mention poster, Johns Hopkins Genetic Research Day Mar. 2015

TEACHING EXPERIENCE:
Teaching Assistant, Johns Hopkins Bloomberg School of Public Health
- Introduction to Genetic Epidemiology (2010, 2013)
- Epidemiologic Methods 3 (2011)
- Epidemiologic Methods 4 (2011)
- Epidemiologic Methods 1 (2012)
- Principles of Genetic Epidemiology 2 (2012)
- Principles of Genetic Epidemiology 3 (2013)

FUNDING:
Ruth L. Kirschstein National Research Service Award (CVD T32) Sept 2012-August 2014
Grant #: 5T32HL007024-38

SKILLS:
Programming:
R, UNIX, PLINK, EIGENSOFT, GATK, STATA, Microsoft Office
Leadership:
TA training chair (2013-2014), Johns Hopkins Epidemiology Department
Co-president (2012-2013), Johns Hopkins Epidemiology Student Organization
Social chair (2010-2011), Johns Hopkins Epidemiology Student Organization

COURSEWORK:
Epidemiologic Methods (1-4), Principles of Genetics Epidemiology (1-4), Methods in Biostatistics (1-4), Statistical Computing, PERL for Bioinformatics, Statistics for Genomics, Introduction to Clinical Trials, Causal Inference, Molecular Biology, Human Physiology