Race-Specific Differential DNA Methylation Marks and Gene Comethylation Modules of COPD

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Title: Race-Specific Differential DNA Methylation Marks and Gene Comethylation Modules of COPD

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Abstract:

Rationale: Chronic obstructive pulmonary disease (COPD) is the third-leading cause of death worldwide. Identifying COPD-associated DNA methylation marks in African-Americans may contribute to our understanding of racial disparities in COPD susceptibility. We determined differentially methylated genes and co-methylation network modules associated with COPD in African-Americans from the Pennsylvania Study of Chronic Obstructive Pulmonary Exacerbations (PA-SCOPE) cohort.

Methods: We assessed DNA methylation from peripheral blood samples in 362 African-American smokers in the PA-SCOPE cohort using the Illumina Infinium HumanMethylation27 BeadChip Array. Final analysis included 19302 CpG probes annotated to the nearest gene transcript after quality control. We tested methylation associations with COPD case-control status using mixed linear models. Weighted gene comethylation networks were constructed using WGCNA and network modules were analyzed for association with COPD.

Results: There were five differentially methylated CpG probes significantly associated with COPD among African-Americans at an FDR less than 5%, and seven additional probes that approached significance at an FDR less than 10%. The top ranked gene association was MAML1, which has been shown to affect NOTCH-dependent angiogenesis in murine lung. Network modeling yielded the "yellow" and "blue" comethylation modules which were significantly associated with COPD (p-value 4x10^{-10} and 4x10^{-9}, respectively). The yellow module was enriched for gene sets related to inflammatory pathways known to be relevant to COPD. The blue module contained the top ranked genes in the concurrent differential methylation analysis ( FXDY1/LGI4, gene significance p-value 1.2x10^{-26}; MAML1, p-value 2.0x10^{-26}; CD72, p-value 2.1x10^{-25}; and LPO, p-value 7.2x10^{-25}), and was significantly associated with lung development processes in Gene Ontology gene-set enrichment analysis.

Conclusions: We identified 12 differentially methylated CpG sites associated with COPD that mapped to biologically plausible genes. Network module comethylation patterns have identified candidate genes that may be contributing to racial differences in COPD susceptibility and severity. COPD-associated comethylation modules contained genes
previously associated with lung disease and inflammation and recapitulated known COPD-associated genes. The genes implicated by differential methylation and WGCNA analysis may provide mechanistic targets for explaining disparities in COPD susceptibility and outcomes between African-Americans and non-Hispanic Whites.

Trial Registration: NCT00774176, Registry: ClinicalTrials.gov, URL: www.clinicaltrials.gov, Date Registered: 04 January 2008 (retrospectively registered).

**Keywords:**

Chronic obstructive pulmonary disease, DNA methylation, microarray, weighted gene comethylation network analysis
Background

Chronic obstructive pulmonary disease (COPD) is an incurable lung disease characterized by progressive airflow obstruction involving emphysematous destruction of lung parenchyma and mucus hypersecretion with chronic bronchitis. Over 12 million Americans are affected by COPD, which is the third leading cause of death in the US[1], and projected to become the third leading cause of death worldwide[2]. Recent data suggest that the prevalence of emphysema, chronic bronchitis, and COPD hospitalizations are increasing among African-Americans (AA) [3-5], and that AA may develop COPD at a younger age than non-Hispanic Whites (NHW)[5]. In addition, AA males have one of the highest prevalence rates of smoking (25.5%) among racial groups in the United States[6], leading to a predictable growing burden of lung disease in this group. AA individuals present with similar severity of airflow obstruction as NHW, despite fewer pack-years of smoking[5]. Once they have developed COPD, AA have lower quality of life scores[7]. Despite these alarming trends, COPD has been understudied in African-Americans.

Race is an important contributor to genetic[8] and epigenetic variability, and recent studies have identified epigenetic association signals that differ between racial groups[9]. Similarly, the results of differential methylation association studies of complex traits in single racial-ancestry cohorts may miss epigenetic risk factors in another racial-ancestry cohort, and may not be generalizable to other racial cohorts at all[10, 11]. Recent methylation studies have shown a subset of methylation signals particular to AA smokers[9], but to our knowledge race-specific investigations of epigenetic associations with COPD have not been previously performed. Understanding the epigenetic associations of smoking and COPD in AA current and former smokers may provide insights into race-specific features relevant to COPD-related disparities in AA.

DNA methylation patterns are determined at multiple time points in the life of an individual[12], including in utero during imprinting, tissue-specific methylation during development, and changes in the methylation of genes in response to major environmental exposures. Differential methylation impacts gene regulation, which may lead to clinically relevant changes in disease-related phenotypes. Modules of genes with correlated comethylation profiles may identify groups of genes under similar regulation that are associated with COPD risk. Prior research has identified differential
methylation signals related to tobacco smoke exposure that may influence risk for development of COPD[13-18]. The majority of these studies have focused on NHW subjects as the largest proportion of their cohorts. Our investigation focused on the identification of differential methylation sites associated with COPD as well as COPD-associated comethylation modules in an AA cohort. Our hypothesis was that race-specific patterns of DNA methylation would identify differentially methylated genes or comethylation networks that may contribute to racial disparities in COPD. A better understanding of the epigenetic factors associated with the features of COPD in AA smokers may provide insights into new diagnostic options, drive the discovery and targeting of therapeutics, and improve primary prevention strategies in this susceptible population.

**Results**

After quality control, the PA-SCOPE AA dataset included methylation data on 19302 probes measured in 93 subjects with COPD defined by GOLD spirometry criteria (GOLD I-IV), as well as 269 smoking controls. A quality control schematic is provided in Figure 1. Baseline statistics for the two groups showed expected differences in metrics used to define COPD severity including forced expiratory volume in 1 second as percent predicted (FEV1), forced vital capacity as percent predicted (FVC), and the ratio of FEV1 to FVC (FEV1/FVC), while pack-year history of smoking (PYH) was similar (Table 1).

**Differential DNA Methylation Analysis:** We used linear mixed models to identify COPD-associated CpG sites, using the method of Benjamini and Hochberg to control type I error. Five differentially methylated CpG sites were associated with COPD (Table 2) at FDR < 5%. All of these five CpG sites exhibited hypomethylation in association with the presence of COPD. The mean difference in percent methylation between cases and controls among the top five associated sites ranged from 5.3 to 9.6 %. The top differentially methylated CpG site was cg16361890 (unadjusted p-value 8.188x10^{-8}, percent methylation change -7.8%), which mapped to the *MAML1* gene. Gene annotation for the remaining four differentially methylated CpG sites associated with COPD included *RBFOX2, CD72, GRASP,* and *SH3TC1.*

Seven additional differentially methylated CpG sites were associated with COPD at FDR of less than 10%, (Table 2). These seven additional CpG sites (annotated to *FOLR3, ELOVL3, FAXDC2, FXYD1/LG14, GABRR1, IFNGR2,* and *LPO*) also
exhibited relative hypomethylation in association with COPD. The mean difference in methylation ranged from 7.5 to 11.9% (see Supplemental Figures). These differential methylation results are presented in a volcano plot (Figure 2) and a Manhattan Plot (Figure 3).

**Comparison to NHW Dataset:** We examined the differentially methylated CpG sites from the PA-SCOPE AA cohort in the International COPD Genetics Network (ICGN) NHW cohort methylation data[19]. We qualitatively compared the magnitude of change in differential methylation between AA and NHW using the difference between the test-statistic for differential methylation at each CpG site (PA-SCOPE minus ICGN) from the mixed linear model output. We posited that a large difference in test statistic of differential methylation at a given CpG site between the PA-SCOPE and ICGN studies may indicate a difference in differential methylation between AA and NHW. This difference metric was found to have a normal distribution (mean= -0.316, sd= 1.515) across the ~19000 probes remaining after data cleaning. Seven of the twelve differentially methylated genes identified at FDR less than 10% in the primary PA-SCOPE analysis were found to have test-statistic-difference values in the lower 2.5 percentile tail of this distribution, suggesting potential race-specific differential methylation at these sites (see Supplemental Figures).

**Weighted Gene Comethylation Network Analysis:**

Weighted Gene Comethylation Network Analysis (WGCNA) was used to create a scale-free comethylation network[20] (see Supplemental Figures). The resultant network contained ten modules (see Figure 4), of which two were significantly correlated with COPD case-control status (labeled “blue” and “yellow” modules). The blue module contained 5009 probes (p-value $4 \times 10^{-9}$ for module association with COPD status), and the yellow module contained 1698 probes (p-value $4 \times 10^{-10}$ for module association with COPD status). The association of the blue and yellow module eigengenes remained statistically significant (p-value $1.6 \times 10^{-4}$ for blue module, $5.5 \times 10^{-5}$ for yellow module) in a logistic regression model of COPD affection status after controlling for age, gender, and pack-years of smoking history.

We investigated the two COPD-associated network modules for genes previously related to COPD. This investigation included genes from the differential methylation analysis results and genes found to be associated with COPD and lung
function measurements in genome-wide association studies (GWAS) (see Table 3). CpG sites marking 11 of the 12 top differential methylation sites found in our differential methylation analysis (GRASP, FXYD1/LGI4, MAML1, FOLR3, CD72, LPO, GABRR1, SH3TC1, RBFOX2, IFNGR2, ELOVL3) were part of the blue module. CpG sites marking the COPD- and lung function-associated genes NOTCH4, SERPINA1, FAM13A, TNS1, PPT2, CHRNA5, PPAP2B, CHRNA3, RARB, CHRNA4, ARMC2, CCDC38, MECOM, ADAMTS19, HHIP, and ZKSCAN3/ZSCAN31 were also all found within the blue module. We limited both the blue and yellow modules to those genes with a stringent module membership (kME value) cutoff of 0.85 for further analysis[20], yielding a gene set of 317 members and 151 members, respectively.

Gene set enrichment analyses of the limited modules were performed using GO Biological Processes ontology, KEGG, and Reactome pathways. The limited blue module of 317 genes was enriched for developmental gene sets, with statistically significant enrichment found in GO pathways such as anatomical structure development (GO:0048856; adjusted p-value $3.6 \times 10^{-10}$), branching morphogenesis of an epithelial tube (GO:0048754; adjusted p-value $1.5 \times 10^{-4}$), lung morphogenesis (GO:0060425; adjusted p-value 0.01), lung lobe morphogenesis (GO:0060425; adjusted p-value 0.0246), lung development (GO:0030324; adjusted p-value ), and lung lobe development (GO:0060428; p-value $1.38 \times 10^{-3}$). The limited yellow module of 151 genes was enriched for immune/inflammatory gene sets, such as response to other organism (GO:0051707; adjusted p-value $8.3 \times 10^{-4}$), immune response (GO:0006955; adjusted p-value $7.3 \times 10^{-3}$), defense response to bacterium (GO:0071219; adjusted p-value $4.2 \times 10^{-3}$), and chemotaxis of natural killer cells, neutrophils, and eosinophils (GO:0035747, 0030593, 0048245, respectively; adjusted p-values $3.5 \times 10^{-2}$ to $7.2 \times 10^{-3}$).

**Discussion**

Within the PASCOPE AA cohort, we identified 5 differentially methylated CpG sites significantly associated with COPD using an FDR of 5%, and 7 additional associations that approached significance using an FDR of 10%. We used WGCNA to identify comethylation modules associated with COPD that were enriched for genes related to lung development and immune response and contained biologically relevant genes associated with COPD and lung function. Differentially methylated CpG sites associated with COPD mapped to genes that were biologically plausible candidates for COPD pathogenesis. Notable functions among these genes included NOTCH4-dependent lung angiogenesis, alveolar...
macrophage response pathways, and airway defense mechanisms targeting bacteria, as detailed below. Our results included genes and CpGs implicated in previous studies of obstructive lung disease and exacerbations, including GRASP and IFNGR2 (previous genetic associations with asthma) as well as FYXD1 (differential methylation associated with response to systemic steroids and to COPD[15, 21]). Only 1 of these 12 differentially methylated CpG sites (cg27461196, mapped to LGI4/FXYD1) was statistically significantly associated with COPD at an FDR of 5% in an independent, larger NHW COPD methylation dataset cleaned and processed in a comparable way. In addition, in a qualitative comparison of the difference in differential methylation of CpG sites between AA and NHW, many of our results were preferentially differentially methylated in AA.

The majority of our significantly associated (FDR <10%) differential methylation CpG sites are located in genes that are biologically plausible genes for lung disease that may affect the pulmonary, immune, and vascular biology of COPD. Many of these genes are expressed in either lung or whole blood based on GTEx data[22].

Two of these genes were associated with COPD in prior studies. Folate Receptor Gamma (FOLR3) was found to be 15- to 20-fold upregulated during stable COPD and acute exacerbations of COPD in previous studies[23], although the mechanistic and functional implications of this upregulation are unclear. Differential methylation of Phospholemman (FXYD1) was shown to be associated with COPD in the ICGN cohort by Qiu et al[15], and this gene was also previously found to be differentially methylated in response to systemic steroid use in COPD[21]. Both of these genes are notably related to acute exacerbations of COPD as well as a preferred treatment modality (systemic steroids) for acute exacerbations. Given that the PASCOPE study was ascertained from subjects who were hospitalized for COPD, the methylation pattern of these genes may be related to acute exacerbations of COPD.

Five CpG sites were annotated to genes related to pulmonary and airway physiology. Lactoperoxidase (LPO) is secreted by submucosal glands in human bronchi and plays a role in human airway host defense against bacteria[24]. Gamma-aminobutyric acid Receptor1 (GABBR1) has been shown to affect alveolar fluid homeostasis in alveolar epithelial type II cells[25]. Upregulated gene expression of Very Long Chain Fatty Acid Elongase3 (ELOVL3) has been proposed to
contribute to dysregulated lipid droplet formation in pulmonary surfactant in response to particulate exposure[26]. Rare missense mutations of GRP1-Associated Scaffold Protein (GRASP) were previously associated with asthma in a Latino cohort[27]. The function of SH3 Domain and Tetratricopeptide Repeats 1 (SH3TC1) has not been adequately described in the lung; however, it is implicated in networks related to bronchial airway epithelial cells and cigarette smoking [28].

An additional three CpG sites were annotated to genes related to immune response and steroid synthesis. Cluster of Differentiation 72 (CD72) is a CD5 co-ligand involved in hypersensitivity reactions and sarcoidosis, highly expressed in pulmonary alveolar macrophages[29]. Fatty Acid Hydroxylase Domain Containing2 (FAXDC2) is implicated in “steroid biosynthesis” through KEGG pathways[30]. Interferon Gamma Receptor 2 (IFNGR2) plays a role in activation of macrophages and regulation of Th1 response to intracellular pathogens, with genetic variants previously associated with atopic asthma[31] and pulmonary tuberculosis.

The final two CpG sites were related to cardiovascular processes. Mastermind-Like1 protein (MAML1) effects angiogenesis during organ development through NOTCH-dependent signaling in murine lung[32]. RNA-binding Protein Fox-1 Homolog 2 (RBFOX2) is a splicing regulator implicated in differentiation of myofibroblasts to skeletal muscle, and diminished expression previously associated with pressure-overload-mediated progression of dilated cardiomyopathy/heart failure[33]; potential impact on airway smooth muscle has not been described.

We present data showing that many of our top COPD-associated CpG sites lie in the lower tail of a histogram of the difference in test-statistic between CpG sites in the African-American PA-SCOPE dataset and the non-Hispanic White ICGN dataset (see Supplemental Data). This finding may represent qualitative evidence that these sites are preferentially differentially methylated in African-Americans and not in non-Hispanic Whites, although this conclusion must be seen as hypothesis-generating only without a separate properly controlled and matched study design that would be free of confounding by technical artifacts related to batch. The potential race-specificity of these differentially methylated CpG sites could be explained by several scenarios. The most mechanistically attractive possibility is that
these CpG sites represent differential methylation events in response to gene-environment interactions. The second mechanistic possibility is that these CpG sites represent blood methylation quantitative trait loci (mQTL) that are influenced by the genetic architecture specific to the population substructure[34] of African-Americans. In both of the preceding scenarios, the differential methylation could in turn impact damage and airflow obstruction through changes in gene expression and protein production, which could present unique targets for intervention. Finally, the differential methylation may simply be a marker of a confounder between methylation state and COPD, tagging a prior exposure that directly contributed to both disease and CpG methylation through distinct mechanisms.

WGCNA identifies modules of co-methylated genes starting from the level of thousands of CpG probes and correlates these modules to phenotypic variables. The network creation and module-building processes of WGCNA are informed purely by gene methylation levels, without consideration of case-control status for COPD. Individual genes within the module can then be related to the module eigengenes by measures of module membership and gene significance to the module. This technique identifies driver genes for the module that may help identify biologically meaningful pathways. In our dataset the yellow and blue modules showed significant association with COPD. Yellow module measures of gene significance were predominantly positive (indicating relative hypermethylation in the presence of COPD) while the blue module contained primarily negative measures of gene significance (indicating relative hypomethylation in the presence of COPD).

Further investigation of the blue module showed a network with biological significance for obstructive lung disease. The module was statistically enriched for pathways related to lung development, and also contained multiple genes previously associated with COPD and lung function. SERPINA1 is the gene responsible for alpha-1-antitrypsin deficiency[35], a known genetic cause of COPD, and this gene was found to be highly significant in the blue module. The blue module also contained multiple genes previously associated with COPD or lung function measurements through GWAS. Many of the CpG sites found in the differential methylation analysis were also found in the blue module with high measures of module membership (indicating importance of the gene to the module) and large measures of gene
significance to COPD. The recapitulation of these CpG sites in the same module as previously known COPD- and lung-function-related genes adds validation to our differential methylation results.

The yellow module, by comparison, contained genes enriched for immune response pathways. Chronic inflammation in response to airway damage from cigarette smoking as well as external pathogens are recognized as integral parts of the pathogenesis of COPD and exacerbations [36-38]. Enrichment for the chemotaxis of effector cells that are known to play a role in COPD pathogenesis (neutrophils[39], eosinophils[40, 41], and natural killer cells[42]) were found using yellow module genes with high module membership values. The PA-SCOPE population was ascertained using subjects with disease exacerbations, so this population may have been enriched for signals associated with acute inflammation and immune response[43, 44].

The PA-SCOPE dataset was a retrospective case-control study and so no direct causation can be implied from results, only associations of CpG sites with disease. DNA methylation in response to smoking is a dynamic process, and effects may be dependent upon recent or remote smoking practices[16, 45]. The methylation dataset for PA-SCOPE did not contain data related to current smoking or time since quitting smoking, and could not assess the effects that these might have on our differential methylation results. Longitudinal data was not available in PA-SCOPE, so further conclusions integrating clinical stability, clinical progression, or other lung function trajectories[46] associated with CpG sites cannot be made using these data. Without paired gene expression data, it is unclear what effect these differentially methylated sites have on expression of the associated gene products. While both PA-SCOPE and ICGN were studies of COPD subjects and smoking controls, differences in ascertainment of the datasets may influence the conclusions. Similarly, the comparison of test-statistic differences between ICGN and PA-SCOPE could be influenced by factors other than racial disparities in differential methylation related to COPD. Batch effects between the PA-SCOPE and ICGN assays, differences in ascertainment and study design, and baseline differences in the two populations other than racial make-up could account for the difference in test statistics among these populations, so we present these data points as qualitative and hypothesis-generating. The Illumina Infinium HumanMethylation27 BeadChip Array interrogates only a subset of CpG sites in the human epigenome, and additional unmeasured sites may be differentially methylated in
association with COPD. Future studies including large populations of both AA and NHW would be needed to further validate both the differential methylation results as well as the race-specificity of the results. The recapitulation of many of our differentially methylated genes in network modules strongly associated with COPD provides some biological validation of the importance of these sites to COPD using a different methodology. While further research is needed to investigate the biological consequences of differential methylation of these genes in experimental models, they represent promising candidate genes for mechanistic investigations of the racial disparity in COPD susceptibility and severity.

**Conclusions**

In conclusion, we performed differential methylation analysis in African-American subjects and identified 12 CpG sites statistically significantly associated with COPD at an FDR less than 10%, of which seven appear more relevant for the AA population compared to a similar NHW population. We also performed weighted gene comethylation network analysis and identified two comethylation modules associated with COPD, one of which included multiple genes related to obstructive lung disease and COPD. This module was enriched for lung-specific gene sets and our results add to insights into molecular mechanisms that may partially explain lung disease disparities in African-Americans. These race-specific DNA methylation marks could reflect differences in exposures among AA and NHW that may influence disease mechanisms that affect COPD susceptibility. Molecular mechanisms for racial disparities in COPD-related outcomes have not been adequately described from a genetic and mechanistic standpoint, and this investigation is a step towards better describing and finding treatments for the biological mechanisms underlying these disparities.

**Methods**

**Subjects and Data Collection:** Researchers at Temple University and the Pennsylvania Department of Public Health designed the Pennsylvania Study of Chronic Obstructive Pulmonary Exacerbations (PA-SCOPE, ClinicalTrials.gov Identifier: NCT00774176) as a collaborative observational study to identify demographic and genetic factors that
contributed to COPD exacerbations among AA smokers with COPD in urban and rural Pennsylvania. Subject recruitment and data gathering occurred between June 2004 and May 2008. PA-SCOPE data types for each of 371 AA subjects in the methylation cohort consisted of questionnaire data, spirometry and pulmonary function tests, and DNA from peripheral blood samples. Subjects were male and female AA smokers (>20 pack-years) with and without COPD (defined by FEV1/FVC ≤ 70% or FEV1 ≤ 70% predicted) ages 40-80 years. Race was determined by self-report. We excluded subjects with asthma or chronic respiratory diseases other than COPD. Participants provided written consent to participate in this study, and the study was approved by the institutional review boards at all participating institutions (Partners IRB: 2005P000453/BWH).

We assessed DNA samples from 371 COPD cases and smoking controls for genome wide differential methylation using the Illumina (San Diego, CA) Infinium HumanMethylation27 BeadChip (Illumina27K). The Illumina27K array assays 27,758 CpG dinucleotides[47] for quantitative measurements of DNA methylation, covering over 14,000 genes. We performed the assay using the manufacturer’s suggested protocol, including standard controls for bisulfite conversion, amplification, hybridization, and extension. We report the percent methylation values ranging from 0 to 100% (corresponding to beta value of 0 to 1), calculated as the ratio of the fluorescent intensity of the methylated bead type (meth) to the combined locus intensity of methylated and unmethylated bead types (meth + unmeth) plus an offset (beta = meth/(meth+unmeth + 100)). The log2-ratio of the methylated to unmethylated intensities (M-value) was used for association testing[48]. The absolute difference in percent methylation between cases and controls was used to quantify the effect size.

**Quality Control:** The R programming language[49] and the BioConductor[50] suite of software (packages included methylumi[51], GenomicRanges[52], and wateRmelon[53]) were used for data annotation, probe quality control and pruning, and subject-level quality control. The Illumina27K probes were annotated to their nearest gene using hg19 coordinates and investigated for potential sources of probe bias. To eliminate bias due to SNPs underlying probe regions, probes containing a CpG within 5 base pairs upstream or downstream of a known genomic SNP were eliminated. Probes underlying genomic repeat regions were eliminated. Probes interrogating the sex chromosomes
were also eliminated. Subject level quality control eliminated one sample having 1% of sites with a detection p-value greater than 0.05. CpG sites with a beadcount of less than 3 in greater than 5% of samples, and sites having greater than 1% of samples with a detection p-value greater than 0.05 were also removed; 8 subjects’ data were incomplete and were removed for the final analysis.

**Cell Type Deconvolution:** Differential methylation signals arising primarily from cell type composition of whole blood can bias methylation analysis. To control for this bias, we performed cell type deconvolution using the method and software provided by Houseman et al[54].

**Differential DNA Methylation Analysis:** Differential methylation analysis was performed on 362 samples after quality control using the limma package[55]. For the COPD analysis, associations between differentially methylated probes and COPD case-control status were modeled in a logistic mixed model controlling for age, sex, pack-years of smoking, batch number, and cell type deconvolution (covariates accounting for natural killer cells, CD8 T-cells, CD4 T-cells, B-cells, and monocytes). Family-wise type I error was controlled using the method of Benjamini-Hochberg to achieve a genome-wide false discovery rate (FDR) threshold of significance of less than 5%. Additionally, we examined the results that approached statistical significance at less than FDR 10% level for assessment of additional biologically-plausible targets. The absolute difference in the mean percent methylation value was used to quantify the magnitude and direction of effect for differentially methylated CpG sites; positive values of delta beta correspond to relative hypermethylation among the cases.

**Comparison to NHW Dataset:** An analysis of differential DNA methylation sites associated with COPD was performed previously by our group using NHW subjects in the International COPD Genetic Network (ICGN)[15]. Whole blood samples were assayed on the Illumina27k array. This dataset included 692 COPD NHW cases by GOLD spirometry criteria, as well as 437 NHW controls. We passed these data through annotation, quality control, probe control, cell type deconvolution, and differential DNA methylation analysis steps that were identical to those used for the PA-SCOPE dataset. We hypothesized that race-specific disparities in differential methylation of genes may reflect biological
disparities in disease mechanisms or disease-relevant exposures. We used this ICGN NHW dataset to compare differential methylation values to those found in the PA-SCOPE AA cohort. We compared the differential methylation characteristics of CpG sites between AAs and NHWs qualitatively in order to identify sites with strong differential methylation in AA without corresponding differential methylation in NHW. In order to identify those sites at which the difference in differential methylation was qualitatively greatest between AAs and NHWs, we calculated the difference in test statistic (retaining direction of effect) for each CpG site between the PA-SCOPE AA analysis and the ICGN NHW analysis.

**Weighted Gene Comethylation Network Analysis:** Weighted gene comethylation networks were constructed using the quality controlled PA-SCOPE dataset as input to the WGCNA R-package by Langfelder and Horvath[56], and network modules were analyzed for association with COPD in a signed correlation network. Scale-free properties were achieved with a soft thresholding value of 12, resulting in ten modules. Analysis of the eigengenes of significantly COPD-associated modules (measured as the correlation of the gene's methylation profile with the module eigenvector) was performed to evaluate driver genes for the module (genes whose methylation is most highly correlated to the eigengene methylation). Gene significance is quantified statistically by the Student’s t-test statistic for differential methylation between COPD cases and controls, and larger values of gene significance indicate more significant p-values for the gene’s significance to COPD. Results were inspected for genes in the differential methylation top results, as well as for known obstructive lung disease associations from GWAS studies of lung function[57-59] and COPD[60-65]. For each significantly associated eigengene, logistic regression models of COPD case-control status were constructed using the module eigengenes and clinical covariates of age, gender, and pack-years of smoking history to evaluate the robustness of the eigengene association to COPD. COPD-associated WGCNA modules were limited to genes with a stringent module membership (kME value) cutoff of >0.85, as per previously published methods[20]. Gene set enrichment analysis on these limited modules was performed using ConsensusPathDB[66] to compare the COPD-associated module genes to evaluate for enrichment in the Gene Ontology (GO) Biological Processes ontology, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Reactome pathways using hypergeometric testing controlled for multiple testing using the false discovery rate method applied to the number of included genes.
Declarations

List of Abbreviations:

AA - African-American
COPD - Chronic obstructive pulmonary disease
CpG - Cytosine phosphate-bond guanine dinucleotide
FEV1 - Forced expiratory volume in one second
FVC - Forced vital capacity
GO - Gene Ontology
ICGN - International COPD Genetics Network
KEGG - Kyoto Encyclopedia of Genes and Genomes
mQTL - Methylation quantitative trait loci
NHW - non-Hispanic White
PA-SCOPE - Pennsylvania Study of Chronic Obstructive Pulmonary Exacerbations
PYH - Pack-year history of smoking
WGCNA - Weighted gene comethylation network analysis

Ethics Approval and Consent to Participate:

Participants provided written consent to participate in this study, and the study was approved by the institutional review boards at all participating institutions (Partners IRB: 2005P000453/BWH).

Consent for Publication:

Not Applicable

Availability of Data and Material:

The PA-SCOPE dataset is not currently available on dbGaP or related public data institutions.
**Competing Interests:**
Robert Busch, Weiliang Qiu, Jarrett Morrow, Jessica Lasky-Su, Gerard Criner, and Dawn DeMeo declare that they have no conflicts of interest or competing interests related to the content of this manuscript.

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**Authors' Contributions:**
All of the authors listed have contributed sufficiently to the project to be included as authors by ICMJE guidelines, and all those who are qualified to be authors are listed in the author byline. We have included statements on funding sources and acknowledgements of the study teams for each included study. The authors have approved the manuscript and its submission, and agreed to be accountable for the content of the work.

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**Prior Publication Declaration:**

This manuscript is original, has not been published previously, and is not under consideration at any other journal. By BMC editorial policies, limited findings from this paper have been published as abstracts/posters at the ATS and ASHG academic meetings:

Busch R, Qiu W, Criner G, DeMeo DL. Race-Specific Differential DNA Methylation Marks of COPD; Abstract #444/F. Presented as a Poster at the 65th Annual Meeting of The American Society of Human Genetics Annual Meeting, October 9, 2015, Baltimore, MD.

References:


34. Smith AK, Kilaru V, Kocak M, Almli LM, Mercer KB, Ressler KJ, Tylavsky FA, Conneely KN: Methylation quantitative trait loci (meQTLs) are consistently detected across ancestry, developmental stage, and tissue type. BMC Genomics 2014, 15:145.


Illustrations and Figures:

Figure 1: Subject- and Probe-Level Quality Control Chart. Quality control of the PA-SCOPE methylation dataset included Probe-level controls and Subject-level quality controls (see Methods for details). Final analysis included 93 COPD cases and 269 smoking controls.
Differentially Methylated CpG Probes Associated with COPD

Figure 2: Differentially Methylated CpG Probes Associated with COPD. Differential methylation analysis revealed 12 CpG sites in 12 genes significantly associated with COPD with an FDR-corrected p-value less than 0.10. Difference in mean percent methylation represents the difference in mean methylation between COPD cases and smoking controls. The y-axis represents the negative log of the association p-value from linear mixed models adjusted for age, pack years of smoking, assay batch, and cell type. The name of the nearest gene is included with each of the top five CpG results.
**Figure 3: Manhattan Plot of Differential Methylation Analysis Results.** Differential methylation analysis results presented by chromosomal location (x-axis). The y-axis represents the negative log of the association p-value from linear mixed models adjusted for age, pack years of smoking, assay batch, and cell type. The name of the nearest gene is included with each of the top five CpG results.
Figure 4: WCGNA Module Trait Relationship Heatmap: Heatmap showing comethylation module correlation with phenotypic trait and associated p-value for these correlations within PA-SCOPE. Positive or negative correlation magnitude with COPD is presented with p-value for the correlation with COPD in parenthesis. The yellow and blue modules were both significantly associated with COPD affection status, labeled "COPD".
### Tables and Captions:

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Controls</th>
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</thead>
<tbody>
<tr>
<td>N=</td>
<td>93</td>
<td>269</td>
</tr>
<tr>
<td>Age (years)</td>
<td>61.4 ± 7.7</td>
<td>48.9 ± 6.4</td>
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<tr>
<td>FEV1/FVC Ratio</td>
<td>0.434 ± 0.123</td>
<td>0.799 ± 0.053</td>
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<tr>
<td>FEV1 percent predicted</td>
<td>36.8 ± 15.4</td>
<td>89.9 ± 12.5</td>
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<tr>
<td>FVC percent predicted</td>
<td>67.5 ± 22.4</td>
<td>90.7 ± 12.3</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>50 (53.8)</td>
<td>195 (72.5)</td>
</tr>
<tr>
<td>Pack-Years of Smoking</td>
<td>42.2 ± 22.1</td>
<td>39.2 ± 20.5</td>
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</table>

**Table 1. Baseline Statistics Among African-Americans in PA-SCOPE.** Data is presented as count with proportion in parentheses or mean with standard deviation. FEV1 is the forced expiratory volume in one second, and FVC is the forced vital capacity.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>CpG Probe</th>
<th>Nearest Gene</th>
<th>Mean Change in Percent Methylation</th>
<th>p-value</th>
<th>FDR Adjusted p-value</th>
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<tbody>
<tr>
<td>5</td>
<td>cg16361890</td>
<td>MAML1</td>
<td>-7.8</td>
<td>8.19E-08</td>
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<td>22</td>
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<td>1.20E-07</td>
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<td>9</td>
<td>cg12971694</td>
<td>CD72</td>
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<td>12</td>
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<td>GRASP</td>
<td>-7.8</td>
<td>7.27E-06</td>
<td>0.029</td>
</tr>
<tr>
<td>4</td>
<td>cg02635407</td>
<td>SH3TC1</td>
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<tr>
<td>11</td>
<td>cg25634666</td>
<td>FOLR3</td>
<td>-10.8</td>
<td>3.23E-05</td>
<td>0.080</td>
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<tr>
<td>10</td>
<td>cg18390025</td>
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<td>19</td>
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</table>

**Table 2. Differentially Methylated CpG Probes Associated with COPD Among African-Americans in PA-SCOPE.** Data are presented in decreasing order of significance. Mean change in percent methylation represents the mean difference in percent methylation between cases and controls; p-values were adjusted for multiple comparisons using the method of Benjamini and Hochberg.
<table>
<thead>
<tr>
<th>CpG site</th>
<th>Nearest Gene</th>
<th>Gene Significance to COPD</th>
<th>Gene Significance p-value</th>
<th>Module Membership to Blue Module</th>
<th>Module Membership p-value</th>
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<td>4.24E-01</td>
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<td>3.32E-39</td>
</tr>
<tr>
<td>cg21750589</td>
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<td>-0.04</td>
<td>4.95E-01</td>
<td>0.26</td>
<td>4.78E-07</td>
</tr>
</tbody>
</table>

Table 3: COPD-related Genes from WGCNA blue comethylation module. Summary of "gene significance to phenotype" values and "blue module membership" values of genes previously associated with COPD and lung function through GWAS, as well as differentially methylated sites discovered in the differential methylation analysis.
Supplemental Data:
Race-Specific Differential DNA Methylation Marks and Gene Comethylation Modules of COPD

Authors: Robert Busch, MD¹, Weiliang Qiu, PhD¹, Jessica Lasky-Su, PhD¹, Jarrett Morrow, PhD¹, Gerard Criner, MD², and Dawn DeMeo, MD MPH¹

Affiliations: ¹Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital, Boston, MA/USA; ²Temple Lung Center, Temple University Health System, Philadelphia, PA/USA

Supplemental Figures:

**Supplemental Figure 1: Comparison to NHW Dataset.** For each CpG site, test statistics were compared between the PA-SCOPE and ICGN datasets. The difference in test statistic was normally distributed over the 19302 probes analyzed. Notably, many of the CpG sites found to be differentially methylated in association with COPD in PA-SCOPE were in the lower tail of this distribution.
Supplemental Figure 2: Network Topology Heatmap. Heatmap representation of the topological overlap-based dissimilarity matrix calculated from PA-SCOPE weighted gene comethylation network analysis. Hierarchical clustering was used to construct clustering dendrograms to separate CpG sites into modules based on comethylation correlation. Module assignment colors based on clustering are represented along the x- and y-axis.
Supplemental Figure 3: Differential Methylation Boxplots. Boxplots showing the mean beta (absolute methylation difference ranging from 0 to 1, corresponding to 0 to 100% methylated) for COPD cases and controls for each of the twelve statistically significantly differentially methylated CpG sites reported in the Results section. These boxplots do not account for covariate adjustment.