Methods

Data Collection

Some demographic information was collected via questionnaires from the Agricultural Health Study (AHS) (e.g., sex, smoking history, site [i.e., Iowa, North Carolina]). In the Agricultural Lung Health Study (ALHS), field technicians visited individuals' home to collect blood samples and measure height and weight to calculate body mass index (kg/m2). A computer-assisted telephone interview (CATI) was conducted after the home visit. The CATI collected information on oral corticosteroid use, asthma control, as well as smoking history, which was used to update their smoking status (never, former, current) and packyear from the AHS. Date of enrollment was used to categorize participants to a season of enrollment: spring (enrolled March 21 to June 20), summer (enrolled June 21 to September 20), fall (enrolled September 21 to December 21), or winter (enrolled December 22 to March 20).

Asthma Control

Individuals with asthma were asked to complete the Asthma Control Questionnaire (ACQ), a six-item questionnaire about their asthma severity during the past two weeks (frequency of night-time waking, symptoms of night-time waking, activity limitation, shortness of breath, wheezing, and use of bronchodilator) (1). Individuals provided responses on a 7-point scale from 0 (no impairment) to 6 (maximum impairment). Pre-bronchodilator percent predicted FEV1 collected by the field technicians during the home visit was also categorized on a 7-point scale from 0 to 6. For those who responded to the six questions and had a percent predicted FEV1, a mean ACQ score was calculated. Individuals with an ACQ score < 1.5 were considered to have controlled asthma, whereas individuals with an ACQ score ≥ 1.5 were considered to have uncontrolled asthma.

Asthma Polygenic Risk Score

A weighted polygenic risk score was calculated using the results from the multi-ancestry, fixed-effects model from the largest genome-wide meta-analysis of asthma (2). SNPs with p-values $<5\times10\text{-}8$ were clumped using PLINK ($r2 = 0.5$) to identify independent loci within ±250 kb (3). 98 SNPs remained after clumping to calculate the polygenic risk score.

Post-hoc Analyses of Differentially Methylated CpGs

Additional analyses were conducted for FDR significant CpG sites. Because sex may contribute to the pathogenesis of asthma, we repeated the analyses and added an interaction term between sex and the methylation value at each differentially methylated CpG site. To examine if methylation differs by use of oral inhaled corticosteroid (yes versus no), we conducted case-only analyses, modeling use of oral inhaled corticosteroid as the outcome and adjusted for the covariates used in the main model. Similar analyses were conducted with asthma control (uncontrolled versus controlled) as the outcome.

Gene Annotation

Because Illumina does not update the annotation in their manifest file, CpG sites were mapped to genes using HOMER v4.9.1 and the human genome database (hg19, v5.10) provided by HOMER (4). Each CpG site was mapped to the closest transcription start site (TSS) based on the RefSeq annotation, using the "annotatePeaks.pl" script with default setting. CpG sites within 100 kb from the TSS were annotated to the gene from the Gene Symbol column.

Enrichment of functional genomic features

CpG sites were localized to four genomic features: CpG islands, CpG island shores, promoters, and transcription factor binding sites. The genomic locations for CpG islands were obtained from the CpG islands track in the regulation section of the University of California, Santa Cruz (UCSC) Genome Browser. CpG island shores were defined as 2 kb regions adjacent to the CpG islands. Promoters were defined as the regions 1 kb upstream and downstream of all RefSeq protein-coding genes.

We obtained the transcription factor binding sites from the transcription factor ChIP track within the ENCODE regulation super-track in the regulation section of the UCSC Genome Browser, based on Factorbook Motifs and

ENCODE data for 161 factors in 91 cell types. Enrichment was tested using a two-sided Fisher's exact test. A genomic feature was considered significant using a Bonferroni threshold ($p<0.0125$). An enrichment score was calculated as the log2 ratio of the percentage of significant CpG sites in a feature over the percentage of total CpG sites in the feature.

Using the summary mode option in eFORGE TF, we uploaded the FDR significant CpG sites (Sample = fLung) to identify enriched transcription factor motifs (FDR<0.05). Because eFORGE TF reads a maximum of 1,000 probes, the top 1,000 significant CpG sites were uploaded if the epigenome-wide analyses identified over 1,000 FDR significant CpG sites.

In eFORGE v2.0, data were analyzed from the Consolidated Roadmap Epigenomics consortium.

Expression Quantitative Trait Methylation (eQTM)

Because studies with methylation from the EPIC array and gene expression data are currently unavailable, eQTMs were conducted for differentially methylated CpG sites that are in the 450K array. Methylation from the Illumina 450K array and gene expression data were available in 3,075 samples in the Biobank-based integrative omics study (BIOS) consortium from the following cohorts: Leiden Longevity Study, LifeLines Study, Rotterdam Study, and Netherland Twin Study (5). cis-eQTMs were conducted for gene expression transcripts within ±250 kb of each CpG site (168 CpGs for non-atopic asthma, 346 CpGs for atopic asthma). In each cohort, the gene expression was regressed on methylation M value (log2 ratio of methylated versus unmethylated probe intensities), using the limma package in R. Analyses were adjusted for age, sex, lymphocyte proportion, monocyte proportion, and RNA flow cell number. Model inflation was corrected using the "bacon" method (6). The results from each cohort were meta-analyzed using an inverse variance-weighted fixed-effects model in METAL (7).

Identify Genes Implicated with Asthma in Previous EWAS or GWAS

Reese et al. performed a literature review up to January 12, 2018 to identify genes that have been reported to be differentially methylated in relation to asthma (8). A list of these genes is available in a supplemental table [Appendix, Table E8]. Their list also includes genes from a large GWAS of asthma.

We updated Reese et al.'s list of genes by running their PubMed search to identify studies published between January 12, 2018 to August 9, 2019. The following search terms were used:

((((("Asthma"[Majr])) OR (((((airways hyper responsiveness[Title/Abstract]) OR airway reactivity[Title/Abstract]) OR bronchodilator response[Title/Abstract]) OR asthma[Title/Abstract]) OR wheez*[Title/Abstract] OR FENO[Title/Abstract]))) AND ((((("Methylation"[Majr]) OR "DNA Methylation"[Majr])) OR ((methylation[Title/Abstract]) OR DNA methylation[Title/Abstract])))

We included the reported asthma genes downloaded from the GWAS Catalog on October 11, 2019.

Search for Druggable Targets

Genes that had not been previous been associated with asthma (i.e., novel genes) were annotated to the ChEMBL database (v25, released on January 2, 2019) to identify approved drugs or drugs in development that target the gene (9).

Replication of Findings in Other Studies

Chicago

This study recruited asthmatic and non-asthmatic adults (mean age 39 and 38 years, respectively) undergoing bronchoscopy between March 2010 and March 2014 at the University of Chicago (10). Asthma cases had to have a current doctor's diagnosis of asthma, no conflicting pulmonary diagnoses, and were using asthma medications. Controls did not have current or previous asthma diagnosis and had normal spirometry and methacholine challenge tests.

Methylation was measured in endobronchial brushings obtained during bronchoscopy, using the Infinium HumanMethylation 450K BeadChip. Probes meeting the following criteria were excluded: 1) probes located on the sex chromosome, 2) probes with a detection p-value >0.01 in 75% of samples, 3) probes mapping to more than one location in a bisulfite-converted genome, or 4) probes overlapping with location of known SNPs. Methylation data were processed using minfi (11). Probe-type bias was corrected using the SWAN method (12). Chip-level effects were regressed out using COMBAT (13). Residual methylation β values were used in the analyses.

Analyses were conducted using limma, adjusting for gender, age, current smoker (yes/no), and ethnicity (African-American, European-American, other). There were 74 asthmatics and 41 non-asthmatics with methylation data. The analyses for have been published (10).

Epigenetic Variation and Childhood Asthma in Puerto Ricans (EVA-PR)

EVA-PR is a case-control study of subjects aged 9-20 years from San Juan, Puerto Rico (14). Cases were considered to have asthma if they had physician-diagnosed asthma and at least 1 episode of wheeze in the previous year. Methylation was measured from nasal epithelial samples. Serum allergen-specific IgE was measured for 5 common aeroallergens in Puerto Rico: house dust mite, cockroach, cat dander, dog dander, and mouse urinary protein. Participants were considered to have atopy if they had at least one specific IgE ≥0.35 IU/mL. In their analyses, EVA-PR had 67 non-atopic asthmatics and 169 atopic asthmatics. The referent group was non-atopic, non-asthmatic controls (n=104).

Methylation was measured using the HumanMethylation 450K BeadChip. DNA was bisulfite-converted using EZ-96 Bisulfite DNA Clean-up Kit (Zymo Research, Orange, CA). Methylation data was processed using minifi (11). We removed samples with low detection values (>10 CpG sites with detection p-value >0.01). The β-values were calculated for each CpG site. ENmix was used for background correction and normalization (15). The following criteria were used to exclude CpG probes: probes with a multimodal distribution, cross-reactive and SNP-containing probes, probes in the sex chromosome, low-quality probes (>10% samples with detection p-value >0.01), probes with a mean β-value <0.1 or >0.9 or extreme β-values in >80% samples.

Methylation was modeled using M values ($M = log2(\frac{\beta}{1-\beta})$). The following logistic regression models were used for their analyses.

Atopic asthma = methylation + age + sex + latent factors + A
Non-atopic asthma = methylation + age + sex + latent factors + A

Where:

- latent factors are from the surrogate variable analysis (sva package) to capture unknown data heterogeneity
- A = top 5 principal components from genotype data

The results from the atopic asthma model have been previously published (14). The same model was applied for non-atopic asthma.

Inner-City Asthma Consortium (ICAC)

The ICAC recruited children ages 10-12 years across 6 sites from which census tracts contained ≥20% of households at less than US government poverty level. Methylation from nasal brushings were measured from samples with ≥80% ciliated epithelial cells.

Methylation measured in 36 cases with asthma and with atopy and 36 controls (i.e., no asthma and no atopy), using the HumanMethylation 450K BeadChip. Methylation data were processed using minfi (11) and normalized using SWAN (12). Probes with localized to known SNPs in European and African populations were excluded (16). Normalized M values were used in the linear regression model, using limma.

Methylation = asthma + age + sex + race/ethnicity + technical variables + batch effects

The results of this study have previously been published (17).

Pregnancy and Childhood Epigenetic (PACE) Consortium

The PACE consortium conducted a meta-analysis of childhood asthma in relation to DNA methylation in blood from children ages 7-17 years. The meta-analysis consisted of 6 studies (BAMSE EpiGene, BAMSE MeDALL, CHOP, GALA II, ICAC, NFBC 1986, PIAMA, Raine study, and STOPPA). A total of 631 asthmatic cases and 2,862 non-cases were analyzed. Studies did not differentiate by atopy status. All studies used untransformed β values for the methylation values. Generally, the cohorts used the following logistic regression model:

Childhood asthma = methylation + maternal age + sustained maternal smoking during pregnancy + maternal asthma + maternal socioeconomic status + child's sex + 7 cell type proportions (monocyte, NK, B cell, CD4, CD8, eosinophil, neutrophil)

Cohorts adjusted for batch effects using ComBat, sva, or including a batch variable in their models. Replication look-up was performed using the results of this PACE meta-analysis paper (8).

Prevention and Incidence of Asthma and Mite Allergy (PIAMA)

PIAMA is a birth cohort of children born in 1996-1997 in the Netherlands (18). Details of the cohort have been published previously (18). The recruitment took place in prenatal clinics. In total, 10,232 pregnant women completed a validated screening questionnaire at their prenatal health care clinic (n=52 clinics). Based on this screening, 7,862 women were invited to participate, of whom 4,146 women agreed and gave informed consent. The study started with 3,963 newborns. Questionnaire based follow-up of the children took place at 3 months of age, annually from 1 to 8 years of age, and at 12, 14, and 16 or 17 years of age. The Medical Ethical Committees of the participating institutes approved the study, and all participants gave written informed consent. Nasal epithelial cells were collected at age 16 years.

Children were considered to have asthma if they had ever been diagnosed with asthma and either wheezed in the last 12 months or used medication for respiratory or lung problems. Serum specific IgE were measured for the following aeroallergens: house dust mite, cat, dactylis (grass), and birch. Atopy was defined as having at least one specific IgE ≥0.35 IU/mL.

Methylation was measured using the Infinium HumanMethylation 450K BeadChip. Methylation data were pre-processed using minfi (11). Samples with call rate <99% were removed. We used the 65 SNP probes to check the concordance between paired DNA samples (samples with blood and nasal methylation from the same participant). Paired samples with Pearson correlation <0.90 were excluded. Probes were also excluded for following criteria: probes on the sex chromosomes, probes mapping to multiple loci, 65 SNP probes, or probes containing SNPs at the target CpG sites with a MAF>5%. Background correction and normalization was conducted using DASEN (19). Trimmed M-values were used in the analyses.

The following model was used for the analysis:

Methylation = atopic asthma + age + center of brushing + gender + batch

Project Viva

Project Viva is a prospective cohort study where mothers were recruited between 1999 and 2002 at their first prenatal visit at Atrius Harvard Vanguard Medical Associates. Mothers were eligible to participate if they were fluent in English, gestational age <22 weeks at first prenatal visit, and singleton pregnancy. Nasal swabs were collected at mean age 12.9 years.

Children had current asthma if the mother reported a doctor's diagnosis of asthma since birth on the early teen questionnaire plus report of wheeze or asthma medication in the past year on early teen follow-up. Children without asthma had no asthma diagnosis, no wheeze, and no asthma medication use.

Total IgE was measured using ImmunoCAP assay (Phadia, Uppsala, Sweden) for common indoor allergens (*Dermatophagoides farinae*, cat and dog dander), mold allergens (*Alternaria* or *Aspergillus* species), and outdoor allergens (rye grass, ragweed, oak, and silver birch). Children with any specific IgE level > 0.35 IU/mL were sensitive to environmental allergens.

DNA methylation was measured in DNA extracted from nasal samples, using the Infinium MethylationEPIC BeadChip. Sample plates and chips were randomized to ensure balance by sex, current asthma status, current allergic rhinitis, and race. Methylation data were pre-processed using minfi (11). Samples were excluded for the following criteria: low-quality samples (intensities <10.5), mismatch recorded sex, mixed genotype distributions on the measured SNP probes), and technical duplicates. Probes were excluded if detection p-value was >0.05 for 5% or more of the samples, probes on the sex chromosomes, non-CpG probes, probes with SNPs at the single base extension (minor allele frequency [MAF] ≥ 5%), probes containing an SNP (MAF ≥ 5%), probes with a SNP at the CpG site (MAF ≥ 5%), and cross-reactive probes (20). Data were preprocessed using the functional normalization with 3 PCs from the control probes to adjust for technical variability (21). Probe-type bias was adjusted using RELIC from the ENmix package (22). Sample plate batch effects were adjusted using ComBat (13).

Project Viva ran two separate asthma analyses. One analysis compared 65 asthmatic children to 398 non-asthmatic children. The other analysis compared 36 asthmatic children with environment IgE sensitization to 265 non-asthmatic children without environmental IgE sensitization.

All analyses adjusted for child's race/ethnicity, sex, age at sample collection, BMI z-sore, maternal education, smokers living in the house, sine and cosine of season at sample collection, and cellular heterogeneity (10 PCs from ReFACToR (23)).

The analyses for asthma and atopic asthma were have been published (24). The full results were downloaded from https://figshare.com/articles/The_Nasal_Methylome_as_a_Biomarker_of_Asthma_and_Airway_Inflammation_in_Children/8285612/1.

Saguenay–Lac-Saint-Jean (SLSJ) asthma familial cohort

The families (1394 individuals distributed in 271 families) included in the SLSJ asthma cohort were recruited through probands with documented allergic asthma (25). To be included in the study, a family needs to fulfill these criteria: the two parents must be available for clinical assessment, one parent must be unaffected and all grand-parents must be of French-Canadian origin. Clinical evaluation (measures of lung function: forced expiratory volume in 1 s (FEV_1) and methacholine challenge (PC_{20})), white blood cell counts, skin prick test for allergy and a standardized questionnaire were completed for all individuals. New blood sampling was performed on 24 individuals, 16 individuals with asthma and eight individuals without asthma, in order to isolate eosinophils to measure methylation profile.

Isolation of eosinophils from blood samples

Eosinophils were isolated from 200ml blood samples from 24 samples, a subset of the SLSJ asthma cohort, following protocols already described (26, 27). The first step was to remove platelet-rich plasma by centrifugation and to use dextran to remove the erythrocytes by sedimentation. A lymphocyte separation medium was used to withdraw mononuclear cells by density gradient. After separation of mononuclear and granulocyte cells, hypotonic lysis with water was performed to remove remaining erythrocytes from the granulocyte cells and negative selection with anti-CD16 MicroBeads and magnetic cell sorter allowed to isolate the eosinophils (Miltenyi Biotec, Auburn, CA, USA). DNA were extracted using the DNeasy Blood and Tissue kit following the company's instruction (QIAGEN, Toronto, Canada).

Methylation analysis
Methylation levels were obtained from Illumina Human Methylation 450k BeadChip. Data filtering and normalization were performed with RnBeads package in R. Probes with low signals considering detection p value > 0.01 as threshold and probes located on SNP were first removed from analysis. Normalization was performed using the "swan" method and background subtraction was done using "methylumi.noob" method.

Covariates and statistical model
Logistic regressions were performed in R using glm (family = binomial) with sex as covariate.

asthma ~ methylation + sex

Asthma is defined as: Present or past documented clinical history of asthma and was coded as 0 = unaffected and 1 = affected.

These analyses were conducted for a previous paper (8).

SAPALDIA

The Swiss Cohort Study on Air Pollution and Lung and Heart Diseases in Adults (SAPALDIA) is a prospective cohort study, enrolling participants from eight regions in 1991. Followed-up occurred in 2001-2003 and 2010-2011. A nested case-control study of adult asthma was conducted in participants who were followed-up in 2010-2011.

Cases had self-reported asthma, self-reported age of onset later than 16 years, never reported having chronic obstructive pulmonary disease (COPD), and were non-smokers for at least 10 years before the blood draw and interview. Controls were randomly selected among participants who never reported having asthma, never used asthma medication, never wheezed without a cold in the last 1 months, and never had 3 or more asthma-related symptoms in the last 12 months.

Atopy was defined using skin prick test against nine common inhalant allergens (Phazet, Pharmacia®, Uppsala, Sweden) performed at baseline examination (28). Subjects with positive skin prick response to at least one allergen (cat, mould (*Cladosporium*), timothy grass pollen, Parietaria pollen, house dust mite, mould (*Alternaria*), birch pollen and dog) were classified as atopic.

Height and weight were measured to compute body mass index. Participants reported their highest education level and categorized into low (primary school), middle (secondary/middle school or apprenticeship), or high (college/university). Packyears was calculated from the self-reported number of cigarettes smoked per day and smoking history.

Methylation was measured in DNA from peripheral blood that was collected in 2010-2011, using the Infinium HumanMethylation 450K BeadChip. Each chip had a similar proportion of samples from different centers, case-control status, and confounding factors to minimize batch effects. Methylation data were preprocessed using minfi (11). Samples with sex mismatches and call rate <0.95 were excluded. We used Noob for background correction and dye-bias correction (29). Methylation levels were expressed as β values. β values were set to missing if the detection p-value was $>10^{-16}$. Probes were excluded if they were on the sex chromosome, had a call rate <0.95, hybridized to multiple genomic locations or overlap with known SNPs with a MAF >1%. Probe-type bias was corrected using beta-mixture quantile normalization (BMIQ) (30). Principal component analysis was conducted on the 220 control probes to obtain the first 30 principal components to adjust for batch effects (31).

Houseman estimated cell types were calculated using the Reinius reference panel (32, 33). Analyses were conducted using logistic regression for non-atopic and atopic asthma, separately.

Non-atopic asthma = Methylation + BMI + Age + Sex + Education + Area + Packyear + Benchtime + Bcell + CD4T + CD8T + NK + Mono + Eos + PC1-30

Atopic asthma = Methylation + BMI + Age + Sex + Education + Area + Packyear + Benchtime + Bcell + CD4T + CD8T + NK + Mono + Eos + PC1-30

Figure E1. Workflow of epigenome-wide association study of adult asthma

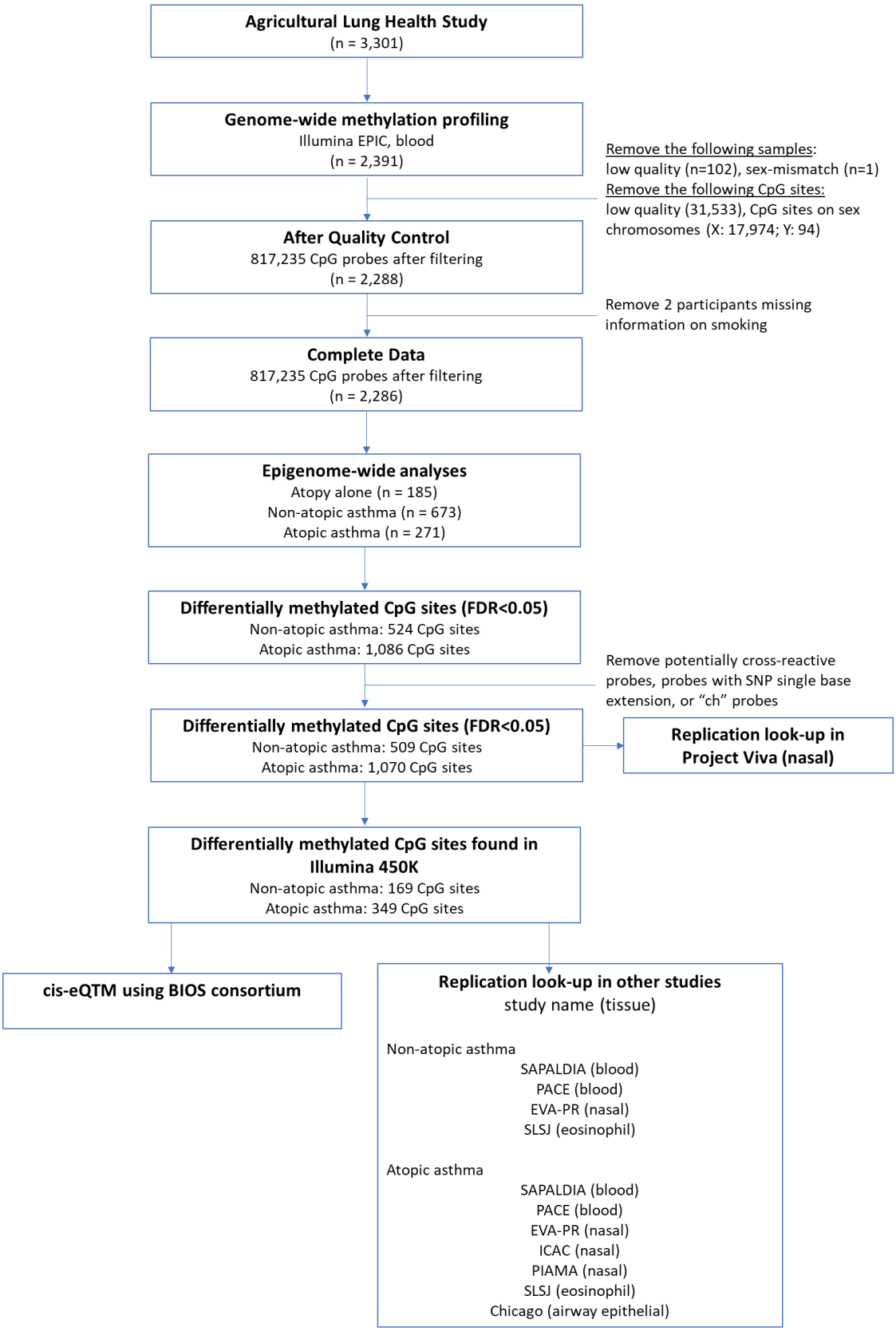
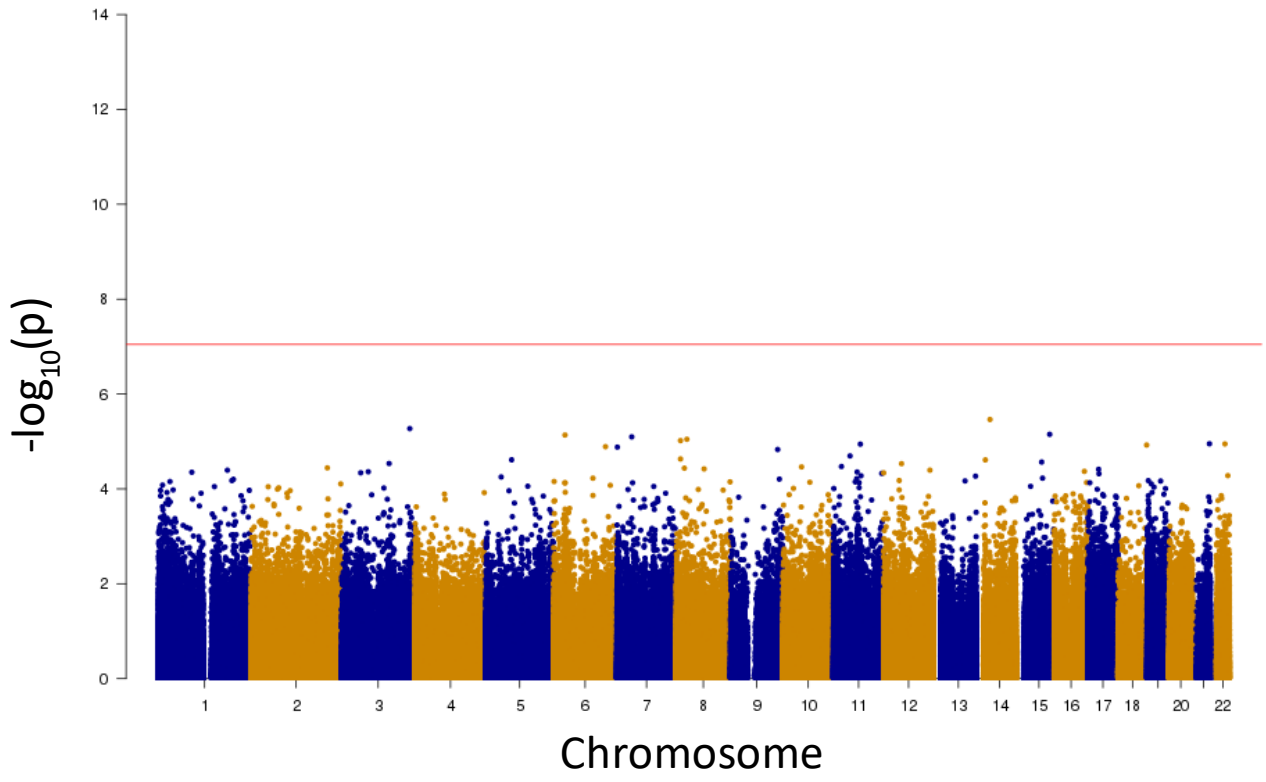


Figure E2. Manhattan plot of epigenome-wide analyses of atopy alone.

(a) Manhattan plot shows that none of the CpG sites were significant using FWER (red line).



(b) QQ-plot showing the observed -$\log_{10}$ p-value versus the expected -$\log_{10}$ p-value for atopy alone. Lambda = 0.97

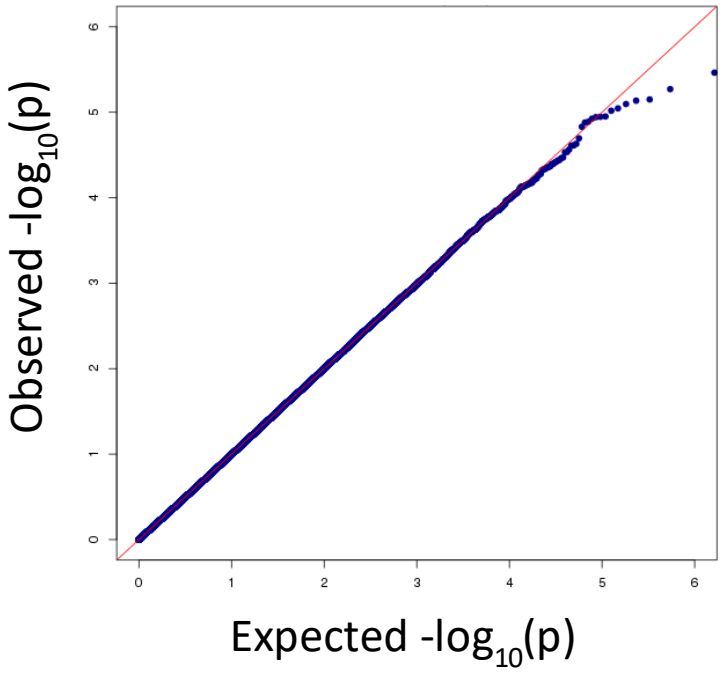
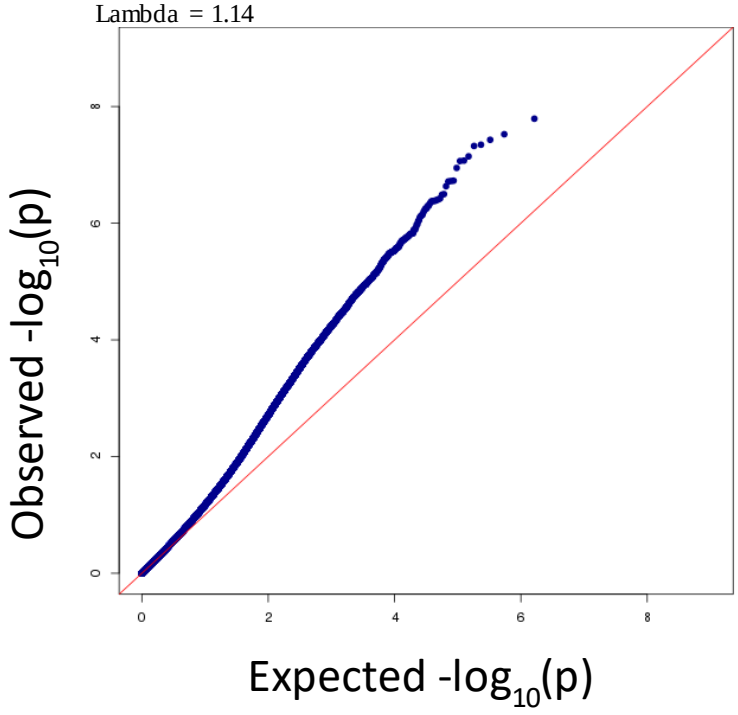


Figure E3. QQ-plot and volcano plot for the epigenome-wide analysis of non-atopic asthma.

(a) QQ-plot of the observed -log10 p-value versus the expected -log10 p-value for non-atopic asthma. Lambda = 1.14



(b) Volcano plot of the observed -log10 p-value versus beta coefficient for non-atopic asthma. Points above the red line are FWER significant. Points above the blue line are FDR significant.

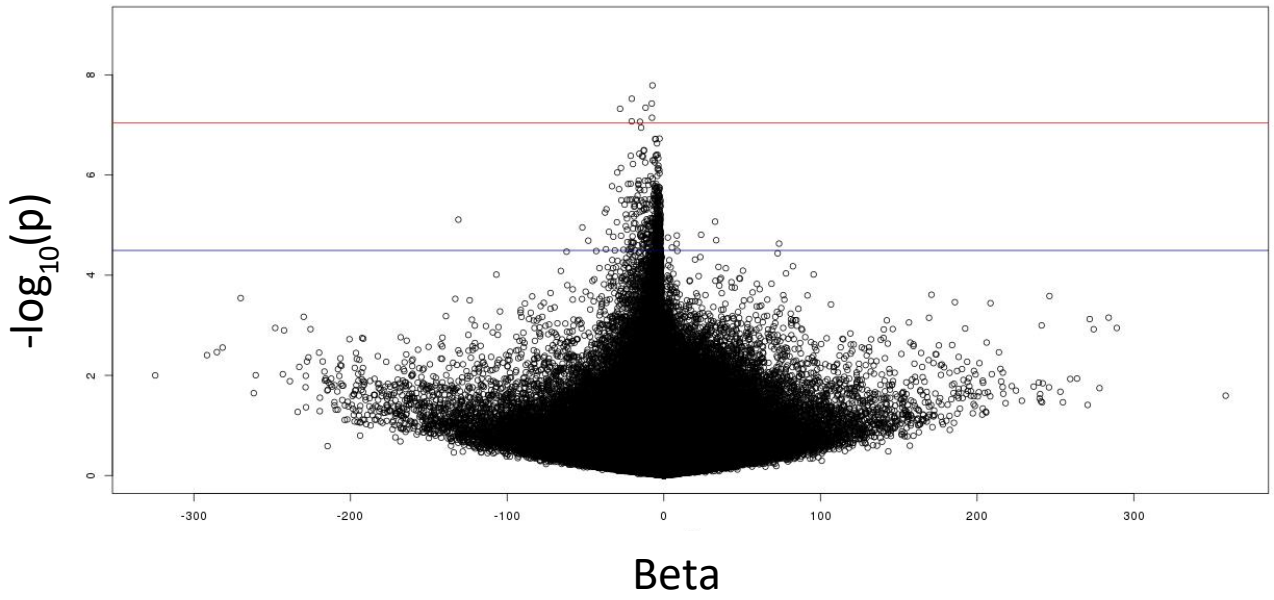
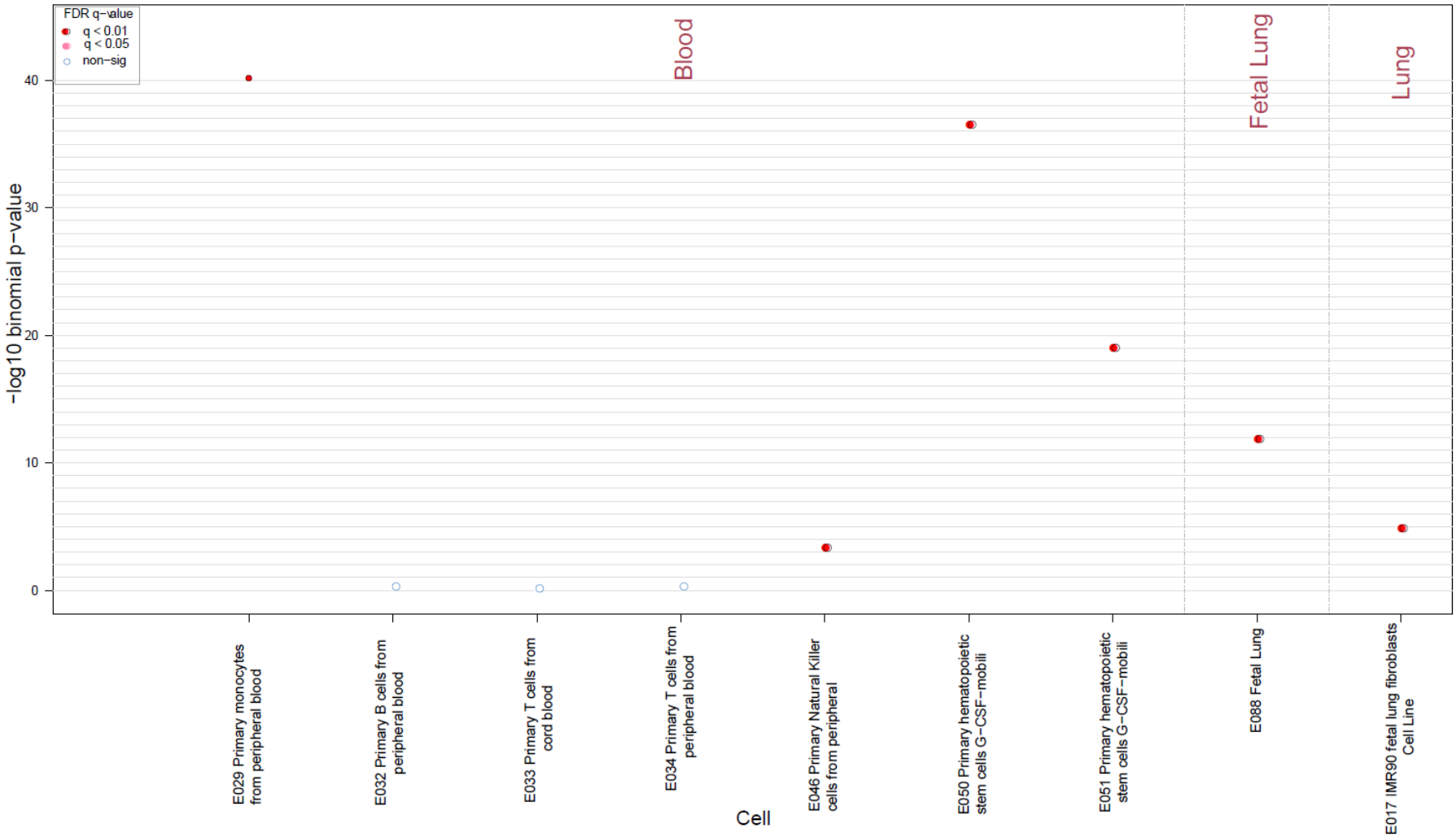
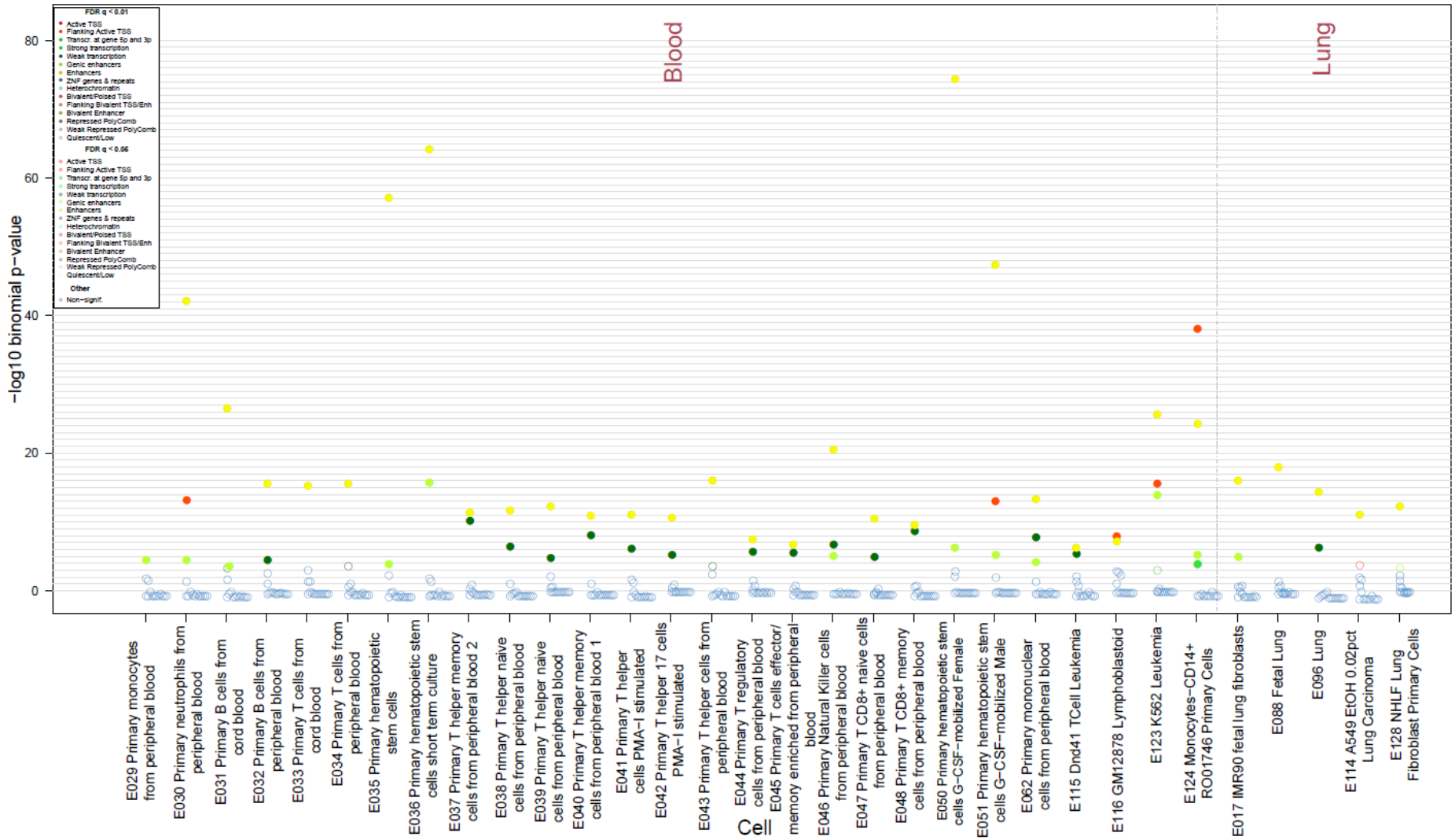


Figure E4. Tissue- and cell-specific enrichment pattern in CpG sites significantly associated (FDR<0.05) with non-atopic asthma, using eFORGE

(a) DNase I hypersensitive sites (probably transcription factor binding sites) enrichment in cell lines from the Roadmap Epigenomics consortium



(b) Chromatin state enrichment in cell lines from the Roadmap Epigenomics consortium



(c) Histone mark enrichment in cell lines from the Roadmap Epigenomics consortium

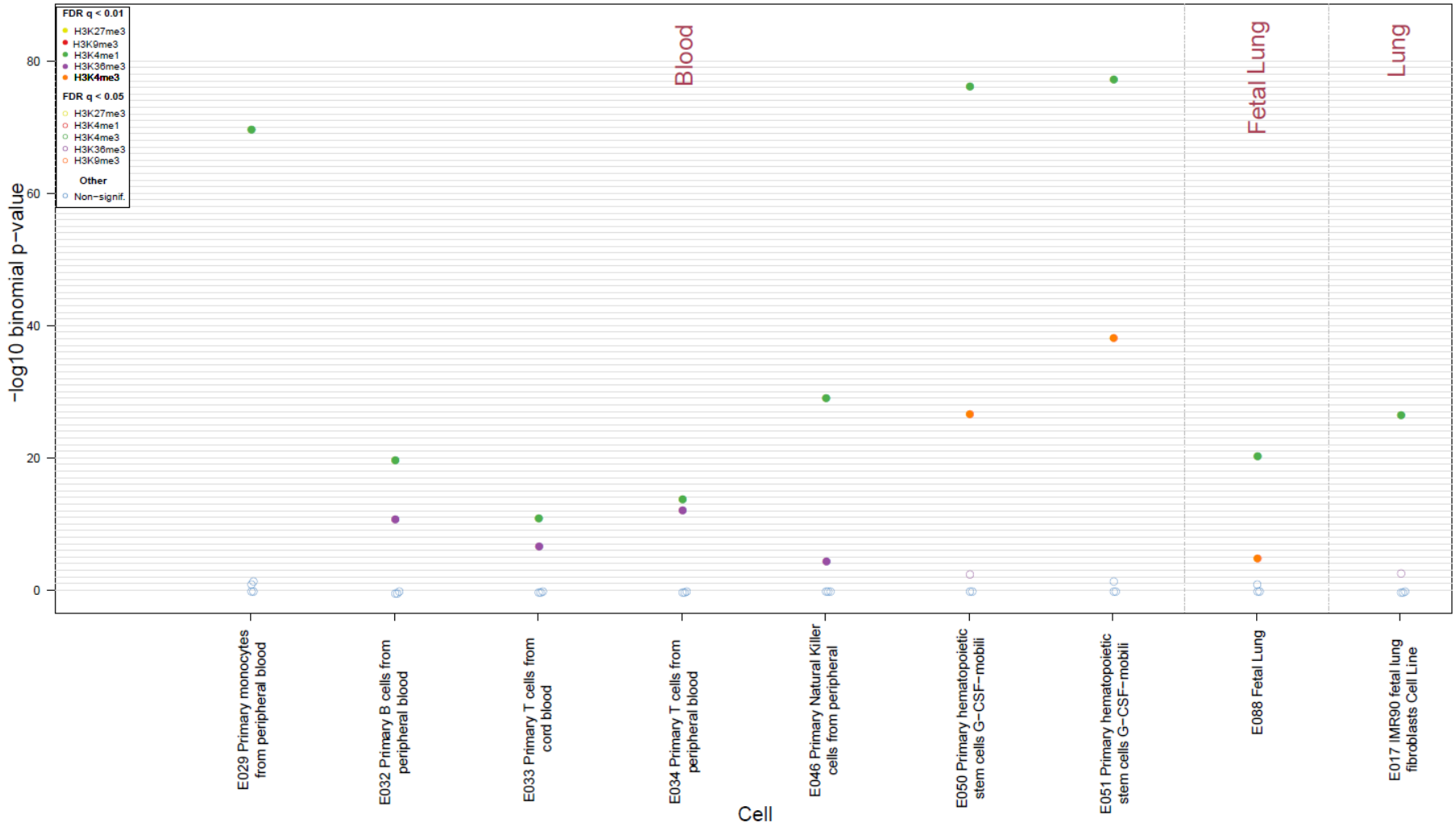


Figure E5. Heatmap of potentially biologically relevant pathways by atopic and non-atopic asthma.

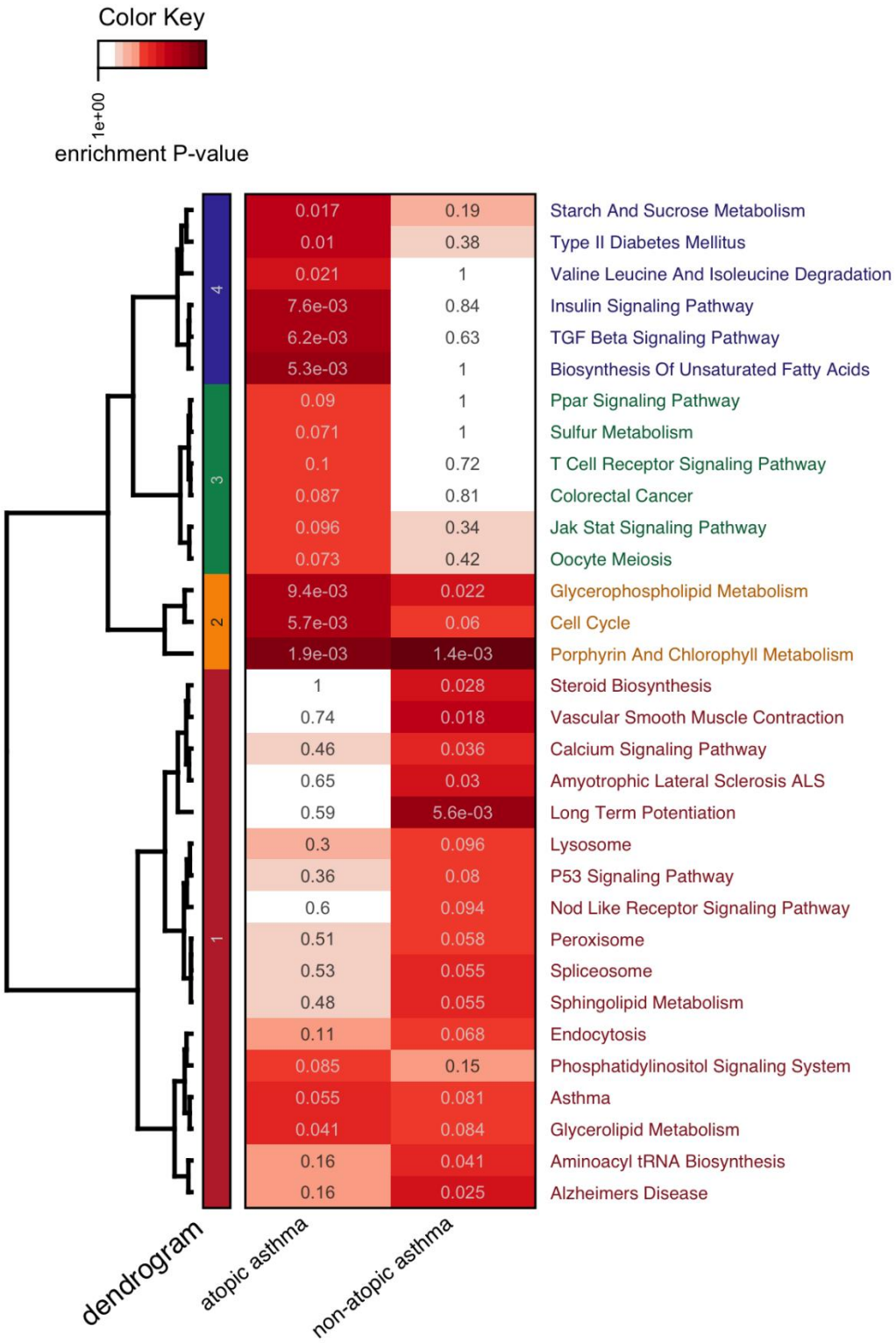


Figure E6. Heatmap of potentially biologically relevant pathways by significant genes and atopic and non-atopic asthma.

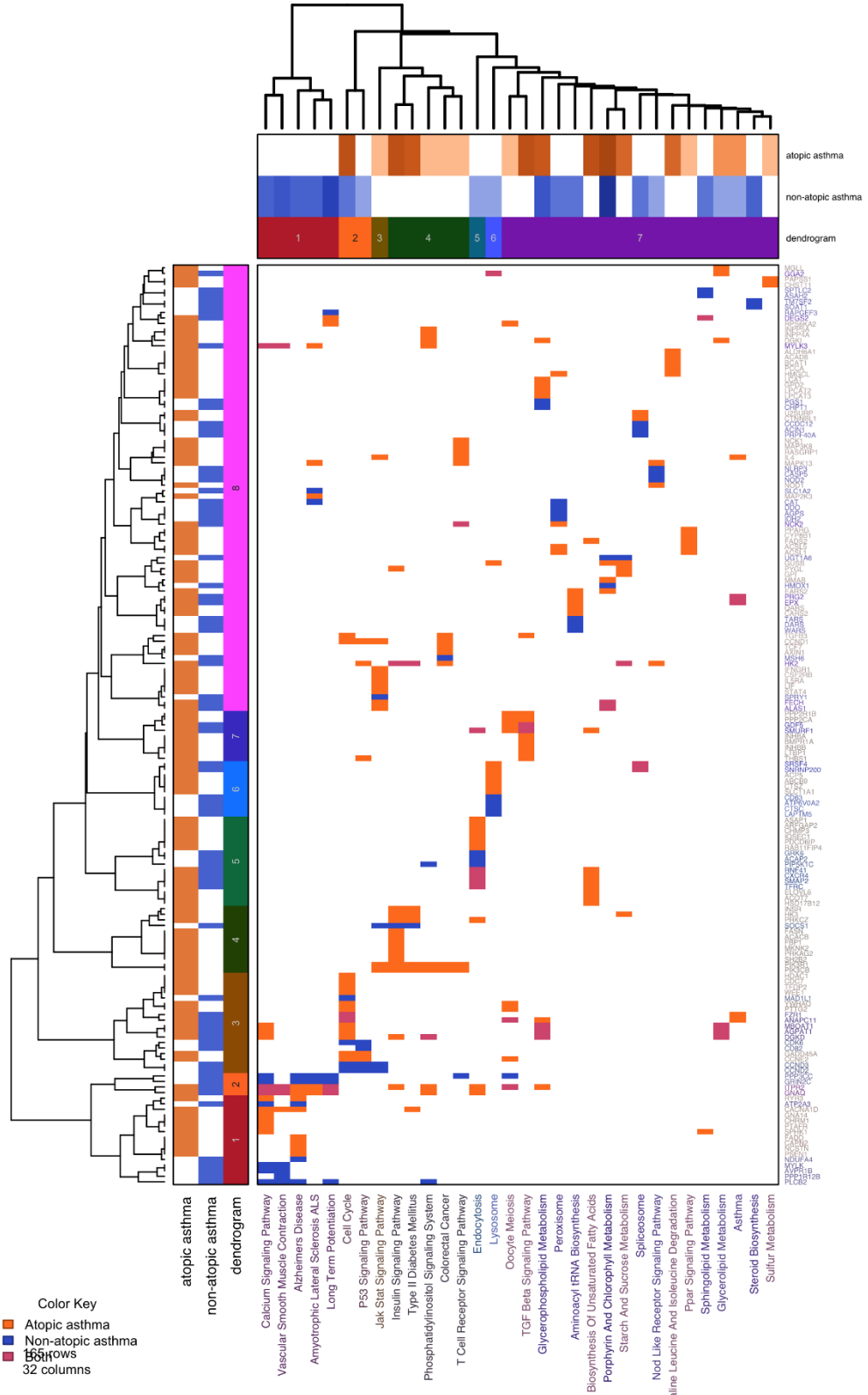
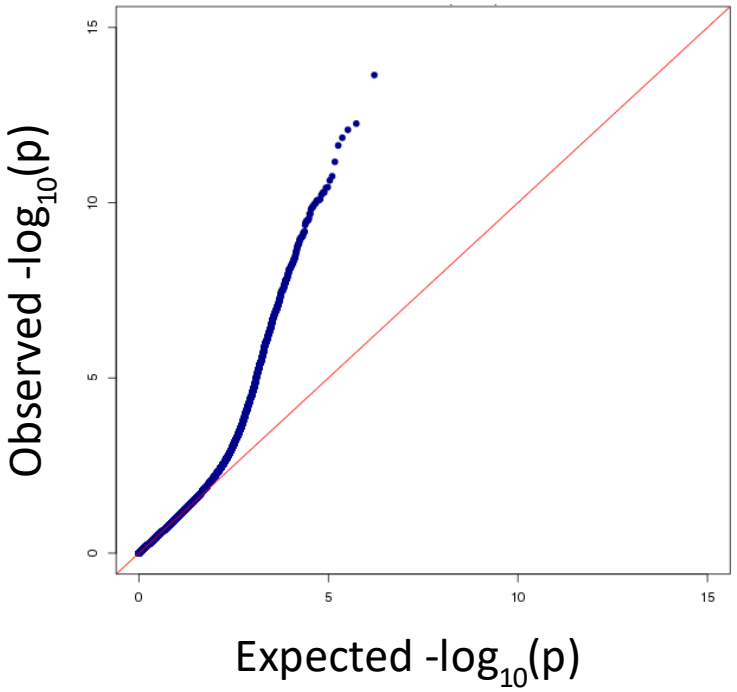


Figure E7. QQ-plot and volcano plot for the epigenome-wide analysis of atopic asthma.

(a) QQ-plot of the observed -log10 p-value versus the expected -log10 p-value in atopic asthma. Lambda = 0.98.



(b) Volcano plot of the observed -log10 p-value versus beta coefficient in atopic asthma. Points above the red line are FWER significant. Points above the blue line are FDR significant.

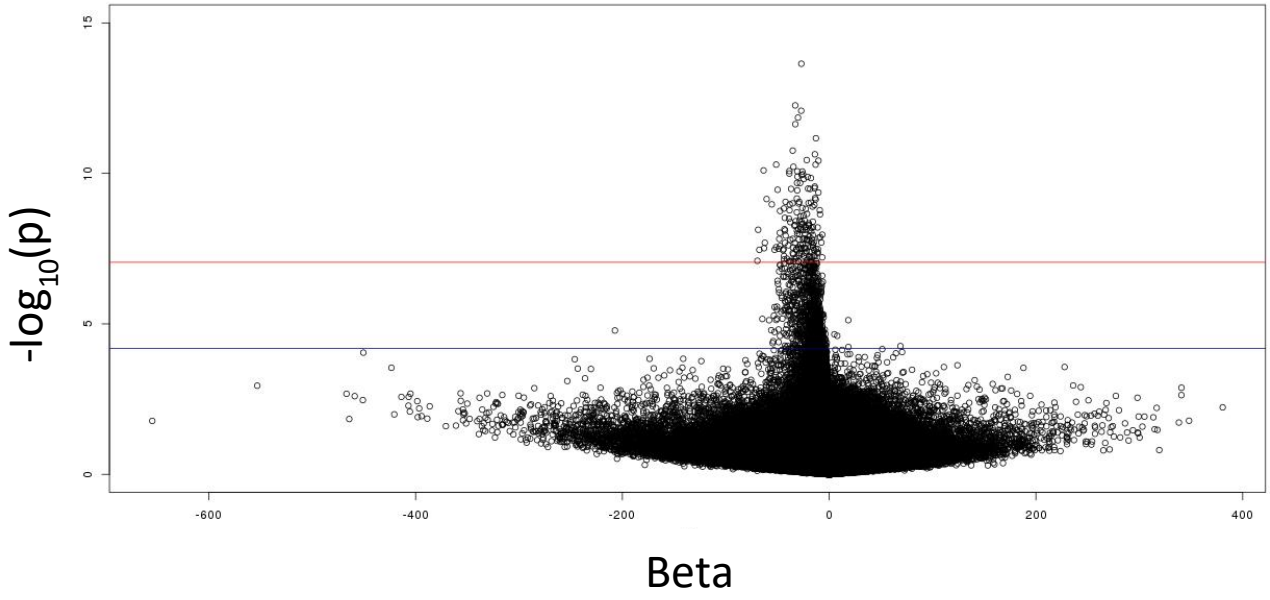
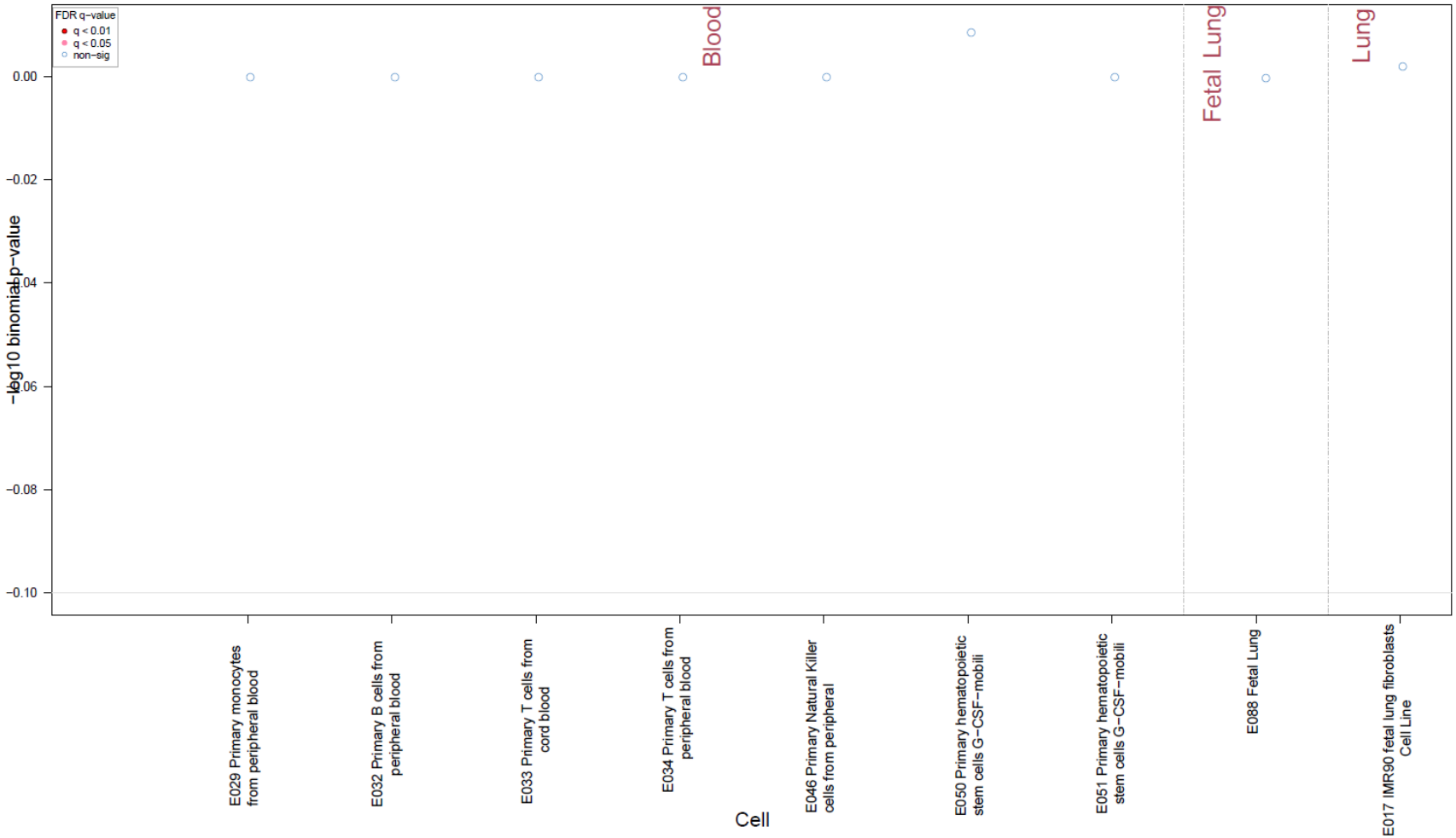
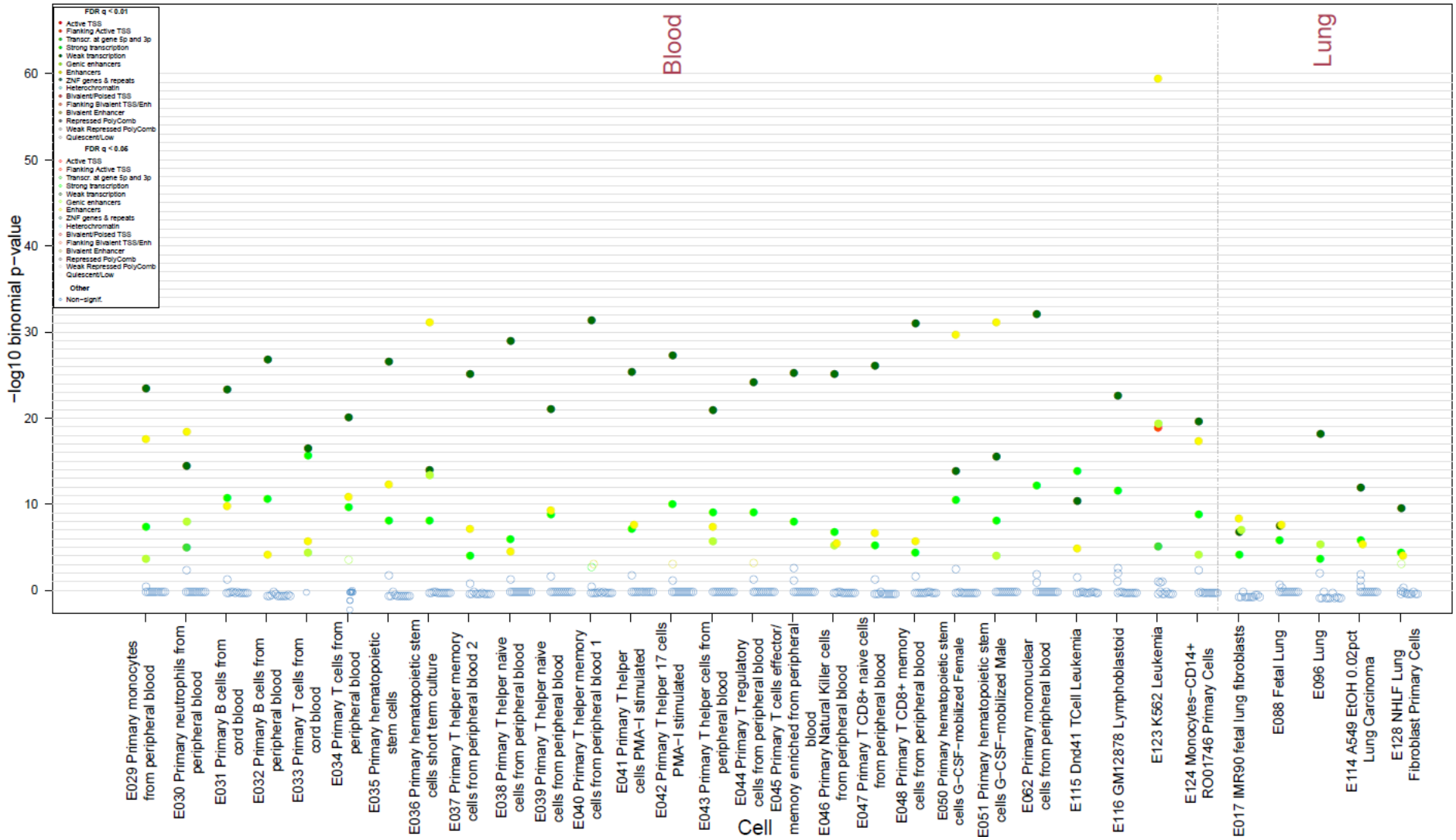


Figure E8. Tissue- and cell-specific enrichment pattern in CpG sites significantly associated (FDR<0.05) with atopic asthma, using eFORGE

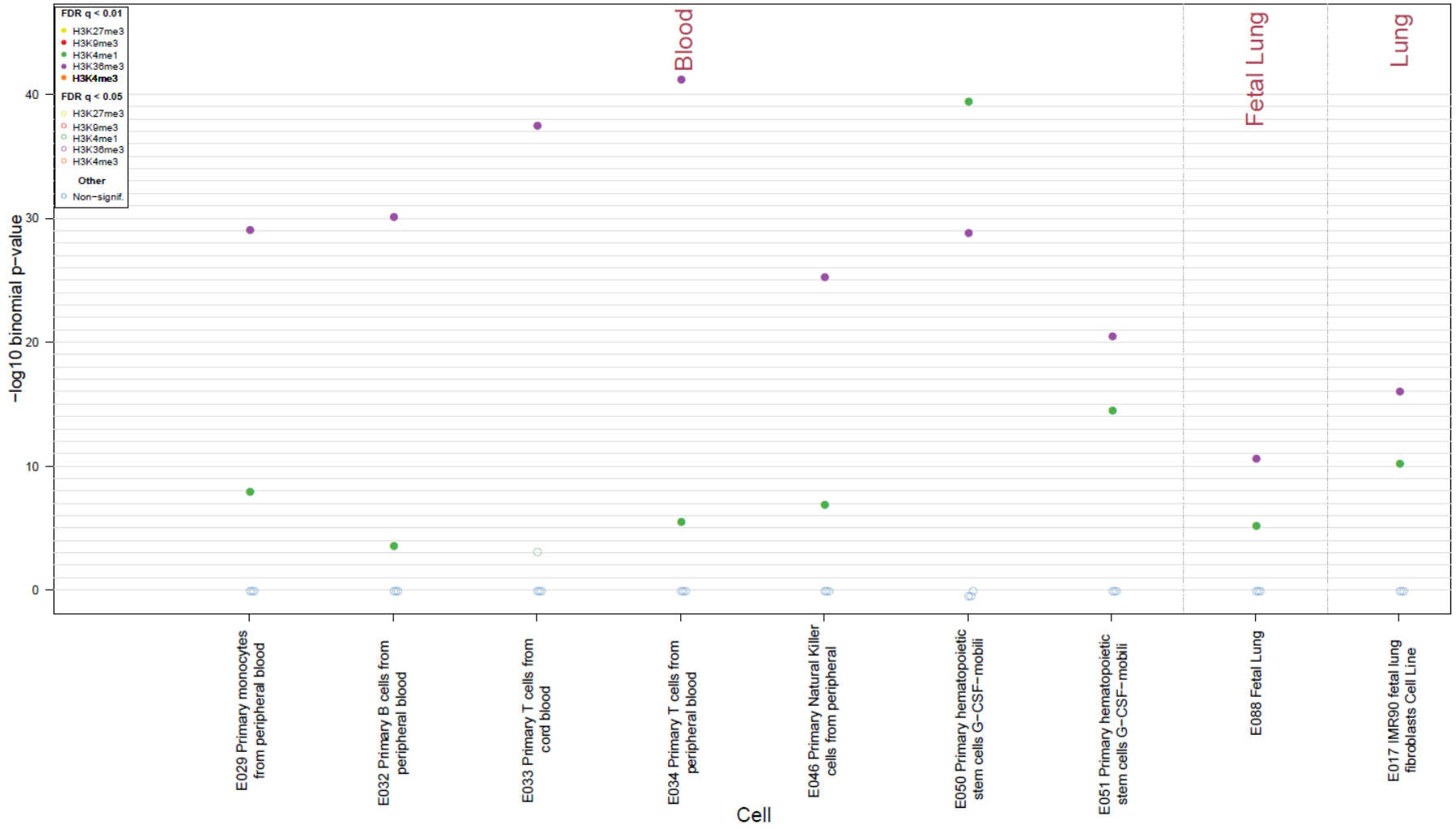
(a) DNase I hypersensitive sites (probably transcription factor binding sites) enrichment in cell lines from the Roadmap Epigenomics consortium



(b) Chromatin state enrichment in cell lines from the Roadmap Epigenomics consortium



(c) Histone mark enrichment in cell lines from the Roadmap Epigenomics consortium



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