



# Co-methylation analysis in lung tissue identifies pathways for fetal origins of COPD

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**Study of fetal and adult lung tissue DNA methylation networks reveals pathways of lung disease that may be primed during *in utero* development and with maternal smoke exposure, supporting early-life origins of COPD** <https://bit.ly/2yzFkds>

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**ABSTRACT** COPD likely has developmental origins; however, the underlying molecular mechanisms are not fully identified. Investigation of lung tissue-specific epigenetic modifications such as DNA methylation using network approaches might facilitate insights linking *in utero* smoke (IUS) exposure and risk for COPD in adulthood.

We performed genome-wide methylation profiling for adult lung DNA from 160 surgical samples and 78 fetal lung DNA samples isolated from discarded tissue at 8–18 weeks of gestation. Co-methylation networks were constructed to identify preserved modules that shared methylation patterns in fetal and adult lung tissues and associations with fetal IUS exposure, gestational age and COPD.

Weighted correlation networks highlighted preserved and co-methylated modules for both fetal and adult lung data associated with fetal IUS exposure, COPD and lower adult lung function. These modules were significantly enriched for genes involved in embryonic organ development and specific inflammation-related pathways, including Hippo, phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT), Wnt, mitogen-activated protein kinase and transforming growth factor- $\beta$  signalling. Gestational age-associated modules were remarkably preserved for COPD and lung function, and were also annotated to genes enriched for the Wnt and PI3K/AKT pathways.

Epigenetic network perturbations in fetal lung tissue exposed to IUS and of early lung development recapitulated in adult lung tissue from ex-smokers with COPD. Overlapping fetal and adult lung tissue network modules highlighted putative disease pathways supportive of exposure-related and age-associated developmental origins of COPD.

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## Introduction

COPD remains a major cause of mortality worldwide [1–3], and is caused by genetic and epigenetic risk factors [4, 5] that communicate through biological networks. Early-life factors associated with reduced maximally attained lung function [6] and accelerated lung function decline [5] might predispose to adult lung diseases such as COPD [7–9]. Investigating these factors has become a greater focus [10], as these insights may impact diagnostic, therapeutic and preventative interventions. Maternal smoking during pregnancy is prevalent, at 14–26% in the US population [11–13] and 10–35% worldwide [12, 14]. This poses severe risks to the developing fetus and fetal lung [15], including intrauterine growth abnormalities [16, 17], pre-term birth [18], low birthweight [19] and altered immune responses [20, 21]. DNA methylation has been suggested as a potential mediator between maternal smoking and low birthweight [22]. A longitudinal study identified persistently perturbed methylation marks associated with maternal smoking during pregnancy [23]. While the detrimental effects of parental smoke exposure on early-life development [24] and its associations with bronchopulmonary dysplasia [25] are widely recognised, links to asthma [26] and adult chronic diseases including COPD have been emerging more recently [27–32]. There is also evidence that maternal smoking is a risk factor for severe, early-onset COPD [33]. A recent epigenome-wide meta-analysis identified more than 6000 significant differentially methylated CpG sites in cord blood associated with maternal smoking and lung developmental processes [34]. A recent meta-analysis of 1688 children in five cohorts identified differentially methylated regions in cord blood DNA, associated with lower lung function in newborns and asthma and COPD in later life [35].

Identifying early COPD and associated mechanisms is crucial for an improved understanding of lung disease pathophysiology and defining therapies [36]. DNA methylation represents a molecular mark that is critical for pre- and post-natal lung development but perturbable by *in utero* exposures [37]. Such perturbations may set the fetal lung on a trajectory of altered development and risk for adult lung diseases, including COPD. Experimental data suggest that DNA methylation changes are dynamic and tissue specific during early development [38]. However, there are limited studies in lung tissue investigating epigenetic links to the developmental origins of COPD. Network approaches can be used to cluster complex biological processes most relevant for age-related respiratory diseases [39, 40]. In this study, we hypothesise that epigenetic signatures of *in utero* smoke (IUS) exposure and age in fetal lung tissue are associated with COPD and lung function in adult lung tissue, and would highlight functionally relevant co-methylation modules supportive of the developmental origins of adult lung disease.

## Methods

### Study samples

The fetal lung DNA samples were isolated from discarded tissue at 8–18 weeks of gestation at pseudoglandular and early canalicular stages of human lung development as previously described [41]. Approval was obtained from the Partners Human Research Committee Institutional Review Board (Boston, MA, USA).

Genome-wide methylation assays were performed using 750 ng bisulfite-treated DNA per sample using the Infinium HumanMethylation450 BeadChip array (Illumina, San Diego, CA, USA) [42] and further data processing was performed using Minfi [43]. Details on sample dropout and filtering of CpGs are described in supplementary table S1. Details on ancestry composition estimates [44] and adjustment for confounding using BACON [45] are included in the supplementary material. Since the fetal lung tissue samples were de-identified, data were only available for gestational age, fetal sex and cotinine levels. Sex was determined using unique Y chromosome microarray probes as previously described [46] and verified using X and Y chromosome methylation. IUS exposure was inferred by measuring placental cotinine concentrations. Exposure was treated as both a continuous and dichotomous predictor variable, with levels of cotinine  $\leq 7.5 \text{ ng}\cdot\text{g}^{-1}$  considered as unexposed (control group) and levels of cotinine  $> 7.5 \text{ ng}\cdot\text{g}^{-1}$  considered as exposed; this level has been reported to have a high sensitivity and specificity for smoke exposure during pregnancy [41]. Further details are available in CHHABRA *et al.* [47]. We used our previously published adult lung tissue methylation results (from the Lung Tissue COPD (LT-COPD) study; n=160: 114 cases and 46 healthy smoker controls) to replicate the fetal lung dataset findings [48].

### Network approach: weighted gene correlation network analysis

An overview of the analytical pipeline is represented in figure 1. For fetal lung data, methylation was the dependent variable (outcome) and IUS exposure was the predictor. Quality control, pre-processing (supplementary table S1) and differential methylation analyses for fetal lung data were performed prior to performing network analyses to make the data suitable for comparison to the adult lung tissue site-specific CpG analysis [48], as detailed in the supplementary material. We further performed an independent network co-methylation analysis in both tissues.

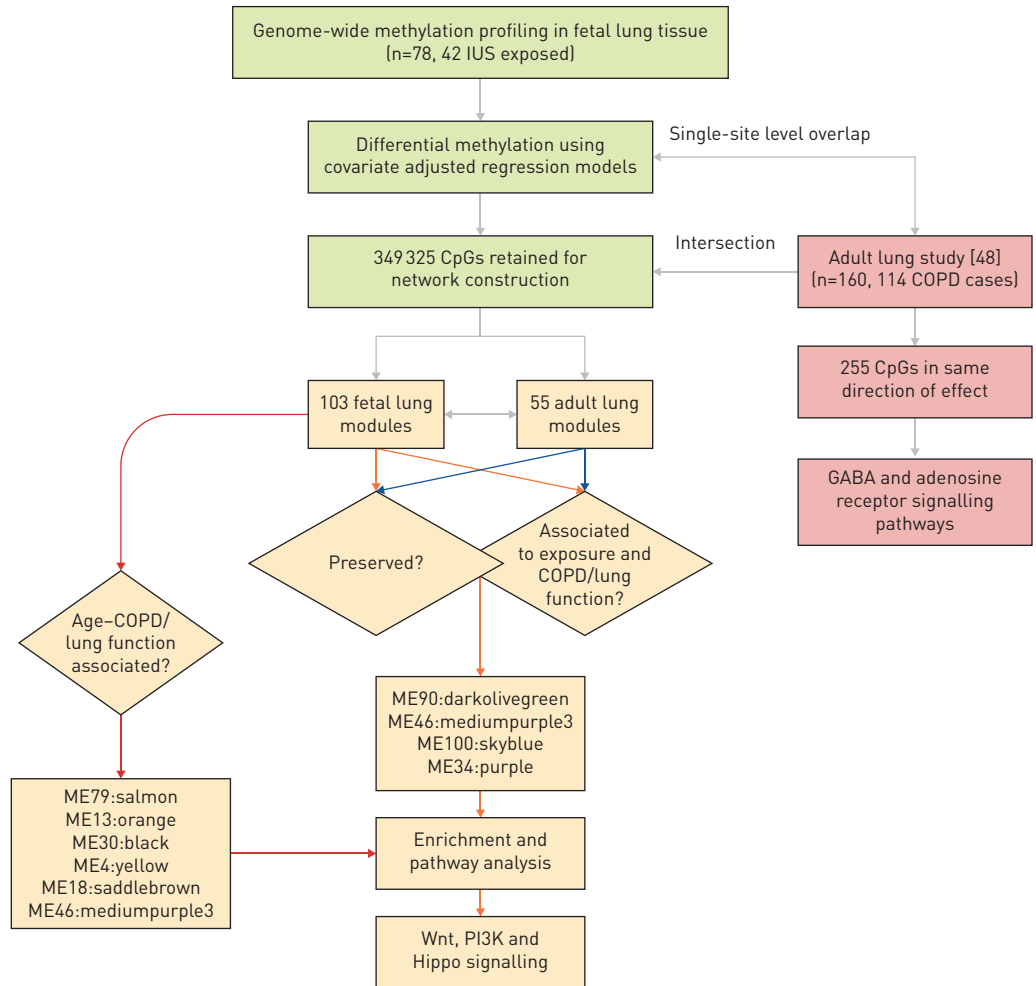


FIGURE 1 Overview of the study flowchart and analytical pipeline for pre-processing and module network creation using weighted correlation network analysis (WGCNA). GABA:  $\gamma$ -aminobutyric acid; ME: module eigengene; PI3K: phosphatidylinositol 3-kinase. ME90:darkolivegreen, ME46:mediumpurple3, ME100:skyblue, ME34:purple, ME79:salmon, ME13:orange, ME30:black, ME4:yellow and ME18:saddlebrown correspond to the resulting modules from the WGCNA output. The colours in boxes represent different stages of the analytical pipeline. Green, yellow and pink colours correspond to the steps for data pre-processing, downstream post-processing, and overlap of fetal and adult lung tissue single-site CpG analysis, respectively. Orange and blue arrows point to the modules and associated findings resulting from fetal lung and adult lung as reference networks, respectively.

The filtered and post-processed 349 355 CpGs from the fetal lung data were evaluated for overlap with the quality controlled CpGs from the LT-COPD study. The overlapping set of 349 325 CpGs from both the fetal lung (reference) and the adult LT-COPD (test) datasets was retained for identifying co-methylated modules (clusters of correlated CpGs) and their associations with phenotypic traits using weighted correlation network analysis (WGCNA) [49]. Modules are named based on associated numbers and colours as output from WGCNA. Phenotype variables including age, forced expiratory volume in 1 s ( $FEV_1$ ), forced vital capacity (FVC),  $FEV_1/FVC$  ratio, quantitative markers of emphysema (low-attenuation areas  $<-950$  HU (LAA-950) and the Hounsfield units value corresponding to the 15th percentile on the density histogram (Perc15)), time since quitting smoking (months) and pack-years (quantitative measure for smoking intensity) were analysed as continuous except for sex, IUS exposure status (1 or 0 with 1=exposed) and COPD status (1 or 0 with 1=COPD cases) for investigating module-trait associations. Module membership (MM) quantifies the contribution of each CpG in the module and allows screening for disease-related hub CpGs/genes. We also evaluated module preservation between fetal and adult lung datasets defined by  $Z_{summary}$  as a measure of connectivity of CpGs within co-methylated modules. As a sensitivity analysis, we used the LT-COPD dataset as the starting reference and repeated the aforementioned analyses to assess whether the LT-COPD-associated modules for COPD, lung function and age recapitulate in fetal lung tissue. Our workflow for the network analysis using WGCNA is shown

in figure 1. Detailed methods and parameters used for reproducibility of the network analysis are described in the supplementary material.

### **Enrichment and pathway analysis**

The R packages missMethyl [50, 51] and g:Profiler [52] were used to perform enrichment analysis for the differentially methylated CpGs and the annotated genes, respectively. Enrichment Map [53] in Cytoscape version 3.6.1 (cytoscape.org) was used to create a network representation of the biological processes enriched in the modules of interest (false discovery rate (FDR) <0.05), using the g:Profiler output including transcription factors, Gene Ontology terms, Reactome and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways.

### **Integration with prior gene expression and genome-wide association study associations**

In line with our hypothesis to identify fetal origins of COPD, we investigated all CpGs with MM  $\geq 0.70$  and with gene annotations for correlations with the previously published COPD differential expression in the LT-COPD dataset [48] and with 82 published COPD genome-wide association studies (GWASs) [54].

## **Results**

### **Support for the fetal origins of COPD using network methods**

Our study included 78 fetal lung (42 IUS exposed and 36 unexposed) samples and 349 355 CpGs that passed the quality control measures (supplementary table S1); of those, 349 325 CpGs overlapped with the LT-COPD dataset. We first evaluated the 535 significant differentially methylated CpG–COPD associations (FDR <5% and difference in mean methylation >1%) published from our LT-COPD study [48] and investigated their methylation patterns in the fetal lung epigenome-wide study (EWAS) findings (supplementary figures S1–S4 and supplementary tables S2–S4). Overlapping CpGs highlighted robust overlap for genes (permutation-based  $p$ -value =  $9.9 \times 10^{-4}$ ) (supplementary figure S5) including *CHRM1*. *CHRM1* had the highest methylation difference in the LT-COPD dataset (cg06289725: FDR  $1.81 \times 10^{-6}$ ; 10% hypermethylated in COPD cases) and nominal significance and same direction of effect in the fetal lung data with a relative hypermethylation of 1.9% in smoke-exposed compared with unexposed fetal lung samples (supplementary figure S6; detailed in the supplementary material).

### **IUS exposure–COPD associations and preserved modules**

We performed an independent network analysis using WGCNA for the overlapping set of 349 325 CpGs from the fetal lung methylation data and identified 319 366 CpGs assigned to 103 co-methylation modules (supplementary figure S7a). A brief framework for selecting preserved module–trait associations between fetal and adult lung datasets, focusing on lung tissue modules with high network preservation, is highlighted in table 1.

We identified seven modules, *i.e.* ME90:darkolivegreen, ME49:darkseagreen, ME97:indianred3, ME46:mediumpurple3, ME100:skyblue, ME34:purple and ME6:plum1, associated with (nominal  $p \leq 0.05$ ) either measure of IUS exposure (binary or continuous) (figure 2a and supplementary table S5). ME46:mediumpurple3, ME100:skyblue and ME34:purple were associated with both measures of exposure (figure 2a). Projecting the fetal lung module structure as reference onto the adult lung tissue methylation data (supplementary figure S7b), we observed that these IUS exposure modules identified in fetal lung associate with either COPD or with FEV<sub>1</sub> and FEV<sub>1</sub>/FVC in adult lung tissue subjects (figure 2b). The ME100:skyblue module had a relatively suggestive association for COPD, but they all remained significant for FEV<sub>1</sub> at FDR <0.05 (figure 2b and supplementary table S6). Based on  $Z_{\text{summary}}$ , three of the seven exposure modules, *i.e.* ME34:purple, ME90:darkolivegreen and ME100:skyblue, were highly preserved and ME46:mediumpurple3 was moderately preserved between both fetal lung and COPD datasets that we refer to as exposure–COPD modules (table 1, supplementary figure S8 and supplementary table S7). Consistent with the associations, eigengene values of the ME90:darkolivegreen module were relatively increased and positively correlated with higher cotinine values in IUS-exposed compared with unexposed fetal lung samples (supplementary figure S9a and b). The largest positive eigengene values of the ME90:darkolivegreen module were driven by lower FEV<sub>1</sub> values in COPD adults compared with controls, with pack-years pointing towards more recent smoking cessation (supplementary figure S9c–e). For the ME34:purple and ME100:skyblue modules, eigengene values were significantly correlated with IUS-exposed subjects in the fetal lung dataset and lower FEV<sub>1</sub> in the LT-COPD dataset (supplementary figures S10 and S11). Based on the MM criteria, *FOXB1* (MM=0.881), *TSSK6* (MM=0.804) and *SLC26A8* (MM=0.871) were the top genes in the ME34:purple, ME90:darkolivegreen and ME100:skyblue modules, respectively (supplementary table S8).

TABLE 1 Preserved module associations for gestational age, *in utero* smoke exposure and COPD/lung function with their annotated pathways

Modules	CpGs	Fetal lung associations		LT-COPD associations	Preservation (score) <sup>#</sup>	Pathways (number of annotated genes) <sup>†</sup>	Genes with highest MM
		Binary	Continuous				
Preserved and exposure-COPD/lung function-associated modules							
ME34:purple	5024	Yes	Yes	FEV <sub>1</sub>	High (18)	Hippo (n=44), Wnt (n=89), MAPK (n=13), protein serine/threonine kinase (n=69) and TGF-β (n=41)	FOXB1
ME90:darkolivegreen	543	No	Yes	Sex, FEV <sub>1</sub> , FVC	High (15)	snRNP, scaRNP, snoRNP and telomerase biogenesis (n=4)	TSSK6
ME100:skyblue	712	Yes	Yes	FEV <sub>1</sub> , FVC, FEV <sub>1</sub> /FVC	High (10)	ERBB4 (n=5) and cell-cell signalling (n=10)	SLC26A8
Preserved and age-COPD/lung function-associated modules							
ME13:orange	878	No	No	COPD, FEV <sub>1</sub> , FVC, FEV <sub>1</sub> /FVC, months quit	High (36)	Wnt signalling (n=23), PI3K/AKT (n=19) and MAPK (n=17) network	ZNF704
ME79:salmon	3348	No	No	COPD, FEV <sub>1</sub> , FVC, FEV <sub>1</sub> /FVC, months quit	High (35)	Protein serine/threonine kinase (n=44), Wnt (n=53), PI3K (n=16), ERBB (n=21), TGF-β (n=23) and BMP (n=24) signalling	PRDM8
ME30:black	10 135	No	No	COPD, FEV <sub>1</sub> , FVC, FEV <sub>1</sub> /FVC	High (18)	Protein serine/threonine kinase signalling (n=83), Wnt signalling (n=133), BMP (n=45), PI3K (n=38) and GABA (n=9) signalling	PCOLCE
ME4:yellow	29 032	No	No	COPD, FEV <sub>1</sub> , FVC, FEV <sub>1</sub> /FVC, months quit	High (16)	ERBB (n=94), PI3K/AKT (n=66), Wnt (n=191), EGFR (n=74), protein serine/threonine kinase (n=150), TGF-β (n=91) and IFN-γ-mediated (n=50) signalling	RNF213
ME18:saddlebrown	682	No	No	COPD, FEV <sub>1</sub> , FVC, FEV <sub>1</sub> /FVC	High (13)	Trans-synaptic and synaptic signalling (n=22)	HNF1A
Exposure and age-COPD/lung function-associated and preserved modules							
ME46:mediumpurple3 <sup>+</sup>	284	Yes	Yes	COPD, FEV <sub>1</sub> , FVC, FEV <sub>1</sub> /FVC	Moderate (7)	RNP biogenesis (n=11), PI3K/AKT (n=8), MHC mediation (n=8),Wnt signalling (n=5), ER-phagosome (n=4) and MAPK (n=6) signalling	HARS2, GUK1

The table highlights associations with exposure in fetal lung and COPD/lung function in the Lung Tissue COPD (LT-COPD) study dataset, associations with gestational age in fetal lung and COPD/lung function in the LT-COPD dataset, and associations with both gestational age and exposure in fetal lung and COPD/lung function in the LT-COPD dataset. MM: module membership; FEV<sub>1</sub>: forced expiratory volume in 1 s; MAPK: mitogen-activated protein kinase; TGF: transforming growth factor; FVC: forced vital capacity; RNP: ribonucleoprotein; snRNP: small nuclear RNP; scaRNP: small Cajal body-specific RNP; snoRNP: small nucleolar RNP; PI3K/AKT: phosphatidylinositol 3-kinase/protein kinase B; BMP: bone morphogenetic protein; GABA:  $\gamma$ -aminobutyric acid; EGFR: epidermal growth factor receptor; IFN: interferon; MHC: major histocompatibility complex; ER: endoplasmic reticulum. <sup>#</sup>: a preservation score ( $Z_{\text{summary}}$ ) as defined by the default weighted correlation network analysis criteria [49] was used to assess the density and connectivity patterns between CpGs in a module.  $Z_{\text{summary}}$  2–<10 implies weak to moderate preservation and  $Z_{\text{summary}} \geq 10$  implies strong and high preservation. Additionally, modules with  $Z_{\text{summary}}$  6–<10 were considered moderately preserved. Only high or moderately preserved modules are included and the scores are rounded without decimal places. <sup>†</sup>: genes in the highlighted pathways for each module are detailed in supplementary table S19. \*: module associated with both exposure and gestational age in fetal lung and COPD/lung function in the LT-COPD dataset.

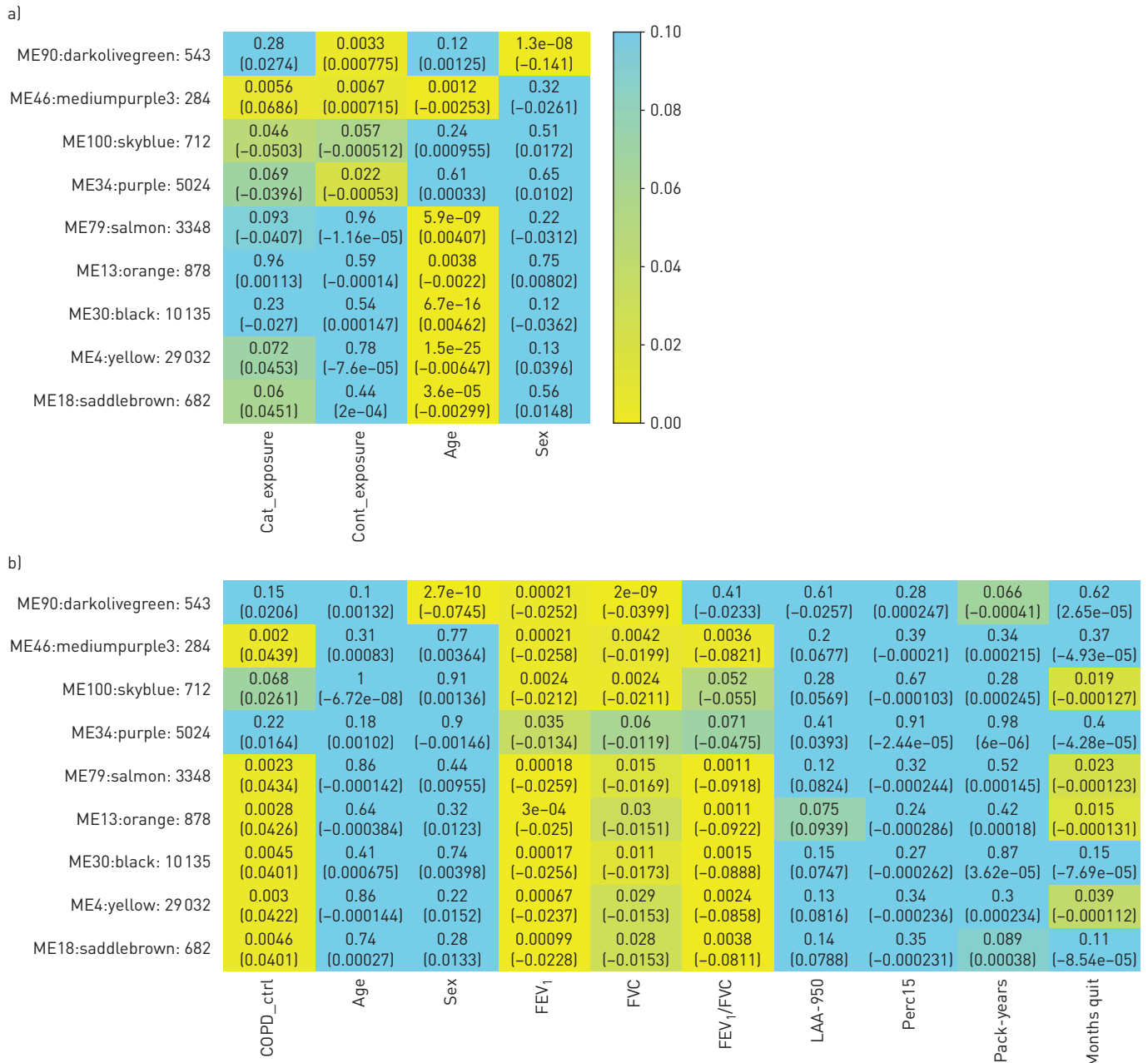


FIGURE 2 Module-trait association heatmaps for the selected nine *in utero* smoke (IUS) exposure and gestational age modules associated with COPD and lung function phenotypes highlighting fetal origins for COPD at the network level. Cat\_exposure: IUS exposure (exposed *versus* unexposed); Cont\_exposure: IUS exposure (continuous cotinine variable); COPD\_ctrl: COPD (cases *versus* controls); FEV<sub>1</sub>: forced expiratory volume in 1 s; FVC: forced vital capacity; LAA-950: low-attenuation areas <-950 HU; Perc15: Hounsfield units value corresponding to the 15th percentile on the density histogram. a) Association of modules in the fetal lung network with the traits of interest in the fetal lung data. b) Association of Lung Tissue COPD dataset traits of interest projected on to the fetal lung module network as reference. The y-axis shows the module labels (module eigengene number:colour), followed by the number of CpGs in the respective module. The panels highlight modules associated with IUS exposure, gestational age, COPD and lung function phenotypes. The blue and yellow colours in the boxes represent the scale for significance (nominal  $p < 0.05$ ), with blue being not significant and yellow being highly significant. The mean  $\beta$ -effect estimate per module corresponding to the trait is shown below in parentheses. Missing data: FEV<sub>1</sub> and FVC were missing for 13 subjects, LAA-950 and Perc15 were missing for 55 subjects, and months since quitting smoking was missing for one subject. Subjects with missing data were removed prior to running models for these predictors.

#### Fetal age-COPD modules suggestive of developmental perturbations

18 modules were associated with fetal age but not cotinine exposure (supplementary figure S7a (FDR <0.05) and supplementary table S5). 15 of those were associated with COPD in adult lung tissue



(supplementary figure S7b and supplementary table S6). Most of the fetal age-associated modules were also associated with lung function phenotypes ( $FEV_1$  and  $FEV_1/FVC$ ) (supplementary table S6). Based on  $Z_{\text{summary}}$ , the highly preserved age-associated modules (figure 2a and b) included ME13:orange, ME79:salmon, ME30:black, ME4:yellow and ME18:saddlebrown, with ME46:mediumpurple3 as moderately preserved (table 1, supplementary figure S8 and supplementary table S7). The highest preserved ME13:orange module also had a suggestive association with quantitative computed tomography measures of emphysema (LAA-950;  $p=0.075$ ) (figure 2b). Based on the MM criteria, *PRDM8* (MM=0.91), *ZNF704* (MM=0.92), *PCOLCE* (MM=0.92), *RNF213* (MM=0.96) and *HNF1A* (MM=0.79) were the top genes annotated to CpGs in the ME79:salmon, ME13:orange, ME30:black, ME4:yellow and ME18:saddlebrown modules, respectively (supplementary table S9).

#### Module connecting exposure and fetal development to COPD

The ME46:mediumpurple3 module showed moderate preservation (supplementary table S7), and its association with cotinine exposure and age in fetal lung (nominal  $p<0.05$ ) (supplementary table S5) and COPD and all lung function phenotypes in the LT-COPD dataset (figure 2 and table 1) made it an interesting module to study further. The associations with fetal age and COPD,  $FEV_1$  and  $FEV_1/FVC$  in the LT-COPD dataset remained significant at FDR  $<0.05$  (supplementary table S6).

Consistent with those associations, the ME46:mediumpurple3 module was strongly associated with IUS exposure and the methylation levels of this module were overall 1.4% higher among IUS-exposed compared with unexposed fetal lung samples (figure 3a). Moreover, smoke-exposed samples having high cotinine levels were positively correlated with the ME46:mediumpurple3 module eigengene ( $R=0.30$ ,  $p=8.4\times 10^{-3}$ ) (figure 3b). Notably, the methylation levels were also overall 1.1% higher in COPD cases (figure 3c) and this positive eigengene association was correlated with lower  $FEV_1$  values ( $R=-0.32$ ,  $p=1.0\times 10^{-4}$ ) (figure 3d), but not associated with cigarette smoke exposure/pack-years ( $R=0.09$ ,  $p=0.22$ ) (figure 3e). Based on the MM criteria, *HARS2* (MM=0.79) and *GUK1* (MM=0.79) were the top genes in the ME46:mediumpurple3 module (supplementary table S8). Seven significant hypermethylated CpGs were annotated to the transcription start site of multisite gene *STK19* within the module (supplementary figure S12).

#### Enrichment and pathway analysis

Among the preserved and IUS exposure–COPD or lung function-associated modules, the ME34:purple module was enriched in several developmental processes and pathways, including Hippo, Wnt, mitogen-activated protein kinase (MAPK) and transforming growth factor (TGF)- $\beta$  signalling as well as several transcription factors (supplementary table S10). The ME90:darkolivegreen module (supplementary figure S13) was primarily associated with positive regulation of telomerase RNA localisation to Cajal bodies (supplementary table S11). The ME100:skyblue module (supplementary figure S14 and supplementary table S11) was enriched for cell–cell communication and *ERBB4* signalling with members of the Hippo pathway and cadherin gene family. Fetal age–COPD-associated modules (ME79:salmon, ME13:orange, ME30:black, ME4:yellow and ME18:saddlebrown) were mostly large and demonstrated significant enrichment (supplementary tables S12–S16 and supplementary figure S15) related to development, proliferation, differentiation and morphogenesis. Significantly enriched pathways included regulation of the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) network, ERBB receptor family and Wnt signalling, and signalling by receptor tyrosine kinases and MAPK. Interestingly, most interconnected and enriched terms in the ME13:orange module included Wnt signalling and the PI3K/AKT signalling network (supplementary table S15 and figure 4). The ME46:mediumpurple3 module (supplementary table S11 and supplementary figure S16) was enriched for viral pathways (FDR adjusted  $p=6.58\times 10^{-7}$ ), ribonucleoprotein biogenesis (FDR adjusted  $p=4.09\times 10^{-5}$ ), DNA repair (FDR adjusted  $p=8.44\times 10^{-4}$ ) and PI3K/AKT signalling (FDR adjusted  $p=1.81\times 10^{-3}$ ).

#### Adult lung network projection onto fetal lung methylation

To assess the robustness of our findings, we repeated our analyses using the overlapping set of 349325 CpGs from the adult LT-COPD dataset projecting the adult lung methylation network structure as the starting reference on the fetal lung methylation data (test) and identified 55 co-methylation modules (supplementary figures S17 and S18, and supplementary table S17). Most modules were associated with fetal age, lung function and COPD, but none significantly associated with both IUS exposure and COPD. Enriched pathways for those modules included epidermal growth factor receptor tyrosine kinase inhibitor resistance, ERBB signalling downregulation and PI3K/AKT. This analysis further supports that adult lung network perturbations might recapitulate and have origins in fetal development-associated epigenetic perturbations, especially with enrichment in the PI3K/AKT signalling pathway.

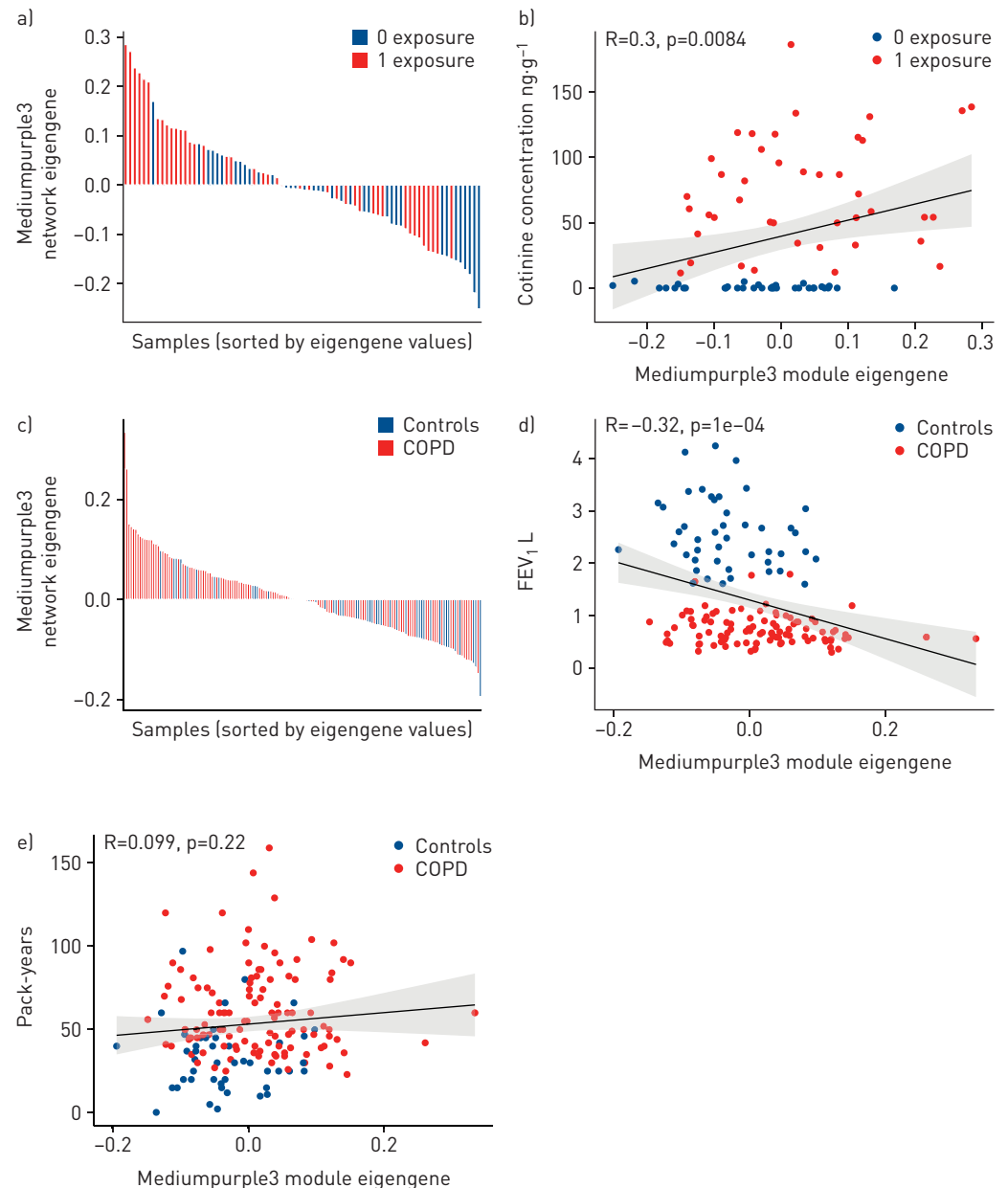


FIGURE 3 Correlation plots between the ME46:mediumpurple3 methylation eigengene and traits of interest. COPD: chronic obstructive pulmonary disease; FEV<sub>1</sub>: forced expiratory volume in 1 s; IUS: *in utero* smoke. Eigengene values of the ME46:mediumpurple3 module were a) robustly increased and b) associated with higher cotinine values in IUS-exposed fetal lung samples compared with unexposed samples. For exposure, 0 corresponds to unexposed samples and 1 corresponds to IUS-exposed samples. Eigengene values of the ME46:mediumpurple3 module were c) robustly increased and d) significantly correlated with lower FEV<sub>1</sub> values in COPD adults compared with smoker controls, but e) not associated with cigarette smoke intensity (pack-years). For b), d) and e), p-values and correlations are computed using Pearson's method and the regression lines are plotted for the overall data along with the 95% confidence intervals.

#### Integration with prior gene expression and GWAS associations

We identified 14236 CpGs at an MM subthreshold of  $\geq 0.70$  annotated to 7364 unique genes across all modules (supplementary table S18). Of those, 100 CpGs from the ME34:purple, ME79:salmon, ME30:black, ME13:orange and ME4:yellow modules mapped to 62 unique differentially expressed genes (FDR  $< 0.05$ ) in the LT-COPD dataset. The highest MM was associated with the CpG site cg07495027 mapped to *EDNRB* in the ME4:yellow module (supplementary table S18). The strongest correlation between CpG methylation and gene expression was observed for *EEF1B2* (correlation  $-0.42$ ,  $p=9.9 \times 10^{-8}$ ) and *ABL1* (correlation  $0.34$ ,  $p=2.4 \times 10^{-5}$ ). Interestingly, 38 unique high MM genes had an overlap with the 82



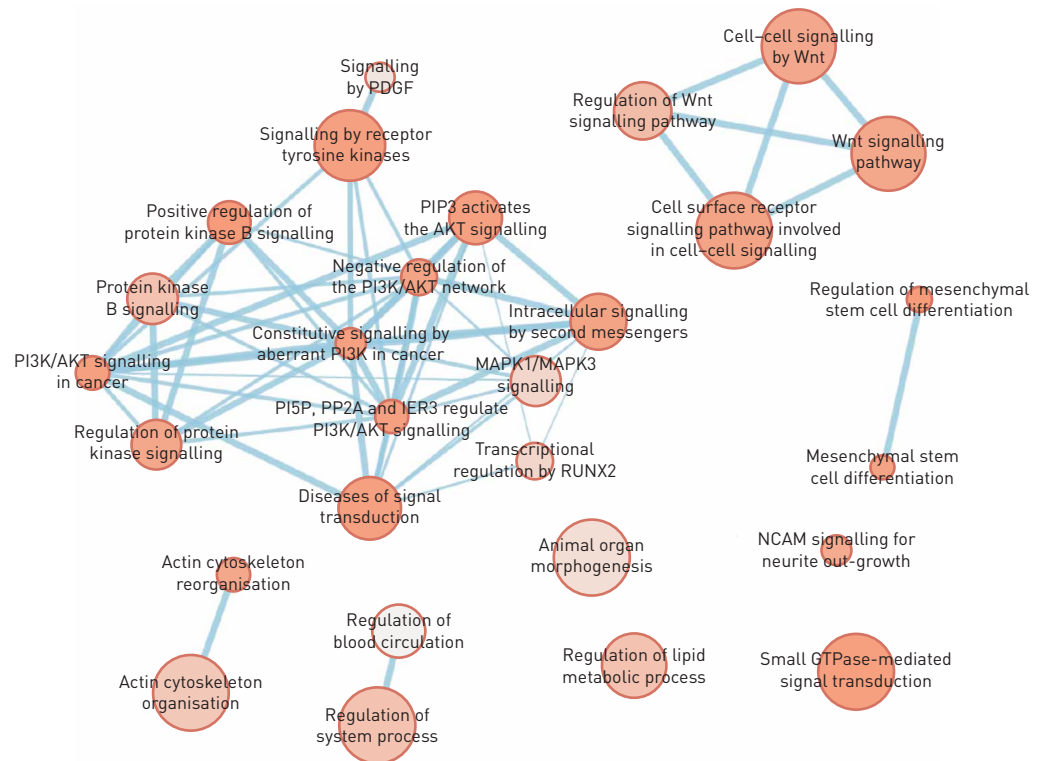


FIGURE 4 Reactome analysis reveals phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) and Wnt signalling pathway networks for the ME13:orange module [false discovery rate <0.05]. PDGF: platelet-derived growth factor; PIP3: phosphatidylinositol 3,4,5-trisphosphate; MAPK: mitogen-activated protein kinase; PI5P: phosphatidylinositol 5-phosphate; PP2A: protein phosphatase 2A; IER3: immediate early response 3; NCAM: neural cell adhesion molecule. Node size corresponds to the number of significant genes in the enriched term (supplementary table S15). Node colour corresponds to the significance of the term (false discovery rate <0.05). Edge thickness corresponds to the number of genes that overlap between two connected terms/gene sets.

previously identified COPD GWAS associations (supplementary table S18). Several genes were also shared between modules with maximum presence for *ASAP2*, *IER3* and *THRA* across modules.

## Discussion

Network analyses may reveal deeper insights into the developmental origins of disease. Addressing the fetal origins hypothesis, we utilised a network-driven approach to focus on smaller gene clusters in relevant functional pathways that may influence risk for adult COPD. The network analysis suggested that the early origins of COPD may be related to both the developmental- and exposure-related perturbations in gene networks, not single genes working in isolation.

Pathway analysis of IUS exposure, fetal age and COPD-associated module genes (ME46:mediumpurple3) as well as the most highly preserved module genes (ME13:orange) included annotation to the PI3K pathway. The PI3K/AKT pathway not only plays a crucial role in human disease [55] but also represents a therapeutic target for ageing [56], lung dysfunction, asthma pathogenesis [57] and treatment of COPD [58]. Most of the mentioned Hippo pathway components interact, including *Mst1/2*, *Sav1*, *Lats1/2*, *Mob1* or *Yap*. Their homozygous deletion causes embryonic lethality [59, 60]. The Hippo signalling pathway regulates lung development/alveolarisation [61, 62], and closely interacts with TGF- $\beta$  [63], Wnt, Notch and Hedgehog signalling pathways. Furthermore, the Hippo pathway is implicated in progression of lung diseases, including asthma and COPD [64, 65], and may therefore be a crucial link for fetal origins of COPD. The Wnt pathway is annotated to almost all of the highlighted modules. Wnt/ $\beta$ -catenin is known for its critical role in embryonic and fetal lung development [66]. IUS exposure has been reported to be associated with downregulation of genes in Wnt signalling and impaired lung growth in the developing fetus [67]. We have previously identified Wnt signalling genes associated with lower lung function in asthmatic subjects [68]. The Wnt/ $\beta$ -catenin pathway has also been implicated in COPD [69–71] and is regulated by *FAM13A*, a COPD susceptibility gene [72]. These findings further support that the disruption of the Wnt pathway in early life may be a prominent contributor to fetal origins of COPD. The role for Wnt in lung ageing has been proposed [73], suggesting this pathway together with the PI3K/AKT pathway

may link fetal exposure and adult lung disease through perturbations in ageing pathways. Highlighted modules revealed pathways to consider for further investigation for fetal origins of COPD. Genes with the highest MM may represent functional COPD susceptibility hubs perturbed in early life. *FOXB1*, the gene with the highest MM in the ME34:purple module, is a transcription factor that has been implicated in embryonic development and was associated with childhood asthma symptoms in a GWAS in Latin American children [74]. The ME34:purple module included multiple enriched pathways (Hippo, PI3K, MAPK, Wnt and TGF- $\beta$ ). The ME90:darkolivegreen module enriched in positive regulation of telomerase activity catalysed by telomerase reverse transcriptase enzyme has often been linked to chromosome maintenance [75, 76]. Its negative association with pack-years and sex may point to sex-specific activation of repair mechanisms after smoking cessation. The ME100:skyblue module was enriched for several members of the cadherin family implicated in lung diseases such as asthma [77] and COPD [78]. *ERBB4* signalling highlighted through the ME100:skyblue module included the *YAP1* gene, a component of the Hippo pathway [60]. *ERBB4* deletion is associated with delayed fetal development [79]. *ERBB4* demonstrated increased gene expression from nonsmokers to smokers with and without COPD and inverse association with FEV<sub>1</sub>, suggesting links to COPD severity [80]. Integration with previously reported gene expression and GWAS associations highlighted candidate genes such as *EDNRB* and *IER3*. Increased *EDNRB* expression has been associated with emphysema, FEV<sub>1</sub>/FVC [81] and COPD severity [82]. *Ier3* knockout in mouse models was associated with immune dysfunction and interference with several pathways, including PI3K/AKT [83].

The biologically plausible and statistically significant pathways we identified using correlation networks are in contrast to the limited findings of the single CpG site analysis. Our findings of many differentially methylated CpGs with a small magnitude of effect is, however, consistent with previously published large EWASs associated with maternal smoking [84]. Notably, the  $\gamma$ -aminobutyric acid (GABA) and adenosine receptor signalling pathways were identified from overlapping fetal and adult lung EWAS results. Nicotine exposure has been reported previously to increase GABA signalling in airway epithelium [85], while adenosine receptors are known for their anti-inflammatory properties and therapeutic potential for the treatment of asthma and COPD [86].

A key strength of the study includes interrogating epigenetic profiles in the fetal lung tissue ascertained during airway branching morphogenesis. These samples are particularly relevant to test the developmental origins of COPD hypothesis. Abnormalities in bronchial airways during this stage can lead to decreased airflow and slowed growth of airways, limiting maximally attained lung function. Analytical strengths of our study include methylation profiles in both fetal and adult lung tissue using the same platform and leveraging a network-based approach for both relevant tissues. Due to complex underlying mechanisms, it remains a challenge to identify significant single-site associations while retaining statistical power, often with effect sizes of small magnitude, which is consistent with previously published large epigenome-wide studies [84]. Previous studies have identified and compared differentially methylated CpGs associated with COPD in whole blood and lung tissue. In light of the considerable heterogeneity across studies and varying statistical analyses [87], our systematic evaluation of previously validated COPD EWAS associations found in blood [48, 88–90] did not identify any consistent overlap with our high MM CpGs.

We have therefore provided extensive details about the network modules to facilitate future research and collaboration in this less explored area. We acknowledge that our study has several limitations, including the sample sizes of the datasets and the use of lung homogenates. Moreover, prospective studies with expanded coverage of methylation sites such as from the Illumina EPIC array and bisulfite sequencing may support validation and improve our understanding of the identified associations significantly. Furthermore, dichotomising continuous variables such as cotinine facilitates interpretation and is consistent with IUS exposure as measured in many studies. However, this may lead to loss of information, especially with small datasets. Therefore, to be consistent with the literature, but also to more precisely model exposure, we reported module–trait associations using both dichotomous and continuous measures of cotinine. In addition, we have limited phenotype data associated with the fetal lung samples. To address this, we directly measured cotinine and given the timing of sample collection presumed elevated cotinine is related to maternal smoking and not nicotine replacement therapy or e-cigarette use. Efforts linking fetal tissue findings with cord blood will increase the opportunity to fully leverage cord blood datasets and understand the fetal origins of adult lung disease. Future research on the developmental contributions to COPD should focus on single-cell analyses to further understand fetal programming in the lung relevant to adult tissue.

### Conclusions

In summary, our co-methylation network evaluation resulted in the identification of gene modules linking fetal lung tissue IUS exposure and fetal age to adult lung function and COPD. Additional studies and functional validation will support causal links between fetal methylation alterations in pathways including

Hippo, Wnt and PI3K/AKT and risk for and development of COPD. As we move towards precision medicine, understanding the impact of early-life molecular trajectories will provide unprecedented insight into complex adult lung diseases and reveal ways to leverage the plasticity of the epigenome towards primordial prevention, lung health and resilience despite early-life programming.

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