Maternal Smoking and CC-16: Implications for Lung Development and COPD Across the Lifespan

Joselyn Rojas-Quintero, MSc, MD[1,2#]

Rosa Faner, PhD[3#]

Chia-Ying Chiu, MPH[4#]

Jeff T. Kue, BS[1]

Yun Zhang, PhD[1]

David Sanz Rubio, PhD[1,5]

Adrianne S. Colborg, MD[6]

Constanze A. Jakwerth, PhD[7,8]

Carsten B. Schmidt-Weber, PhD[7,8]

Anke-Hilse Maitland-van der Zee, PhD[9]

Mahmoud I Abdel-Aziz, PhD[9]

Aprile L. Pilon, PhD[10]

Caroline A. Owen, MD, PhD[2]

Erin J. Plosa, MD[11]

Gregory L. Kinney, PhD[12]

Sharon Mc-Grath Morrow, MD[13]

Mariacarolina G. Gazzaneo, MD[14]

Nahir Cortes Santiago, MD[14]

Krithika Lingappan, MSc, MD, PhD[15]

Julie Ledford, PhD[16]

Jason Spence, PhD[17]

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

Jennifer M. Sucre, MD[11]

Maor Sauler, MD[18]

Tianshi David Wu, MD[1]

Alvar Agusti, MD[3]

Asa Wheelock, PhD[19]

Sabina Illi, PhD[7,20]

Erika von Mutius, MD[7,20,21]

Russell P. Bowler, MD, PhD[22]

Bartolome Celli, MD[2]

Steven H. Abman, MD[23*]

J. Michael Wells, MD, MSPH[4,24*]

Francesca Polverino, MD, PhD[1,2*]✉

and ALLIANCE study group[25], ECLIPSE[26] and COPDGene investigators[27]

[1]Pulmonary Division, Department of Medicine, Baylor College of Medicine. Houston, TX, USA.

[2]Pulmonary and Critical Care Medicine Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA.

[3]University of Barcelona, IDIBAPS, Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBER), Barcelona, Spain.

[4]Department of Medicine, Division of Pulmonary, Allergy, and Critical Care Medicine; UAB Lung Health Center, University of Alabama at Birmingham, Birmingham, AL, USA.

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

[5]IIS Aragón, Trastornos Respiratorios Sueño. Zaragoza, ES 50009. Miguel Servet University Hospital, Zaragoza, ES 50009.

[6]Texas Children's Hospital. Pediatric Pulmonary Division. Houston, TX, USA.

[7]Center of Allergy and Environment (ZAUM), School of Medicine and Health, Technical University of Munich, and Helmholtz Munich, Munich, Germany.

[8]Comprehensive Pneumology Center Munich (CPC-M); Member of the German Center for Lung Research (DZL), Germany.

[9]Department of Pulmonary Medicine, Amsterdam UMC, Amsterdam, Netherlands.

[10]Trove Therapeutics, Inc. & APCBio Innovations, Inc., Rockville, MD, USA.

[11]Department of Pediatrics, Vanderbilt University, Nashville, TN, USA.

[12]Colorado School of Public Health, Department of Epidemiology, University of Colorado Anschutz Medical Campus, Aurora, CO, USA.

[13]Division of Pulmonary and Sleep Medicine, Children's Hospital of Philadelphia, Philadelphia, PA, USA.

[14]Department of Pathology and Immunology, Texas Children's Hospital, Houston, TX, USA.

[15]Division of Neonatology. Children's Hospital of Philadelphia. Perelman School of Medicine at the University of Pennsylvania. Philadelphia, PA, USA.

[16]Asthma and Airway Disease Research Center, University of Arizona, Tucson, AZ, USA.

[17]Department of Internal Medicine, University of Michigan Medical School, Ann Harbor, USA.

[18]Division of Pulmonary, Critical Care and Sleep Medicine, Yale University, New Haven, CT, USA.

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

[19]Respiratory Medicine Unit, Department of Medicine Solna and Center for Molecular Medicine, Karolinska Institute, Stockholm, Sweden.

[20]Institute of Asthma- and Allergy Prevention (IAP), Helmholtz Center Munich, German Research Center for Environmental Health (GmbH), Neuherberg, Germany.

[21]Department of Pediatric Allergology, Dr von Hauner Children's Hospital, Ludwig-Maximilians University, Munich, Germany.

[22]Department of Genomic Medicine, Cleveland Clinic, Cleveland, Ohio, USA.

[23]Division of Pediatric Pulmonary Medicine, Children's Hospital Colorado, Aurora, CO, USA.

[24]Birmingham VA Healthcare System, Birmingham, AL, USA.

[25]Members of the ALLIANCE Study Group are listed in Online Supplement.

[26]Members of the ECLIPSE investigators are listed in Online Supplement.

[27]Members of the COPDGene investigators are listed in Online Supplement.

[#]Co-First Authors contributed equally to this body of work.

*Co-Senior Authors contributed equally to this body of work.

☒**CORRESPONDING AUTHOR**: Francesca Polverino. Department of Medicine, Baylor College of Medicine, Houston, TX, 77030. Email: fpolverino@copdnet.org

RUNNING TITLE: Maternal smoking exposure, CC16, and COPD.

Blue-202504-0854OC: 3848 words

DISCLOSURES

RF is consultant for Astra-Zeneca, and reports grants from Astra-Zeneca, and Sanofi-Regeneron outside the current work. ALP has a conflict in that she is a Founder and employee of APCBio Innovations, Inc. and Trove Therapeutics, Inc. developing rhCC10 as a therapeutic for respiratory disease. CAO is currently an employee in Early Clinical Development Respiratory and Immunology at AstraZeneca Biopharmaceuticals R&D, Gaithersburg, MD outside the submitted work. JMW reports grants and contracts to his institution from Medscape, Verona Pharma, Grifols, Sanofi, and he has served as a consultant for AstraZeneca, Takeda, Bavarian Nordic, Krystal Biotech, Sanofi, and Verona Pharma outside the submitted work. CAJ reports grants from Federal Ministry of Education and Research (BMBF) for the German Center for Lung Research (DZL), during the conduct of the study; also grants from: Federal German Ministry of Education and Research (BMBF ABROGATE), European Institute of Technology (ADAPT), Zeller, Else-Kröner-Fresinius-Stiftung, DFG Exzellenzinitiative TUM International Graduate School of Science and Engineerin (JADS-Project: PANORAMA), DFG Graduiertenkolleg RTG2668 (Project A1, Project-ID: 435874434) outside the submitted work. CSW reports grants from Federal Ministry of Research and Education (BMBF), during the conduct of the study. He received personal fees from Sanofi, Leti and research grants from Zeller and Allergopharma, outside the submitted work. EvM receives royalties/consulting fees from Elsevier GmbH, Georg Thieme Verlag, Springer-Verlag GmbH, Elsevier Ltd., Springer Nature Group, Deutscher Apotheker Verlag, Chinese University of Hong Kong, European Commission, AstraZeneca, Imperial College London, OM Pharma S.A., and Clarivate; no conflicts relevant to this manuscript. EvM receives grants from Bavarian State Ministry of

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

Health and Care for “URS Study”, Go Bio Initial Grant, BMBF, European Research Council Award, and “BEAR Study”, O.M. Pharma S.A. EvM is a member of the following boards: BEAMS External Scientific Advisory Board, Editorial Board of “The Journal of Allergy and Clinical Immunology: In Practice”, Scientific Advisory Board of the Children’s Respiratory and Environmental Workgroup, International Scientific & Societal Advisory Board of Utrecht Life Sciences at University of Utrecht, Member of External Review Panel of the Faculty of Veterinary Science, University of Utrecht, Selection Committee for the Gottfried Wilhelm Leibniz Programme, International Advisory Board of “The Lancet Respiratory Medicine”, Scientific Advisory Board of the Canadian Healthy Infant Longitudinal Development study, McMaster University, Hamilton, Canada, Pediatric Scientific Advisory Board Iceland, Abbott Allergy Risk Reduction Advisory Board, Scientific Advisory Board for French Prevention National Research Programme, and Scientific Advisory Panel for The Natasha Allergy Research Foundation. FP has served as consultant and/or has received grants from for Amgen, Astra-Zeneca, Chiesi, Apogee Therapeutics, GlaxoSmithKline, Genentech, Verona Pharma, Sanofi-Regeneron. FP is spokesperson for the American Lung Association, section editor for the ERJ, and program chair for the ATS RCMB. FP has received funding from Victory Houston. The rest of the authors have declared that no conflict of interest exists.

AUTHOR CONTRIBUTION

JRQ, CAO, BC, SHA, JMW, FP conceived and designed the research. JRQ, ASC, DSR, and JTK performed the experiments. JRQ, C-YC, YZ, SI, CAJ, CBSW, AHMvdZ, MIAA, RF, TDW, ALP, CAO, SMGM, EJP, MGG, NCS, JMS, GLK, AA, AW, EvM, MS, RB, FP,

ALLIANCE, ECLIPSE and COPDGene analyzed data and interpreted the results. JRQ, KL, JS, JL, BC, SHA, JMW and FP prepared figures and drafted the manuscript. All the authors edited, revised, and approved the manuscript.

FUNDING

Project was supported by the Flight Attendants' Medical Research Institute (YFAC14004), the Parker B. Francis Foundation, the Baylor College of Medicine unrestricted funds to FP, and the NIH HL149744 (FP). RF is supported by Serra Húnter, and ICREA programs. The Genetic Epidemiology of COPD Program is supported by NIH grants U01 HL089897 and U01 HL089856. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NHLBI or the NIH. COPDGene is also supported by the COPD Foundation through contributions made to an industry advisory board comprising AstraZeneca, Boehringer-Ingelheim, Genentech, GlaxoSmithKline, Novartis, Pfizer, Siemens, and Sunovion. The ALLIANCE study was developed by the German Center of Lung Research (DZL) and funded by the Federal Ministry of Education and Research (BMBF), by grants from the Bavarian State Office for Health and Food Safety (LGL; K1-2497-GLB-20-V4) of the "Gesund.Leben.Bayern." initiative of the Bavarian State Ministry of Health and Care and from the Deutsche Forschungsgemeinschaft RTG2668 (Project A5, Project-ID: 435874434) to C.B. Schmidt-Weber.

At a Glance Commentary

Current Scientific Knowledge on the Subject: Maternal smoking reduces the expression of CC16 in childhood and throughout the lifespan.

Maternal smoking, via loss of CC16 expression, can alter lung development and predispose to childhood asthma/wheezing and fast emphysema progression in subjects with COPD.

What This Study Adds to the Field: We provide evidence for the role of CC16 protein in lung development and its capacity to modify the trajectory of lung function in children exposed to maternal smoking.

Artificial Intelligence Disclaimer: No artificial intelligence tools were used in writing this manuscript.

This article has an online data supplement, which is accessible at the Supplements tab.

ABSTRACT

Rationale: Early-life lung function trajectories predict long-term respiratory health, including COPD risk. Club Cell protein 16 (CC16) is a key determinant of lung health, with low levels associated with impaired lung development, reduced lung function, and COPD. Cigarette smoking lowers CC16, but it is unknown whether maternal smoking leads to persistent CC16 deficiency from early life, thereby disrupting lung development and predisposing to COPD risk and progression

Methods: CC16 expression was analyzed across 4 human cohorts, in plasma samples (COPDGene [n=1,062] and ECLIPSE [n=2,164]), nasal brushings (ALLIANCE [n=63]), and peripheral lung sections (LTRC [n=44]) from participants with and without a history of maternal smoking exposure. Lung histology and respiratory mechanics were assessed in WT and *Cc16*$^{-/-}$ mice with and without maternal smoking exposure. Recombinant human (rh)CC16 effects on lung maturation were assessed in embryonic murine lung explants.

Results: Maternal smoking was linked to reduced circulating and airway CC16 in COPD patients, controls, and a preclinical murine COPD model. In human adults, lower CC16 correlated with accelerated lung function decline and emphysema progression, while in children it was associated with obstructive physiology and early small airway impairment. In both mice and humans, maternal smoking–induced CC16 reduction was accompanied by greater epithelial injury (fibrosis, inflammation, apoptosis, oxidative stress). In murine explants, smoking impaired lung branching, whereas rhCC16 restored branching via α2-integrin binding

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

Conclusions: Maternal smoking reduces CC16 levels, disrupting lung development in ways that predispose to lifelong impairment of lung function and worse COPD outcomes. Defining the mechanisms by which CC16 regulates lung maturation is essential for establishing reliable outcome measures and designing trials aimed at preventing early COPD.

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

INTRODUCTION

Longitudinal studies demonstrate that lung function trajectories are established early in life[1,2], and children with lower lung function percentiles[3] tend to have persistent lower lung function into adulthood[4,5]. Individuals with lung "dysanaptic growth" early in life have increased respiratory morbidity and are at increased risk for low lung function in adulthood as airway function declines with age[5-7]. Maternal smoking is associated with abnormal long-lasting structural and functional lung changes, increased respiratory illness, and higher asthma risk in children[5,8-10], all through adulthood[4,5].

Club Cell secretory protein-16 (CC16 or *SCGB1A1*) is secreted by airway Club cells into airway fluid, and can also be detected in circulation, sputum, nasal fluid, and urine[11,12]. CC16 has anti-inflammatory, antifibrotic, and anti-aging[13-15] properties, and these protective effects occur, at least in part, via α2 integrin binding[16]. Murine *Cc16*-deficiency leads to spontaneous emphysema over time[13]. Cigarette smoking (CS) downregulates CC16 expression in animal models and COPD[15,17]. Low CC16 levels have been linked to childhood lung impairment, and reduced peak lung function, accelerated lung function decline and airflow limitation in the adult, key features of COPD[13-15,17]. Importantly, we have shown that recombinant human (rh)CC16 replacement therapy protects adult murine lungs against CS-induced COPD pathologies[14].

However, the role of CC16 in lung development during maternal smoking exposure remains poorly understood. Using a pediatric cohort, three COPD cohorts, and a preclinical maternal smoking model, we determined that: a) CC16 promotes lung development via α2-integrin binding; b) CC16 deficiency heightens lung susceptibility to

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

insults like maternal smoking; c) maternal smoking reduces CC16 levels in lungs and blood, persisting into adulthood; and d) low CC16 levels hasten lung function decline, contributing to COPD.

MATERIALS AND METHODS. See Online Supplement

HUMAN STUDIES

COPDgene. The COPDGene study (NCT00608764) is a prospective, multicenter cohort study of current and former smokers enriched for individuals with COPD (n=1,062). Study design, ethics approvals, and emphysema methodology quantification (%LAA-$_{950}$) have been previously described[18,19]. Maternal smoking was assessed by self-reported response to "*Did your mother smoke while pregnant*?" (**Supplemental Figure E1**).

ECLIPSE. The ECLIPSE study (NCT00292552) was a prospective, international, controlled, observational cohort study (n=2,164 patients) with complete medical history, plasma metabolomics, lung function assessment and follow-up at 3-year follow-up[20,21]; **Supplemental Figure E2**. CC16 in plasma was measured both at baseline and 1-year follow-up. Design, ethics approvals, funding sources, and metabolomic methodology have been previously published[20,21].

ALLIANCE. The All-Age Asthma Cohort (ALLIANCE, NCT02496468/NCT02419274) is a multicenter pediatric asthma cohort from the German Center for Lung Research[22,23]; all local ethics committees approved the study protocol[22,23]. Cohort enrolled children with and without history of wheezing/asthma, and recorded history of either *maternal smoking* or *passive smoking exposure*[22,23]; **Supplemental Figure E3**. Nasal brushings were collected for RNA analysis, and *SCGB1A1* RNA was quantified[23,24]. Lung function measurements and nasal brushings were performed at some but not all visits.

Lung Tissue Research Consortium (LTRC). De-identified formalin-fixed paraffin-embedded (FFPE) lung sections from never- (NSC), and ever-smoking controls (SC), and COPD patients were obtained from LTRC[25] (**Supplemental Figure E4**). Maternal smoking was assessed by self-reported response to "*Did your mother smoke while pregnant*?". Conventional immunofluorescence (IF) was performed to assess CC16, Active Caspase-3 (apoptosis marker), Ki-67 (cell replication marker), and EPCAM or Pan-Cytokeratin (epithelial cell markers); **Supplemental Table E1**. Quantification was done using MetaMorph®[13-15].

MURINE STUDIES

See Online Supplement for additional methods. IACUC approval was obtained from Brigham and Women's Hospital/Harvard Medical School (MA, USA), University of Colorado Anschutz Medical Center (CO, USA) and Baylor College of Medicine (TX, USA).

Maternal smoking exposures in mice: WT C57BL/6 and *Cc16*$^{-/-}$ mice were housed in a barrier facility under pathogen-free conditions, with access to normal chow diet and water *ad libitum*. WT and *Cc16*$^{-/-}$ breeding pairs were exposed to room air or mixed CS at ~150 ppm with Teague smoking chamber[14,26]. CS exposure was done 6 days/week, 2 hours daily, throughout copulation and pregnancy. CS-exposure stopped at birth to mimic *in utero* maternal smoking exposure in humans. Newborn pups were raised in room air. The weanlings were humanely euthanized to harvest lungs at post-natal day-1 (P1), -7, -14, and -28 (**Supplemental Figure E5**).

Lung morphometry and pathology: FFPE lung sections from P28 WT and *Cc16*$^{-/-}$ weanlings, with and without *in utero* maternal smoking exposure, were assessed. Gills'-stained lung sections were used to measure alveolar chord length[14,15] and radial alveolar counts (RAC)[27]. Masson's Trichrome[14,15] was used to assess small airway remodeling (SAR) and extracellular matrix deposition (ECM) around vessels, and serial sections were used to quantify macrophages, lymphocytes, and neutrophils using MetaMorph®[13-15]. Additional serial lung sections were immunostained (IF) for Cc16, active caspase-3, Ki-67, 4-hydroxy-nonenal (4HNE, oxidative stress marker), cytochrome P450-2F2 (Cyp2F2, Club Cell marker), and Epcam or Pan-CK; **Supplemental Table E1**. Cell subtypes quantification in airway and parenchyma was done with MetaMorph®[13-15].

Respiratory mechanics: P28 WT and *Cc16*$^{-/-}$ weanlings, with and without *in utero* maternal smoking exposure, were anesthetized, tracheas were cannulated and respiratory mechanics was assessed (Flexivent®, Scireq Inc, Canada)[13-15,26].

CC16 RECEPTOR STUDIES: ALPHA 2 INTEGRIN

IF and co-immunoprecipitation (Co-IP): IF for Cc16 and α2-integrin was performed on WT P28 FFPE lung sections; **Supplemental Table E1**. Pulldown using embryonic lung explants was done against rhCC16 bound to α2 while membrane immunoblotting was done first with anti- α2 antibody, followed by anti-SCGB1A1 antibody to evaluate density of the ~10 kDa bands.

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

Lung organogenesis. To assess the potential role of CS and CC16-α2 integrin binding in lung development, we treated murine lung explants at embryonic day (E)15 dams as follows: ±CS-extract (2%-CSE), ±10 μg/mL rhCC16[14], ±1 μM of BTT-3303 (α2 integrin inhibitor[16]) for 5 days and assessed branchial budding as previously described[28].

STATISTICAL ANALYSES

COPDGene: Analyses were performed in SAS, with figures generated in R. In essence, the following models were built to determine the associations between baseline lnCC16 and $\%FEV_{1pred}$ (or changes in $\%LAA_{-950}$). Baseline plasma CC16 levels were natural-log-transformed (lnCC16) to correct for skewness. Bivariate analyses assessed associations between lnCC16 and maternal smoking exposure, followed by generalized linear models adjusting for age, sex, race, smoking status, pack-years, and annual income. Additional models evaluated lnCC16 relationships with $\%FEV_{1pred}$ (GLI global values) and quantitative emphysema ($\%LAA_{-950}$) on High-Resolution Computed Tomography (HRCT). Longitudinal changes in $\%FEV_{1pred}$ and $\%LAA_{-950}$ were analyzed using Mixed Models for Repeated Measures (MMRM) with the same covariate adjustments. Multiple groups comparison was confirmed with *post-hoc* analyses with Bonferroni test. Correlations were done with Pearson's correlation test.

ECLIPSE: All analyses were performed in R. Serum CC16 levels were transformed to lnCC16 to correct skewness. Annual changes in lnCC16, post-bronchodilator (post-BD) $\%FEV_1$, and emphysema ($\%LAA_{-950}$) on HRCT were calculated. Linear models adjusted for age, sex, pack-years, education, and race, assessed associations between baseline lnCC16 and $\%FEV_{1pred}$ or $\%LAA_{-950}$, and 1-year lnCC16 with $\%FEV_{1pred}$. Multinomial logistic regression, using NSC as the reference, evaluated differences across study groups. $\%FEV_1$ decline was estimated using a mixed model with random slope and intercept to account for baseline variability. Scatter plots to describe the association between lnCC16 and changes in $\%FEV_{1pred}$ and $\%LAA_{-950}$. Clinical characteristics and lnCC16 levels across decline groups were compared using ANOVA, Kruskal–Wallis, t-tests, Mann–Whitney, or χ^2 tests. A two-tailed P-value <0.05 was considered significant. Correlations were done with Pearson's correlation test.

ALLIANCE: CC16 associations with maternal and passive smoking exposure were assessed using bivariate analyses and generalized linear models, adjusted for age, disease status, sex, and study center. Relationships between CC16 and $FEV1/FVC\%_{pred}$ or $FEF25\text{-}75_{\%pred}$ were also evaluated. Longitudinal changes in these lung function measures, in children with ≥2 measurements, were analyzed by calculating differences between first and last values using adjusted models including passive smoking exposure and by applying MMRM with the same covariates in children with ≥1 measurement.

LTRC and murine experiments: statistical analyses were performed using SigmaPlot™. Data were analyzed using one-way ANOVAs for continuous data, followed by post-hoc testing with 2-sided Student's t-tests or Mann-Whitney U tests according to distribution. Two-way ANOVA was performed to test for interaction in continuous outcomes by maternal smoking exposure. Correlations were calculated using Spearman test (ρ). For all the analyses, $P<0.05$ was considered statistically significant.

RESULTS

Human data

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

Maternal smoking is associated with lower circulating CC16 levels and accelerated lung function decline and emphysema progression in COPDGene: Demographic and clinical characteristics for 1,062 subjects with CC16 measurement available[29] are displayed in **Supplemental Table E2**. A third of individuals reported maternal smoking. Mean CC16 concentrations were 8.4±11.2 ng/mL, corresponding to lnCC16 of 1.9±0.62 units. When adjusted for current smoking status, maternal smoking was also associated with lower lnCC16 levels in SC and COPD subjects ($F=10.41$, $P<0.0001$ for cohort; SC: $F=5.10$, $P=0.024$; COPD: $F=23.04$, $P<0.0001$); **Supplemental Table E3**. Maternal smoking history was associated with lower circulating lnCC16 levels in SC and COPD subjects (**Figure 1A**). The scatter plots in **Figure 1B** and **1C** display the unadjusted correlations between CC16 and baseline $\%FEV_{1pred}$ or $\%LAA_{-950}$, respectively. We found a weak correlation between lnCC16 and FEV1% ($r=0.18$, $p<0.0001$) but no correlation between lnCC16 and %LAA-950 ($r=0.004$, $p=0.90$) at baseline. In models adjusted for smoking status, pack-years, income, demographics, and maternal smoking, each standard deviation (SD) decrease in lnCC16 was associated with a 6.3% decrease in $\%FEV_{1pred}$ and a 0.9% increase in $\%LAA_{-950}$ (**Table 1**). As shown in **Figure 1D** and **1E**, we found that lnCC16 at the baseline visit was associated with COPD progression at the 5-year follow-up visit as measured by a change in $\%FEV_{1pred}$ ($P<0.0001$), as well as change in $\%LAA_{-950}$ ($P=0.0014$). These results held in adjusted linear mixed models (**Table 1**).

To contextualize the clinical relevance of the association between lnCC16 and FEV_1, we measured associations between a 1-SD increase in lnCC16 (SD=0.62) on baseline $\%FEV_1$. In this linear regression model, each SD decrease in lnCC16 was associated with a 172 mL decrease in FEV_1 at the Phase 1 visit, which exceeds the established minimal clinically important difference (MCID) of 100 mL for FEV_1[30]. In a separate logistic regression model evaluating associations between lnCC16 and longitudinal $\%FEV_{1pred}$ change meeting the MCID threshold (≥100 mL decline between P1 and P2 in COPDGene), lnCC16 was not significantly associated with the MCID decline [aOR 1.005, 95%CI 0.733, 1.377, P=0.976]. There was no interaction between maternal smoking exposure and current smoking status on CC16 (*data not shown).* Overall, these results suggest that maternal smoking history has a lasting impact on lung health, where lower expression of CC16 impacts lung function severity and COPD progression, even in non-smoking subjects.

Lower circulating CC16 levels are associated with accelerated lung function decline in COPD in ECLIPSE cohort: We used this cohort to confirm the findings in COPDGene. **Supplemental Table E4** shows the association between baseline lnCC16 or lnCC16 at 1-year with post-BD $\%FEV_1$ or $\%LAA_{-950}$ adjusting by sex, education, race and packs/year. We used a multinominal logistic regression to explore these associations across study groups (**Supplemental Table E5**) show the associations of baseline lnCC16 or lnCC16 at 1-year in both SC and COPD vs. NSC, adjusting by covariates. Scatter plots shown in **Supplemental Figure E6A-B** show the associations between lnCC16 and changes in $\%FEV_{1pred}$ or $\%LAA_{-950}$, respectively. To investigate the association of CC16 with $\%FEV_{1pred}$ decline ECLIPSE individuals with COPD were categorized according to their $\%FEV_{1pred}$ decline during the study in those with Substantial Decline (SDe; i.e. >60ml/year) and those with Stable (S, i.e. ±30 ml/year) decline. CC16 levels (baseline and 1-year follow-up) were compared between the non-smoking normal lung function

Controls (C) and SD and S individuals. CC16 levels were higher in controls than in COPD patients, yet higher in those COPD individuals with SD when compared to accelerated lung function decline (**Table 2**).

Nasal CC16 (SCGB1A1) expression is associated with worse lung function in asthmatic children: Among 272 children with nasal *SCGB1A1* quantification (**Supplemental Table E6**), 9.56% were exposed to maternal smoking during pregnancy, and 15.44% were exposed to passive smoking. Children without passive smoking exposure had significantly higher CC16 expression (**Supplemental Table E7A**), and multivariate regression confirmed a significant correlation between passive smoking exposure and lower CC16 expression (**Supplemental Table E7B).** In a sub-cohort of 63 wheezing children (≤6 years) with longitudinal lung function measurements, lower nasal *SCGB1A1* expression was significantly associated with subsequent lower $FEV_1/FVC_{\%pred}$ and $FEF25\text{-}75_{\%pred}$ in adjusted models (**Supplemental Table E8**).

Maternal smoking exposure is linked to decreased airway CC16 expression and epithelial cell abnormalities in adulthood: LTRC study subject demographics are in **Supplemental Table E9.** The results were adjusted by age, sex and pack-years using a regression model (**Supplemental Table E10**). The $\%CC16^{+}$ airway epithelial cells was lower in adult individuals exposed to maternal smoking across the whole population (**Figures 2A-B**). SC and COPD individuals exposed to maternal smoke had significantly fewer proliferating cells and more apoptotic cells in airway (**Figures 2C-D** respectively), parenchyma and vascular endothelial cells (**Supplemental Figures E7A-D** respectively).

CC16 airway levels are positively correlated with $\%FEV_{1pred}$ and inversely correlated with emphysema severity: The $\%CC16^{+}$ cells directly correlated with

%FEV_{1pred} (**Figure 2E**), FEV_1/FVC ratio, DLCO score, while inversely correlated with whole lung %LAA_{-950} (**Supplemental Figures E8A-C** respectively). The correlation between %CC16+ cells and %FEV_1pred remained significant even when analyzed within maternal smoking exposure subgroups (**Supplemental Figures E9A-B** respectively). The %$CC16^+$ cells directly correlated with the number of proliferating cells (**Supplemental Figures E8D-E** respectively) and inversely correlated with the number of apoptotic cells in the airways and alveoli (**Supplemental Figures E8F-G** respectively).

Overall, human data indicate that individuals with a history of maternal smoking tend to have lower pulmonary and circulating CC16 expression, reduced lung function, increased lung epithelial cell death, and emphysematous change (**Figure 2F**).

Murine data

In utero maternal smoking exposure is associated with reduced airway CC16 expression in murine offspring: CC16 expression in Club cells ($Cyp2F2^+$) was quantified in P28 WT weanlings, with and without maternal smoking exposure. Although the number of Club cells was similar in both groups (not shown), offspring exposed to maternal smoking had significantly less CC16 (**Figure 3**).

CC16 protects against maternal smoking-induced alveolar simplification, airway and vascular remodeling: Maternal smoking *$Cc16^{-/-}$* weanlings had significantly lower weights vs. WT (**Supplemental Figure E10**); there were no differences in litter survival (*data not shown*). *$Cc16^{-/-}$* mice had increased alveolar simplification at P28 (**Figure 4A**) and lower RAC (**Figure 4B**) vs. WT counterparts. Moreover, *$Cc16^{-/-}$* mice had also more abundant ECM deposits around vessels at baseline (**Figure 4C-D**), and the

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

same pattern was observed in the small airways (**Figure 4E**). Maternal smoking exposure exacerbated all these lung pathologies in *Cc16*$^{-/-}$ mice. These findings suggest that the absence of CC16 is sufficient to induce alveolar simplification and SAR in the absence of secondary injury, rendering the developing lung more susceptible to maternal smoking insults.

Cc16 deficiency associated with maternal smoking induced- lung function abnormalities, lung inflammation and cell death in offsprings. *Cc16*$^{-/-}$ weanlings exposed to maternal smoking showed a distinctive right shift in the Pressure-Volume loops with increased elastance and peripheral resistance and decreased in *quasi-static* compliance (**Figure 5A-D** respectively). Moreover, *Cc16*$^{-/-}$ weanlings had significantly higher numbers of alveolar macrophages (**Figure 5E**) vs. WT, both with and without *in utero* maternal smoking exposure. The number of lymphocytes increased only in maternal smoking-exposed P28 *Cc16*$^{-/-}$ weanlings (**Figure 5F**); there were no significant differences in neutrophils (*data not shown*).

Cc16$^{-/-}$ weanlings had significantly more apoptotic cells in the airways (**Figures 6A**) vs. WT, regardless of maternal smoking exposure. *Cc16*$^{-/-}$ weanlings exposed to maternal smoking had significantly fewer proliferating cells in the airways (**Figure 6B**) and had also significantly more alveolar cells undergoing oxidative stress vs. WT counterparts (**Figure 6C**). Overall, maternal smoking exposure reduces Cc16 expression, increases the risk for alveolar simplification and fibrotic remodeling of airways and vessels, and is associated with changes in lung function (**Figure 6D**).

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

***CC16 binds to α2β1 integrin receptor in the developing lung**:* In P28 WT FFPE lung sections, the expression of α2 was ubiquitous across the airway epithelium. The Cc16 protein colocalized with α2 on the cell apical regions (**Figure 7A**). To confirm that CC16 binds to α2 in embryonic lung epithelium, we conducted Co-IP using E15 lung explants. After 15- and 30-minutes incubation, CC16 bound to α2 (**Figure 7B**).

As a *proof-of-concept*, we treated E15 murine lung explants with: ±2%-CSE, ±rhCC16 and ±BTT-3303. In **Figure 7C**, representative pictures of E15 murine embryos ±rhCC16. CSE impairs bronchial branching during lung development, while treatment with rhCC16 significantly enhances branching and mitigates the detrimental effects of smoking (**Figure 7D**). However, these protective effects of rhCC16 were absent in the context of α2 deficiency. Overall, the *in vitro* data suggest that Cc16 replacement, via α2 binding, can protect again CSE-induced blunted branching (**Figure 7E**).

DISCUSSION

We show that *in utero* maternal smoking is associated with reduced CC16 in airways and circulation, and low circulating CC16 predicts faster lung function decline and emphysema progression in adulthood. Additionally: a) maternal smoking–related CC16 loss correlates with increased apoptosis and reduced replication of lung cells; b) CC16 deficiency heightens vulnerability to smoking, impairing lung development and function; and c) CC16 supports lung development partly via α2 receptor binding on airway epithelial cells (**Figure 8**).

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

Maternal smoking and CC16

The developing fetal lung is very sensitive to the effects of *in utero* tobacco products[31,32]. Maternal smoking is associated with preterm birth[33,34], childhood wheezing/asthma, and decrements in lung function during throughout childhood leading to early onset COPD[35]. Similarly, low lung CC16 levels are linked to subsequent poor lung development[36]. However, there are still knowledge gaps in understanding how prenatal insults, like maternal smoking, induce lung development arrest and manifest as early onset loss of lung function[3]. Here we show, using complementary human and murine cohorts, that CC16 deficiency causes lung pathology in offspring and increases vulnerability to maternal smoking, which worsens these effects. Maternal (and passive) smoking harms offspring lungs regardless of CC16 status, but its interaction with alveolar simplification suggests a uniquely harmful impact.

Club cells have the highest levels of cytochrome P450 oxidases and play a crucial role in detoxifying xenobiotics in the human lung[37]. Active smoking-induced acute and chronic damage to Club cells causes increased airway permeability and release of CC16 into the bloodstream[12,38,39]. Interestingly, our findings indicate that while maternal smoking exposure does not affect the number of Club cells, it does reduce CC16 expression within these cells (**Figure 3**). This suggests that smoking exposure may impair the functional capacity of Club cells without compromising their survival.

Our findings from well-characterized human cohorts, such as COPDGene, ECLIPSE, and LTRC, reveal that maternal smoking exposures significantly reduce CC16 levels not only in the lungs but also in the bloodstream. Remarkably, these effects persist for decades, as CC16 measurements were taken when participants were in their 5th-6th

decade of life, long after the initial exposure. In ALLIANCE, passive smoking in asthmatic children was associated with lower nasal *SCGB1A1* expression, which correlated with reduced lung function in a wheezing sub-cohort. Altogether, the human data suggests that early-life insults, including but not limited to maternal smoking, have potentially permanent consequences on CC16 expression, ultimately impacting lung structure and function throughout life.

We previously showed that active CS reduces CC16 levels in the lung, especially in COPD[15,40], and that exogenous CC16 deliver protects from active CS exposure[14]. We now propose that insufficient CC16 levels and/or signaling during a critical early window for lung development are enough to trigger postnatal respiratory dysfunction, setting the stage for lung disease in adulthood such as (early-onset?) COPD.

CC16, lung function decline, and COPD

Longitudinal cohort studies have consistently shown that individuals with lower baseline serum CC16 levels experience a more rapid decrease in $\%FEV_{1pred}$ over time[1,13-15,17,41,42], indicating that CC16 deficiency may contribute to impaired lung repair and heightened susceptibility to environmental and inflammatory damage. Moreover, we and others have shown that individuals with COPD or low-peak lung function not only have lower circulating CC16 vs. controls, but also exhibit decreased CC16 levels in their peripheral lungs, bronchoalveolar lavage fluid and sputum[13-15,17]. Here we show that these associations are also present in smoker controls (**Supplemental Table E5**). Vestbo et al.[21] reported that circulating levels of CC16 were associated with $\%FEV_{1pred}$ decline rate at 3-year in ECLIPSE. Here, we further show that this association is more pronounced in individuals with accelerated decline in $\%FEV_{1pred}$, who also exhibit lower baseline levels. This pattern is similarly observed in a second measurement of CC16 at 1-year follow-up, suggesting that an individual's initial CC16 level may influence the trajectory of disease progression.

These findings confirm CC16 as a key biomarker of lung health, with its deficiency predicting and potentially driving airway damage in COPD[20,21,43]. Across COPDGene, ECLIPSE, and ALLIANCE, low CC16 consistently associated with faster lung function decline and, intriguingly, with slower emphysema progression, highlighting a complex link between CC16 and alveolar cell health supported by murine data.

CC16 and integrin receptor signaling

While some integrin-mediated activities are essential during lung development[44], others promote lung pathologies, including airway remodeling, inflammatory cell recruitment, and airspace enlargement[45]. Our novel findings suggest that CC16 promotes bronchial branching through its interaction with α2 subunit, which may subsequently support alveologenesis.

Binding of α2 subunit to specific ECM components, such as collagen[46,47], can modulate these signaling pathways and drive the growth and development of lung tissue. However, direct binding of CC16 to fibronectin prevents fibronectin-collagen binding and kidney fibrosis in *Cc16*$^{-/-}$ mice but no lung phenotype was observed[48], and CC16 doesn't bind directly to collagen *in vitro*[49]. Our published[13,14] data suggest that CC16 is involved in collagen deposition and/or turnover, as *Cc16*-deficient mice have SAR and vascular remodeling, that is inhibited by exogenous CC16 therapy[14]. Thus, CC16's ability to reduce

ECM deposits[13,14] might be related to the inhibition of integrin receptor signaling[12], reducing collagen secretion and/or content in the lung and ECM remodeling. Additional functional studies are needed to better define the effects of CC16-α2 binding in the developing lung.

Limitations

First, the lower levels of CC16 in adults previously exposed to maternal smoking are purely observational. More functional studies are needed to determine the effects of maternal smoking on CC16 levels, and the protective effects of CC16 against pre- and peri-natal injury. For example, *SCGB1A1* is hypermethylated and repressed during active smoking[50], however, we do not know if maternal smoking causes *SCGB1A1* hypermethylation in fetal lungs. Second, we acknowledge the limitations of self-reported maternal smoking exposure data and the potential confounding effects of participants' own smoking histories, including passive smoking history. We also acknowledge that maternal smoking during pregnancy often co-occurs with postnatal smoking and lower socioeconomic status, making it difficult to disentangle the specific effects of *in utero* exposure from other early-life environmental and social factors. Third, the change in CC16 between measurements could not be included in the model to avoid batch effects. Validation in the large-scale ECLIPSE and ALLIANCE cohorts, linking maternal and/or passive smoking history with longitudinal CC16 and lung function data, supports our hypothesis by showing consistent findings across populations, despite incomplete maternal smoking histories. In ALLIANCE, for instance, the absence of a significant correlation between nasal CC16 and maternal smoking likely reflects the small number of exposed children. Fourth, mechanistically, kinetic assays are needed to confirm and

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

characterize the binding of CC16 to α2 integrin. Our study does not explore the exact mechanisms by which CC16 induces lung development, whether by promoting bronchial branching only, or by a direct effect on the alveolar tissue. Future studies on embryonic and fetal lung tissue are needed to fill these knowledge gaps.

Conclusions

Our findings, based on complementary human and murine studies, demonstrate that CC16 deficiency is linked to impaired lung maturation and function, with maternal smoking exposure further exacerbating this phenotype. We also show that prenatal insults, such as maternal smoking, lead to a lasting reduction in circulating CC16 levels, which in turn is associated with a more rapid decline in lung function and accelerated emphysema progression in COPD. These results position CC16 as a promising biomarker of lung dysfunction across the lifespan and highlight its potential, accessible via non-invasive nasal sampling, as a therapeutic target for restoring lung health.

ACKNOWLEDGMENTS

We are thankful for the strategic collaboration between ALLIANCE, ECLIPSE and COPDGene cohorts. Their contributions are invaluable to this body of work. The full list of Collaborators/Study Group members are detailed in Supplement.

REFERENCES

1. Olvera N, Agusti A, Vonk JM, et al. Heterogeneity of reduced FEV(1) in early adulthood: A looking forward, looking backwards analysis. Respirology 2025. DOI: 10.1111/resp.14876.
2. Melen E, Faner R, Allinson JP, et al. Lung-function trajectories: relevance and implementation in clinical practice. Lancet 2024;403(10435):1494-1503. DOI: 10.1016/S0140-6736(24)00016-3.
3. Polverino F, Washko GR, Covar RA, et al. The low flyers: persistent airflow limitation in young adults. Lancet Respir Med 2022. DOI: 10.1016/S2213-2600(22)00250-8.
4. McEvoy CT, Shorey-Kendrick LE, Milner K, et al. Effect of Vitamin C Supplementation for Pregnant Smokers on Offspring Airway Function and Wheeze at Age 5 Years: Follow-up of a Randomized Clinical Trial. JAMA Pediatr 2023;177(1):16-24. DOI: 10.1001/jamapediatrics.2022.4401.
5. McEvoy CT, Spindel ER. Pulmonary Effects of Maternal Smoking on the Fetus and Child: Effects on Lung Development, Respiratory Morbidities, and Life Long Lung Health. Paediatr Respir Rev 2017;21:27-33. DOI: 10.1016/j.prrv.2016.08.005.
6. Harding R, Maritz G. Maternal and fetal origins of lung disease in adulthood. Semin Fetal Neonatal Med 2012;17(2):67-72. DOI: 10.1016/j.siny.2012.01.005.
7. Allinson JP, Chaturvedi N, Wong A, et al. Early childhood lower respiratory tract infection and premature adult death from respiratory disease in Great Britain: a national birth cohort study. Lancet 2023;401(10383):1183-1193. DOI: 10.1016/S0140-6736(23)00131-9.
8. Collaco JM, Aherrera AD, Breysse PN, Winickoff JP, Klein JD, McGrath-Morrow SA. Hair nicotine levels in children with bronchopulmonary dysplasia. Pediatrics 2015;135(3):e678-86. DOI: 10.1542/peds.2014-2501.
9. Tong VT, Dietz PM, Morrow B, et al. Trends in smoking before, during, and after pregnancy--Pregnancy Risk Assessment Monitoring System, United States, 40 sites, 2000-2010. MMWR Surveill Summ 2013;62(6):1-19. (https://www.ncbi.nlm.nih.gov/pubmed/24196750).
10. Gibbs K, Collaco JM, McGrath-Morrow SA. Impact of Tobacco Smoke and Nicotine Exposure on Lung Development. Chest 2016;149(2):552-561. DOI: 10.1378/chest.15-1858.
11. Broeckaert F, Bernard A. Clara cell secretory protein (CC16): characteristics and perspectives as lung peripheral biomarker. Clin Exp Allergy 2000;30(4):469-75. DOI: 10.1046/j.1365-2222.2000.00760.x.
12. Laucho-Contreras ME, Polverino F, Tesfaigzi Y, Pilon A, Celli BR, Owen CA. Club Cell Protein 16 (CC16) Augmentation: A Potential Disease-modifying Approach for Chronic Obstructive Pulmonary Disease (COPD). Expert Opin Ther Targets 2016;20(7):869-883. DOI: 10.1517/14728222.2016.1139084 [doi].
13. Laucho-Contreras ME, Polverino F, Rojas-Quintero J, Wang X, Owen CA. Club cell protein 16 (Cc16) deficiency increases inflamm-aging in the lungs of mice. Physiol Rep 2018;6(15):e13797. DOI: 10.14814/phy2.13797.

14. Rojas-Quintero J, Laucho-Contreras ME, Wang X, et al. CC16 augmentation reduces exaggerated COPD-like disease in Cc16-deficient mice. JCI Insight 2023. DOI: 10.1172/jci.insight.130771.
15. Laucho-Contreras ME, Polverino F, Gupta K, et al. Protective role for club cell secretory protein-16 (CC16) in the development of COPD. Eur Respir J 2015;45(6):1544-1556. DOI: 09031936.00134214 [pii];10.1183/09031936.00134214 [doi].
16. Iannuzo N, Welfley H, Li NC, et al. CC16 drives VLA-2-dependent SPLUNC1 expression. Front Immunol 2023;14:1277582. DOI: 10.3389/fimmu.2023.1277582.
17. Park HY, Churg A, Wright JL, et al. Club cell protein 16 and disease progression in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2013;188(12):1413-1419. DOI: 10.1164/rccm.201305-0892OC [doi].
18. Regan EA, Hokanson JE, Murphy JR, et al. Genetic epidemiology of COPD (COPDGene) study design. COPD 2010;7(1):32-43. DOI: 10.3109/15412550903499522.
19. Occhipinti M, Paoletti M, Crapo JD, et al. Validation of a method to assess emphysema severity by spirometry in the COPDGene study. Respir Res 2020;21(1):103. DOI: 10.1186/s12931-020-01366-4.
20. Vestbo J, Edwards LD, Scanlon PD, et al. Changes in forced expiratory volume in 1 second over time in COPD. N Engl J Med 2011;365(13):1184-1192. DOI: 10.1056/NEJMoa1105482 [doi].
21. Vestbo J, Anderson W, Coxson HO, et al. Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points (ECLIPSE). Eur Respir J 2008;31(4):869-73. DOI: 10.1183/09031936.00111707.
22. Maison N, Omony J, Illi S, et al. T2-high asthma phenotypes across lifespan. Eur Respir J 2022;60(3). DOI: 10.1183/13993003.02288-2021.
23. Fuchs O, Bahmer T, Weckmann M, et al. The all age asthma cohort (ALLIANCE) - from early beginnings to chronic disease: a longitudinal cohort study. BMC Pulm Med 2018;18(1):140. DOI: 10.1186/s12890-018-0705-6.
24. Jakwerth CA, Weckmann M, Illi S, et al. 17q21 Variants Disturb Mucosal Host Defense in Childhood Asthma. Am J Respir Crit Care Med 2024;209(8):947-959. DOI: 10.1164/rccm.202305-0934OC.
25. Diaz AA, Bartholmai B, San Jose Estepar R, et al. Relationship of emphysema and airway disease assessed by CT to exercise capacity in COPD. Respiratory medicine 2010;104(8):1145-51. DOI: 10.1016/j.rmed.2010.02.023.
26. Laucho-Contreras ME, Taylor KL, Mahadeva R, Boukedes SS, Owen CA. Automated measurement of pulmonary emphysema and small airway remodeling in cigarette smoke-exposed mice. J Vis Exp 2015(95):52236. DOI: 10.3791/52236.
27. Betz P, Nerlich A, Bussler J, Hausmann R, Eisenmenger W. Radial alveolar count as a tool for the estimation of fetal age. Int J Legal Med 1997;110(2):52-4. DOI: 10.1007/s004140050030.
28. Abele AN, Taglauer ES, Almeda M, et al. Antenatal mesenchymal stromal cell extracellular vesicle treatment preserves lung development in a model of bronchopulmonary dysplasia due to chorioamnionitis. Am J Physiol Lung Cell Mol Physiol 2022;322(2):L179-L190. DOI: 10.1152/ajplung.00329.2021.

29. Zemans RL, Jacobson S, Keene J, et al. Multiple biomarkers predict disease severity, progression and mortality in COPD. Respir Res 2017;18(1):117. DOI: 10.1186/s12931-017-0597-7.
30. Donohue JF. Minimal clinically important differences in COPD lung function. COPD 2005;2(1):111-24. DOI: 10.1081/copd-200053377.
31. Zhu X, Huang H, Zong Y, Zhang L. SRY-related high-mobility group box 9 (SOX9) alleviates cigarette smoke extract (CSE)-induced inflammatory injury in human bronchial epithelial cells by suppressing stromal interaction molecule 1 (STIM1) expression. Inflamm Res 2022;71(5-6):565-576. DOI: 10.1007/s00011-022-01576-0.
32. Szymanowska-Narloch A, Jassem E, Skrzypski M, et al. Molecular profiles of non-small cell lung cancers in cigarette smoking and never-smoking patients. Adv Med Sci 2013;58(2):196-206. DOI: 10.2478/ams-2013-0025.
33. Keller RL, Feng R, DeMauro SB, et al. Bronchopulmonary Dysplasia and Perinatal Characteristics Predict 1-Year Respiratory Outcomes in Newborns Born at Extremely Low Gestational Age: A Prospective Cohort Study. J Pediatr 2017;187:89-97 e3. DOI: 10.1016/j.jpeds.2017.04.026.
34. Morrow LA, Wagner BD, Ingram DA, et al. Antenatal Determinants of Bronchopulmonary Dysplasia and Late Respiratory Disease in Preterm Infants. Am J Respir Crit Care Med 2017;196(3):364-374. DOI: 10.1164/rccm.201612-2414OC.
35. Bui DS, Lodge CJ, Burgess JA, et al. Childhood predictors of lung function trajectories and future COPD risk: a prospective cohort study from the first to the sixth decade of life. Lancet Respir Med 2018. DOI: 10.1016/S2213-2600(18)30100-0.
36. Ramsay PL, DeMayo FJ, Hegemier SE, Wearden ME, Smith CV, Welty SE. Clara cell secretory protein oxidation and expression in premature infants who develop bronchopulmonary dysplasia. Am J Respir Crit Care Med 2001;164(1):155-61. DOI: 10.1164/ajrccm.164.1.2008022.
37. Tong SS, Hirokata Y, Trush MA, et al. Clara cell damage and inhibition of pulmonary mixed-function oxidase activity by naphthalene. Biochem Biophys Res Commun 1981;100(3):944-50. DOI: 10.1016/0006-291x(81)91914-8.
38. Egron C, Labbe A, Rochette E, Mulliez A, Bernard A, Flore A. Urinary club cell protein 16 (CC16): Utility of its assay during acute bronchiolitis. Pediatr Pulmonol 2020;55(2):490-495. DOI: 10.1002/ppul.24584.
39. Almuntashiri S, Zhu Y, Han Y, Wang X, Somanath PR, Zhang D. Club Cell Secreted Protein CC16: Potential Applications in Prognosis and Therapy for Pulmonary Diseases. J Clin Med 2020;9(12). DOI: 10.3390/jcm9124039.
40. Laucho-Contreras ME, Polverino F, Tesfaigzi Y, Pilon A, Celli BR, Owen CA. Club Cell Protein 16 (CC16) Augmentation: A Potential Disease-modifying Approach for Chronic Obstructive Pulmonary Disease (COPD). Expert Opin Ther Targets 2016;20(7):869-83. DOI: 10.1517/14728222.2016.1139084.
41. Voraphani N, Stern DA, Ledford JG, et al. Circulating CC16 and Asthma: A Population-based, Multicohort Study from Early Childhood through Adult Life. Am J Respir Crit Care Med 2023;208(7):758-769. DOI: 10.1164/rccm.202301-0041OC.

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

42. Zhai J, Stern DA, Sherrill DL, et al. Trajectories and Early Determinants of Circulating CC16 from Birth to Age 32 Years. Am J Respir Crit Care Med 2018;198(2):267-270. DOI: 10.1164/rccm.201712-2398LE.
43. Guerra S, Halonen M, Vasquez MM, et al. Relation between circulating CC16 concentrations, lung function, and development of chronic obstructive pulmonary disease across the lifespan: a prospective study. Lancet Respir Med 2015;3(8):613-620. DOI: S2213-2600(15)00196-4 [pii];10.1016/S2213-2600(15)00196-4 [doi].
44. Plosa EJ, Benjamin JT, Sucre JM, et al. beta1 Integrin regulates adult lung alveolar epithelial cell inflammation. JCI Insight 2020;5(2). DOI: 10.1172/jci.insight.129259.
45. Morris DG, Huang X, Kaminski N, et al. Loss of integrin alpha(v)beta6-mediated TGF-beta activation causes Mmp12-dependent emphysema. Nature 2003;422(6928):169-73. DOI: 10.1038/nature01413.
46. Bahou WF, Potter CL, Mirza H. The VLA-2 (alpha 2 beta 1) I domain functions as a ligand-specific recognition sequence for endothelial cell attachment and spreading: molecular and functional characterization. Blood 1994;84(11):3734-41. (https://www.ncbi.nlm.nih.gov/pubmed/7949129).
47. Bazan-Socha S, Jakiela B, Zuk J, et al. Interactions via alpha(2)beta(1) Cell Integrin May Protect against the Progression of Airway Structural Changes in Asthma. Int J Mol Sci 2021;22(12). DOI: 10.3390/ijms22126315.
48. Zheng F, Kundu GC, Zhang Z, Ward J, DeMayo F, Mukherjee AB. Uteroglobin is essential in preventing immunoglobulin A nephropathy in mice. Nat Med 1999;5(9):1018-25. DOI: 10.1038/12458.
49. Antico G, Lingen MW, Sassano A, et al. Recombinant human uteroglobin/CC10 inhibits the adhesion and migration of primary human endothelial cells via specific and saturable binding to fibronectin. J Cell Physiol 2006;207(2):553-61. DOI: 10.1002/jcp.20604.
50. Buro-Auriemma LJ, Salit J, Hackett NR, et al. Cigarette smoking induces small airway epithelial epigenetic changes with corresponding modulation of gene expression. Hum Mol Genet 2013;22(23):4726-4738. DOI: ddt326 [pii];10.1093/hmg/ddt326 [doi].

Table 1. Circulating CC16 levels are associated with cross-sectional FEV_1, emphysema, and longitudinal changes reflecting COPD severity in COPDgene.

	Per SD decrease in CC16	95% CI	P
Measures of COPD severity at baseline			
$\%FEV_{1pred}$	**-6.3**	**4.4, 8.3**	**<0.0001**
$\%LAA_{-950}$, HU	0.9	-1.5, -0.3	**0.0041**
Progression of COPD at 5-years of follow-up			
$\%FEV_{1pred}$	**-6.5**	**4.5, 8.4**	**<0.0001**
$\%LAA_{-950}$ change	1.1	-1.8, -0.4	**0.0014**

Y = ln_CC16 + age, race, sex, smoking status, smoking pack-year, maternal smoke, and annual income. One SD change in CC16 is 1.92 ng/mL. Abbreviations: $\%FEV_{1pred}$: forced expiratory volume in 1-second predicted; $\%LAA_{-950}$: percent low attenuation area less than -950 Hounsfield units (HU); CC16: Club cell 16 kDa protein.

Table 2. Circulating CC16 is associated with lung function decline in ECLIPSE cohort.

CC16 (mg/ml)	n	Non-smoking control (C) (N=253)	n	Substantial Decline (SDe) (N=817)	n	Stable (S) (N=492)	P values		
							C vs. SD	C vs. S	SD vs. S
Initial Visit									
Median ± IQR	250	6.61 ± 3.14	799	4.8 ± 3.28	480	5.3 ± 3.75	**<0.001**	**<0.001**	**<0.001**
1 year									
Median ± IQR	212	8.8 ± 5.37	791	5.08 ± 5.76	480	6.05 ± 6.3	**<0.001**	**<0.001**	**0.001**

P values shown are of the post-hoc test after the One-Way ANOVA between the three study groups. Abbreviations: CC16 = Club cell protein 16; C= non-smoking controls; S= Stable decline; SDe Substantial Decline; %FEV_{1pred} = forced expiratory volume in 1-second predicted. Significant P values are in **bold**.

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

FIGURE LEGENDS

Figure 1. **Circulating CC16, maternal smoking exposure and lung function in the COPDGene cohort**. In **A**, lnCC16 levels in NSC, SC, and COPD subjects, with and without maternal smoking history are presented. Data were analyzed using one-way ANOVAs followed by pair-wise testing with U-Mann Whitney test. Boxes in the box-plots show the medians and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles. Pearson's (r) correlation between lnCC16 and (**B**) baseline FEV_1 % predicted, (**C**) baseline %LAA-950, (**D**) change in FEV_1 % predicted, and (**E**) change in %LAA-$_{950}$. Abbreviations: lnCC16: natural-log-transformed Club cell 16-kDa protein; NSC: Non-Smoker Controls; SC: Smoker Controls; %FEV_{1pred}: forced expiratory volume in 1-second predicted; %LAA-$_{950}$: percent low attenuation area less than -950 Hounsfield units (HU).

Figure 2. Maternal smoking is associated with reduced CC16 expression and increased airway and alveolar epithelial cell apoptosis in LTRC cohort. FFPE lung sections from NSC, SC and COPD patients (n=44), with and without history of maternal smoking, were immunostained for CC16, Ki-67, active-caspase-3, and EPCAM. %CC16+ cells (**A-B**) were quantified in airways. Data were analyzed using one-way ANOVAs followed by pair-wise testing with Student's t-test. Data were expressed in bars as mean±SEM; n=4-12/group. Adjusted *P<0.05; ***P<0.001 vs. corresponding NSC or the group indicated. %KI-67+ (**C**) and %Act-Casp3+ (**D**) cells were quantified in airways. Data were analyzed using one-way ANOVAs followed by pair-wise testing with U-Mann Whitney test. Boxes in the box-plots show the medians and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles; n=4-12/group. Adjusted *P<0.05; ***P<0.001

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

vs. corresponding NSC or the group indicated. In (**E**), Spearman correlations (ρ) were calculated between %CC16+ cells and %FEV_{1pred} is shown (n=43). In **F**, diagram depicting the main results from the human cohorts.

Figure 3. Maternal smoking exposure reduces the expression of Cc16 in Club cells. FFPE lung sections from no-maternal smoking and maternal smoking P28 WT mice were triple immunostained for EPCAM, Cyp2F2 (Club Cell marker) and Cc16. Quantification of %Cc16+ cells in WT mice with and without maternal smoking exposure was performed. Data were analyzed using one-way ANOVAs followed by pair-wise testing with U-Mann Whitney test. Boxes in the box-plots show the medians and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles; n=4/group. ***P<0.001 vs. indicated group.

Figure 4. Experimental *Cc16* deficiency in mice is associated with poor lung development and lung pathologies in the offspring, exacerbated by maternal smoking. Decreased alveolarization was assessed by the quantification of alveolar chord length and radical alveolar counts (**A** and **B**) in Gill's stained FFPE lung sections from no-maternal smoking and maternal smoking P28 WT and *Cc16*$^{-/-}$ mice. Data were analyzed using one-way ANOVAs followed by pair-wise testing with U-Mann Whitney test. Boxes in the box-plots show the medians and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles, n=3-6/group. *P<0.05; ***P<0.001 vs. corresponding no-maternal smoking or the group indicated. Fibrotic changes were assessed by the quantification of ECM deposits around small airways (**C**) and around blood vessels (**D**). Data were expressed in bars as mean±SEM, n=3-6/group. Data was analyzed using one-way ANOVAs followed by pair-wise testing with Student's t-test. *P<0.05; ***P<0.001 vs. corresponding no-maternal smoking or the group indicated.

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

Figure 5. Experimental *Cc16* deficiency and maternal smoking in mice are associated with a restrictive lung phenotype in the offspring. Lung function was assessed in P28 WT and *Cc16*$^{-/-}$ mice using a Flexivent® machine. In **A**, Pressure-volume (PV) loops are shown. Tissue elastance (**B**), peripheral airway resistance (**C**) and quasi-static compliance (**D**) are shown. Macrophages (**E**) and lymphocytes (**F**) were quantified from FFPE lung sections from no-maternal smoking and maternal smoking P28 WT and *Cc16*$^{-/-}$ mice. Boxes in the box-plots show the medians and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles. Data expressed in bars represent mean±SEM. All data were analyzed using one-way ANOVAs followed by pair-wise testing with Student's t-test or U-Mann Whitney test accordingly, n=3-6/group. *P<0.05; **P<0.01 vs. corresponding no-maternal smoking or the group indicated.

Figure 6. Experimental *Cc16* deficiency and maternal smoking in mice are associated with increase apoptosis and oxidative stress, and decreased proliferation in the offspring lung epithelial cells. FFPE lung sections from no-maternal smoking and maternal smoking P28 WT and *Cc16*$^{-/-}$ mice were immunostained to quantify active caspase-3 (**A**) and Ki-67 in airways (**B**), and 4-NHE alveolar epithelial cells (**C**). Data were analyzed using one-way ANOVAs followed by pair-wise testing with U-Mann Whitney test. Boxes in the box-plots show the medians and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles; n=3-6/group. **P*<0.05; ****P*<0.001 vs. corresponding no-maternal smoking or the group indicated. In **D**, diagram depicting the main results from the murine studies.

Figure 7. CC16 binds to α2 and protects against smoking-induced bronchial branching impairment in the developing lung model. In **A**, representative pictures

showing CC16 (green), α2-integrin (red), and merger panels in WT airways. In **B**, E15 rodent lung explants were incubated with rhCC16 for 15 and 30 minutes and then used for co-immunoprecipitation followed by conventional SDS-PAGE and western blotting. The immunoblotting was done first with antibody anti-α2 integrin (blue arrow), and second with immunoblotting for anti-Scgb1a1 (red arrow). Results reveal that CC16 binds to a2 in the developing lung. In **C**, representative pictures of bronchial branching in E15 rodent lung explants with and without rhCC16. In **D**, E15 murine lung explants were cultured with complete media and treated as follows: 10 μg/mL rhCC16 (red), 1 μM BTT-3033 (gray), 2% cigarette smoking extract (2%-CSE, dark gray) + 10 μg/mL rhCC16 (green), 2%-CSE + 1 μM BTT-3033 (dark yellow), and 2-CSE + 10 μg/mL rhCC16 + 1 μM BTT-3033 (dark cyan); unstimulated lungs were considered as controls (white). Culture media was changed daily and count performed on day 5. Data were expressed in bars as means±standard error; n=3-6/group. *P<0.05; **P<0.01; ***P<0.001 vs. unstimulated or the group indicated. In **E**, diagram depicting the main results from the in vitro studies.

Figure 8. Working hypothesis: Effects of maternal smoking over CC16 expression, lung development and COPD. Subjects without maternal smoking exposure have normal prenatal CC16 expression. This leads to proper lung development and preserved lung function throughout adulthood. However, subjects with maternal smoking exposure show lower CC16 expression, which is associated with accelerated loss of lung function, faster emphysema progression, predisposing the subjects to early-onset COPD and worse COPD outcomes.

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

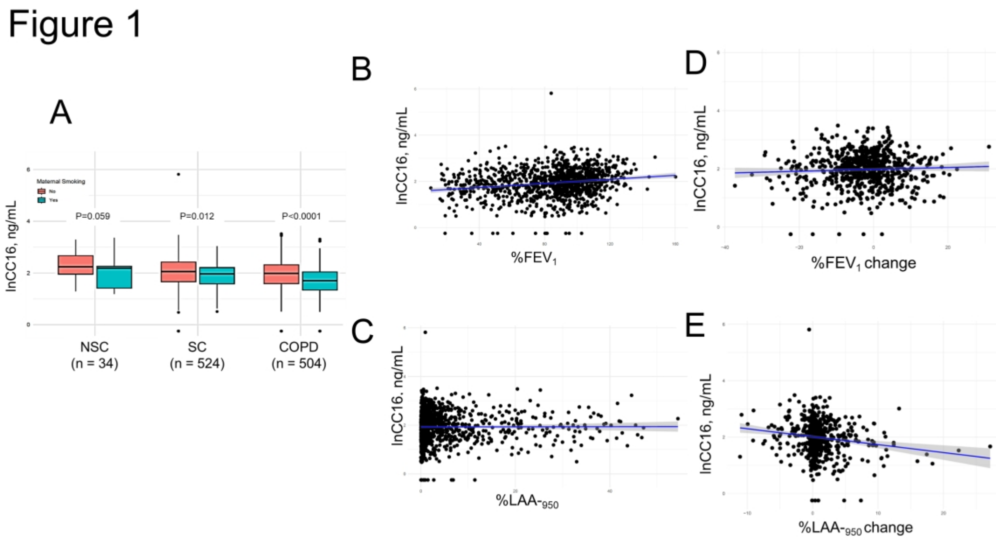


Figure 1. Circulating CC16, maternal smoking exposure and lung function in the COPDGene cohort. In A, lnCC16 levels in NSC, SC, and COPD subjects, with and without maternal smoking history are presented. Data were analyzed using one-way ANOVAs followed by pair-wise testing with U-Mann Whitney test. Boxes in the box-plots show the medians and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles. Pearson's (r) correlation between lnCC16 and (B) baseline FEV1 % predicted, (C) baseline %LAA-950, (D) change in FEV1 % predicted, and (E) change in %LAA-950. Abbreviations: lnCC16: natural-log-transformed Club cell 16-kDa protein; NSC: Non-Smoker Controls; SC: Smoker Controls; %FEV1pred: forced expiratory volume in 1-second predicted; %LAA-950: percent low attenuation area less than -950 Hounsfield units (HU).

861x484mm (236 x 236 DPI)

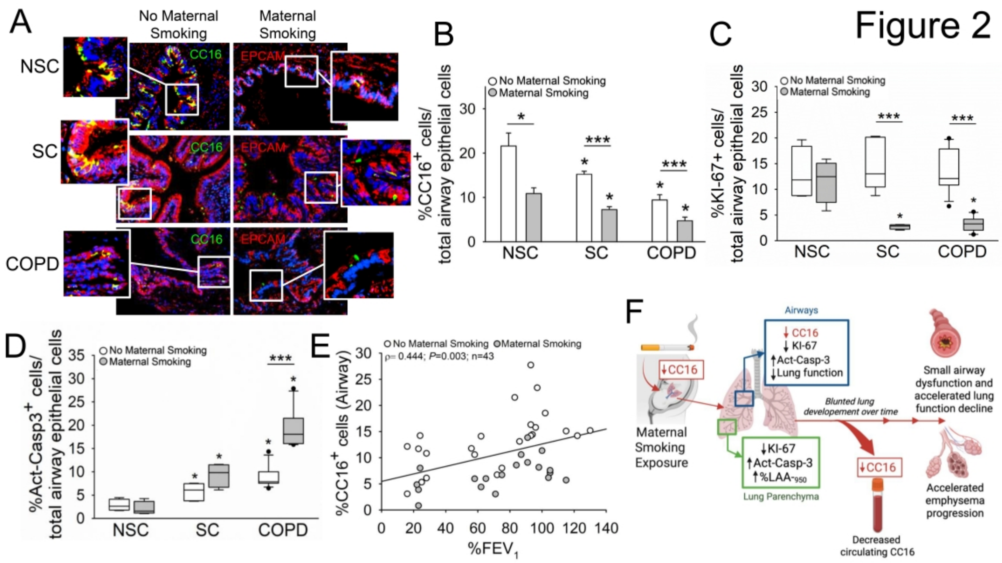


Figure 2. Maternal smoking is associated with reduced CC16 expression and increased airway and alveolar epithelial cell apoptosis in LTRC cohort. FFPE lung sections from NSC, SC and COPD patients (n=44), with and without history of maternal smoking, were immunostained for CC16, Ki-67, active-caspase-3, and EPCAM. %CC16+ cells (A-B) were quantified in airways. Data were analyzed using one-way ANOVAs followed by pair-wise testing with Student's t-test. Data were expressed in bars as mean±SEM; n=4-12/group. Adjusted *P<0.05; ***P<0.001 vs. corresponding NSC or the group indicated. %KI-67+ (C) and %Act-Casp3+ (D) cells were quantified in airways. Data were analyzed using one-way ANOVAs followed by pair-wise testing with U-Mann Whitney test. Boxes in the box-plots show the medians and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles; n=4-12/group. Adjusted *P<0.05; ***P<0.001 vs. corresponding NSC or the group indicated. In (E), Spearman correlations (ρ) were calculated between %CC16+ cells and %FEV1pred is shown (n=43). In F, diagram depicting the main results from the human cohorts.

861x484mm (236 x 236 DPI)

Figure 3

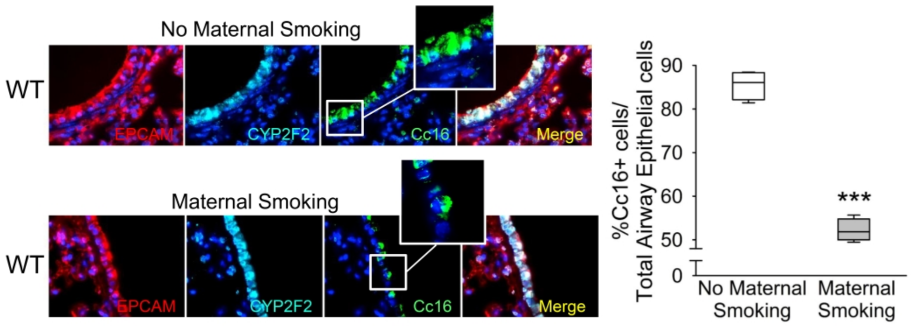


Figure 3. Maternal smoking exposure reduces the expression of Cc16 in Club cells. FFPE lung sections from no-maternal smoking and maternal smoking P28 WT mice were triple immunostained for EPCAM, Cyp2F2 (Club Cell marker) and Cc16. Quantification of %Cc16+ cells in WT mice with and without maternal smoking exposure was performed. Data were analyzed using one-way ANOVAs followed by pair-wise testing with U-Mann Whitney test. Boxes in the box-plots show the medians and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles; n=4/group. ***P<0.001 vs. indicated group.

861x484mm (236 x 236 DPI)

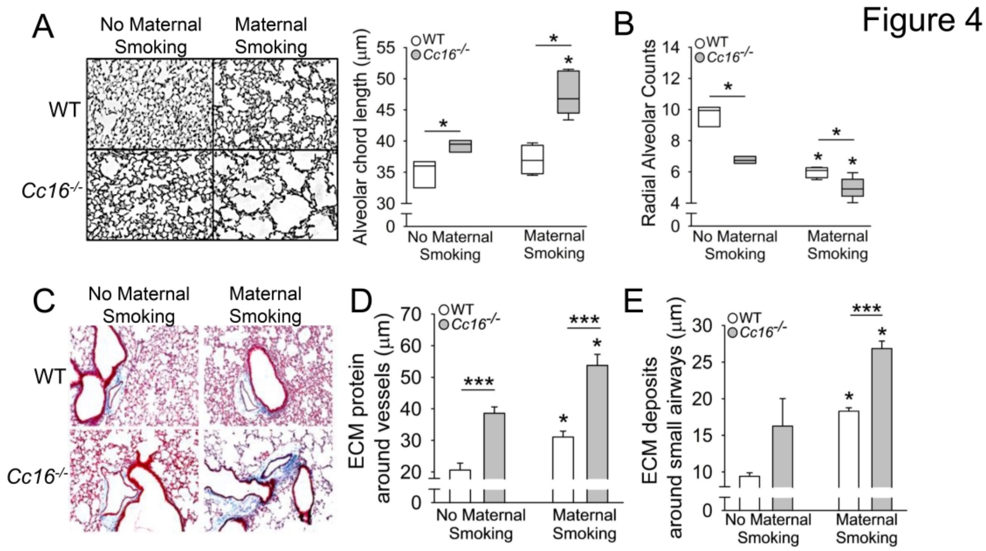


Figure 4. Experimental Cc16 deficiency in mice is associated with poor lung development and lung pathologies in the offspring, exacerbated by maternal smoking. Decreased alveolarization was assessed by the quantification of alveolar chord length and radical alveolar counts (A and B) in Gill's stained FFPE lung sections from no-maternal smoking and maternal smoking P28 WT and Cc16-/- mice. Data were analyzed using one-way ANOVAs followed by pair-wise testing with U-Mann Whitney test. Boxes in the box-plots show the medians and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles, n=3-6/group. *P<0.05; ***P<0.001 vs. corresponding no-maternal smoking or the group indicated. Fibrotic changes were assessed by the quantification of ECM deposits around small airways (C) and around blood vessels (D). Data were expressed in bars as mean±SEM, n=3-6/group. Data was analyzed using one-way ANOVAs followed by pair-wise testing with Student's t-test. *P<0.05; ***P<0.001 vs. corresponding no-maternal smoking or the group indicated.

861x484mm (236 x 236 DPI)

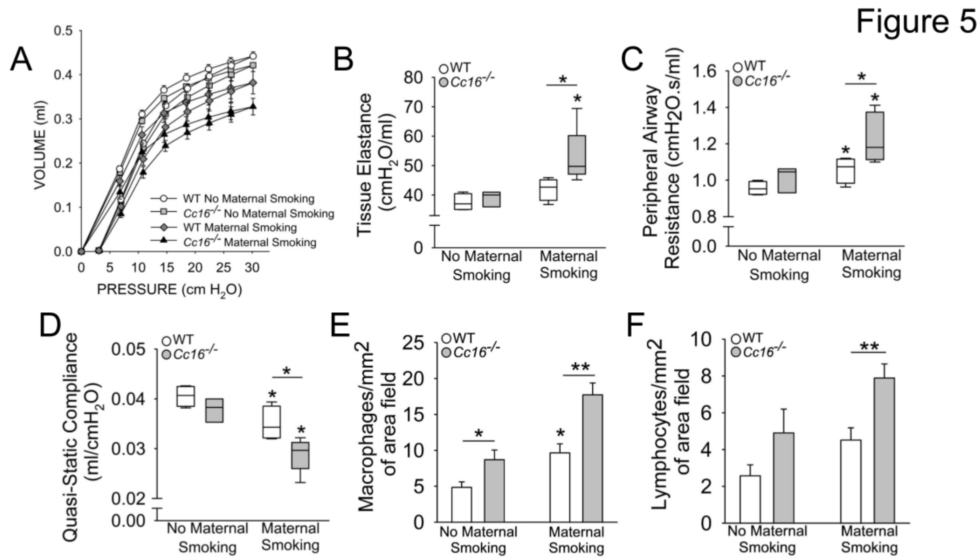


Figure 5. Experimental Cc16 deficiency and maternal smoking in mice are associated with a restrictive lung phenotype in the offspring. Lung function was assessed in P28 WT and Cc16-/- mice using a Flexivent® machine. In A, Pressure-volume (PV) loops are shown. Tissue elastance (B), peripheral airway resistance (C) and quasi-static compliance (D) are shown. Macrophages (E) and lymphocytes (F) were quantified from FFPE lung sections from no-maternal smoking and maternal smoking P28 WT and Cc16-/- mice. Boxes in the box-plots show the medians and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles. Data expressed in bars represent mean±SEM. All data were analyzed using one-way ANOVAs followed by pair-wise testing with Student's t-test or U-Mann Whitney test accordingly, n=3-6/group. *P<0.05; **P<0.01 vs. corresponding no-maternal smoking or the group indicated.

861x484mm (236 x 236 DPI)

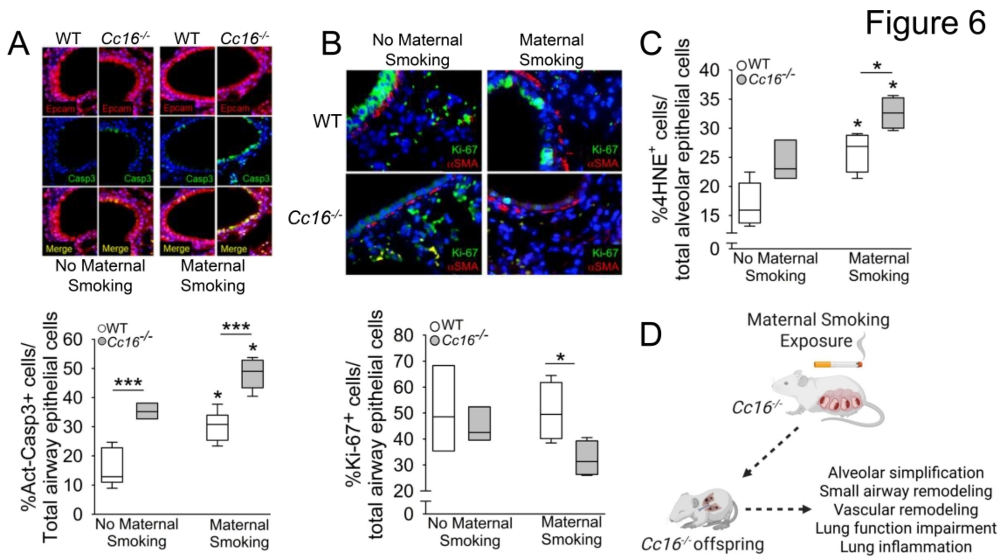


Figure 6. Experimental Cc16 deficiency and maternal smoking in mice are associated with increase apoptosis and oxidative stress, and decreased proliferation in the offspring lung epithelial cells. FFPE lung sections from no-maternal smoking and maternal smoking P28 WT and Cc16-/- mice were immunostained to quantify active caspase-3 (A) and Ki-67 in airways (B), and 4-NHE alveolar epithelial cells (C). Data were analyzed using one-way ANOVAs followed by pair-wise testing with U-Mann Whitney test. Boxes in the box-plots show the medians and 25th and 75th percentiles, and whiskers show the 10th and 90th percentiles; n=3-6/group. *P<0.05; ***P<0.001 vs. corresponding no-maternal smoking or the group indicated. In D, diagram depicting the main results from the murine studies.

861x484mm (236 x 236 DPI)

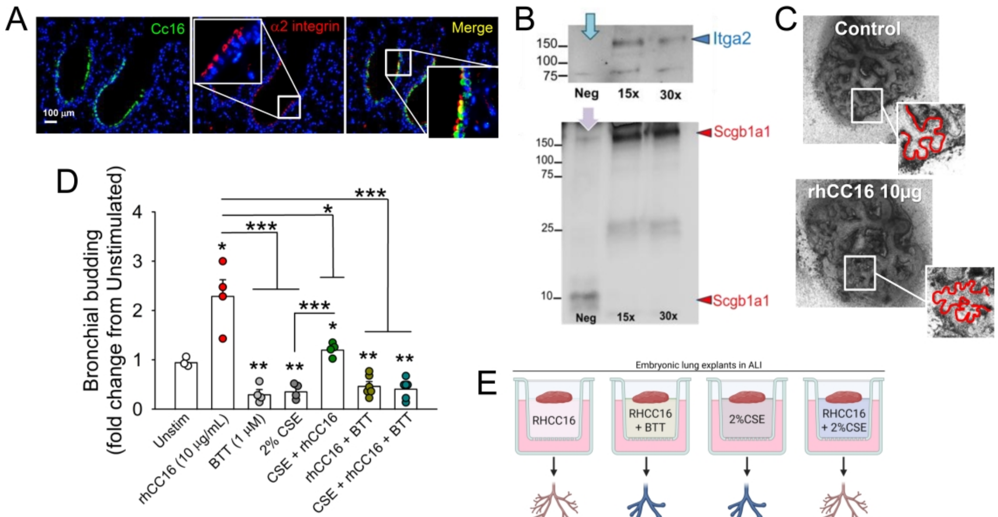


Figure 7

Figure 7. CC16 binds to α2 and protects against smoking-induced bronchial branching impairment in the developing lung model. In A, representative pictures showing CC16 (green), α2-integrin (red), and merger panels in WT airways. In B, E15 rodent lung explants were incubated with rhCC16 for 15 and 30 minutes and then used for co-immunoprecipitation followed by conventional SDS-PAGE and western blotting. The immunoblotting was done first with antibody anti-α2 integrin (blue arrow), and second with immunoblotting for anti-Scgb1a1 (red arrow). Results reveal that CC16 binds to a2 in the developing lung. In C, representative pictures of bronchial branching in E15 rodent lung explants with and without rhCC16. In D, E15 murine lung explants were cultured with complete media and treated as follows: 10 µg/mL rhCC16 (red), 1 µM BTT-3033 (gray), 2% cigarette smoking extract (2%-CSE, dark gray) + 10 µg/mL rhCC16 (green), 2%-CSE + 1 µM BTT-3033 (dark yellow), and 2-CSE + 10 µg/mL rhCC16 + 1 µM BTT-3033 (dark cyan); unstimulated lungs were considered as controls (white). Culture media was changed daily and count performed on day 5. Data were expressed in bars as means±standard error; n=3-6/group. *P<0.05; **P<0.01; ***P<0.001 vs. unstimulated or the group indicated. In E, diagram depicting the main results from the in vitro studies.

861x484mm (236 x 236 DPI)

Figure 8

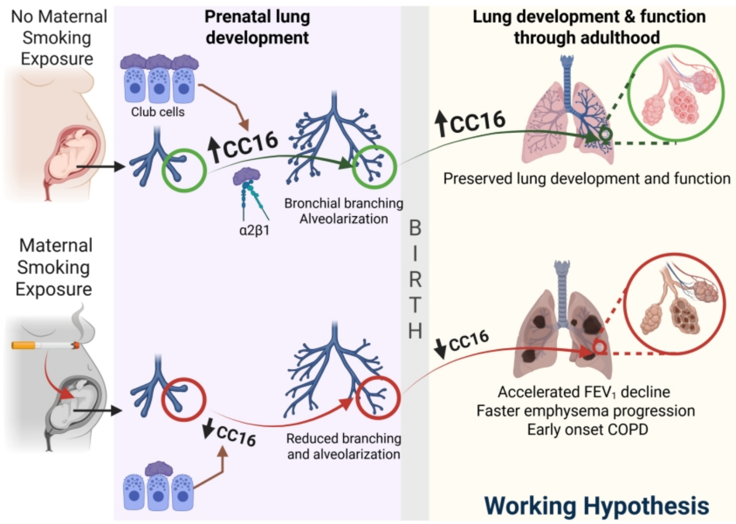


Figure 8. Working hypothesis: Effects of maternal smoking over CC16 expression, lung development and COPD. Subjects without maternal smoking exposure have normal prenatal CC16 expression. This leads to proper lung development and preserved lung function throughout adulthood. However, subjects with maternal smoking exposure show lower CC16 expression, which is associated with accelerated loss of lung function, faster emphysema progression, predisposing the subjects to early-onset COPD and worse COPD outcomes.

861x484mm (236 x 236 DPI)

Maternal Smoking and CC-16: Implications for Lung Development and COPD Across the Lifespan

Joselyn Rojas-Quintero, MSc, MD, Rosa Faner, PhD, Chia-Ying Chiu, MPH, Jeff T. Kue, BS, Yun Zhang, PhD, David Sanz Rubio, PhD, Adrianne S. Colborg, MD, Constanze A. Jakwerth, PhD, Carsten B. Schmidt-Weber, PhD, Anke-Hilse Maitland-van der Zee, PhD, Mahmoud I Abdel-Aziz, PhD, Aprile L. Pilon, PhD, Caroline A. Owen, MD, PhD, Erin J. Plosa, MD, Gregory L. Kinney, PhD, Sharon Mc-Grath Morrow, MD, Mariacarolina G. Gazzaneo, MD, Nahir Cortes Santiago, MD, Krithika Lingappan, MSc, MD, PhD, Julie Ledford, PhD, Jason Spence, PhD, Jennifer M. Sucre, MD, Maor Sauler, MD, Tianshi David Wu, MD, Alvar Agusti, MD, Asa Wheelock, PhD, Sabina Illi, PhD, Erika von Mutius, MD, Russell P. Bowler, MD, PhD, Bartolome Celli, MD, Steven H. Abman, MD, J. Michael Wells, MD, MSPH, Francesca Polverino, MD, PhD, and ALLIANCE study group[25], ECLIPSE[26] and COPDGene investigators

ONLINE DATA SUPPLEMENT

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

Collaborators/Study group members

The members of the **ALLIANCE study group** are (in alphabetical order per center)[1,2]: CPC-M (Munich): Mira Bürk, Sybille Contento, Markus Ege, Kristina Gottschau, Silvia Gschwendtner, Alexander Hose, Lena Lagally, Nicole Maison, Jimmy Omony, Bianca Schaub, Michael Schloter, Lena Ullemeyer, Esther Zeitlmann, Ulrich Zissler; ARCN (Lübeck/Großhansdorf): Mustafa Abdo, Thomas Bahmer, Heike Biller, Folke Brinkmann, Karoline I. Gaede, Clara Haug, Nikolas Jacobs, Anne-Marie Kirsten, Inke R. König, Matthias V. Kopp, Lea Kronziel, Gyde Nissen, Frauke Pedersen, Loana Penner, Klaus F. Rabe, Isabell Ricklefs, Vera Veith, Benjamin Waschki, Henrik Watz, Markus Weckmann; BREATH (Hannover): Marie Bickes, Mifflin-Rae Calvero, David S. DeLuca, Anna-Maria Dittrich, Svenja Gaedcke, Ruth Grychtol, Anika Habener, Gesine Hansen, Christine Happle, Adan Chari Jirmo, Bin Liu, Lennart Riemann, Nicolaus Schwerk; Marburg: Svenja Foth, Inga Jerrentrup, Harald Renz, Chrysanthi Skevaki, Stefanie Weber; Cologne: Miguel A. Alejandre Alcazar, Samira Blau, Silke van Koningsbruggen-Rietschel, Ernst Rietschel, Tobias Trojan, Jan-Christoph Thomassen. Member of the German Center for Lung Research (DZL), Germany.

The ECLIPSE Investigators are[3,4]: Y Ivanov, K Kostov, J Bourbeau, M Fitzgerald, P Hernández, K Killian, R Levy, F Maltais, D O'Donnell, J Krepelka, J Vestbo, E Wouters, D Quinn, P Bakke, M Kosnik, A Agusti, Jaume Sauleda, Y Feschenko, V Gavrisyuk, L Yashina, W MacNee, D Singh, J Wedzicha, A Anzueto, S Braman, R Casaburi, B Celli, G Giessel, M Gotfried, G Greenwald, Rancho Mirage, N Hanania, D Mahler, B Make, S

Rennard, C Rochester, P Scanlon, D Schuller, F Sciurba, A Sharafkhaneh, T Siler, St Charles, E Silverman, A Wanner, R Wise, R ZuWallack.

The COPDGene investigators are[18]: *Administrative Center*: James D. Crapo, MD (PI); Edwin K. Silverman, MD, PhD (PI); Barry J. Make, MD; Elizabeth A. Regan, MD, PhD. *Genetic Analysis Center*: Terri H. Beaty, PhD; Peter J. Castaldi, MD, MSc; Michael H. Cho, MD, MPH; Dawn L. DeMeo, MD, MPH; Adel El Boueiz, MD, MMSc; Marilyn G. Foreman,MD, MS; Auyon Ghosh, MD; Lystra P. Hayden, MD, MMSc; Craig P. Hersh, MD, MPH; Jacqueline Hetmanski, MS; Brian D. Hobbs, MD, MMSc; John E. Hokanson, MPH, PhD; Wonji Kim, PhD; Nan Laird, PhD; Christoph Lange, PhD; Sharon M. Lutz, PhD; Merry-Lynn McDonald, PhD; Dmitry Prokopenko, PhD; Matthew Moll, MD, MPH; Jarrett Morrow, PhD; Dandi Qiao, PhD; Elizabeth A. Regan, MD, PhD; Aabida Saferali, PhD; Phuwanat Sakornsakolpat, MD; Edwin K. Silverman, MD, PhD; Emily S. Wan, MD; Jeong Yun, MD, MPH. *Imaging Center*: Juan Pablo Centeno; Jean-Paul Charbonnier, PhD; Harvey O. Coxson, PhD; Craig J. Galban, PhD; MeiLan K. Han, MD, MS; Eric A. Hoffman, Stephen Humphries, PhD; Francine L. Jacobson, MD, MPH; Philip F. Judy, PhD; Ella A. Kazerooni, MD; Alex Kluiber; David A. Lynch, MB; Pietro Nardelli, PhD; John D. Newell, Jr., MD; Aleena Notary; Andrea Oh, MD; Elizabeth A. Regan, MD, PhD; James C. Ross, PhD; Raul San Jose Estepar, PhD; Joyce Schroeder, MD; Jered Sieren; Berend C. Stoel, PhD; Juerg Tschirren, PhD; Edwin Van Beek, MD, PhD; Bram van Ginneken, PhD; Eva van Rikxoort, PhD; Gonzalo Vegas Sanchez-Ferrero, PhD; Lucas Veitel; George R. Washko, MD; Carla G. Wilson, MS. *PFT QA Center*: Salt Lake City, UT: Robert Jensen, PhD. *Data Coordinating Center and Biostatistics, National Jewish Health,* *Denver, CO*: Douglas Everett, PhD; Jim Crooks, PhD; Katherine Pratte, PhD; Matt Strand, PhD; Carla G. Wilson, MS. *Epidemiology Core, University of Colorado Anschutz Medical Campus, Aurora, CO*: John E. Hokanson, MPH, PhD; Erin Austin, PhD; Gregory Kinney, MPH, PhD; Sharon M. Lutz, PhD; Kendra A. Young, PhD. *Mortality Adjudication Core*: Surya P. Bhatt, MD; Jessica Bon, MD; Alejandro A. Diaz, MD, MPH; MeiLan K. Han, MD, MS; Barry Make, MD; Susan Murray, ScD; Elizabeth Regan, MD; Xavier Soler, MD; Carla G. Wilson, MS. *Biomarker Core*: Russell P. Bowler, MD, PhD; Katerina Kechris, PhD; Farnoush Banaei-Kashani, PhD. *Clinical Centers*: Ann Arbor VA: Jeffrey L. Curtis, MD; Perry G. Pernicano, MD; Baylor College of Medicine, Houston, TX: Nicola Hanania, MD, MS; Mustafa Atik, MD; Aladin Boriek, PhD; Kalpatha Guntupalli, MD; Elizabeth Guy, MD; Amit Parulekar, MD; Brigham and Women's Hospital, Boston, MA: Dawn L. DeMeo, MD, MPH; Craig Hersh, MD, MPH; Francine L. Jacobson, MD, MPH; George Washko, MD; Columbia University, New York, NY: R. Graham Barr, MD, DrPH; John Austin, MD; Belinda D'Souza, MD; Byron Thomashow, MD; Duke University Medical Center, Durham, NC: Neil MacIntyre, Jr., MD; H. Page McAdams, MD; Lacey Washington, MD; HealthPartners Research Institute, Minneapolis, MN: Charlene McEvoy, MD, MPH; Joseph Tashjian, MD, Johns Hopkins University, Baltimore, MD: Robert Wise, MD; Robert Brown, MD; Nadia N. Hansel, MD, MPH; Karen Horton, MD; Allison Lambert, MD, MHS; Nirupama Putcha, MD, MHS; Lundquist Institute for Biomedical Innovation at Harbor UCLA Medical Center, Torrance, CA: Richard Casaburi, PhD, MD; Alessandra Adami, PhD; Matthew Budoff, MD; Hans Fischer, MD; Janos Porszasz, MD, PhD; Harry Rossiter, PhD; William Stringer, MD; Michael E. DeBakey VAMC, Houston, TX: Amir Sharafkhaneh, MD, PhD; Charlie Lan, DO; Minneapolis VA: Christine Wendt, MD; Brian

Bell, MD; Ken M. Kunisaki, MD, MS; Morehouse School of Medicine, Atlanta, GA: Eric L. Flenaugh, MD; Hirut Gebrekristos, PhD; Mario Ponce, MD; Silanath Terpenning, MD; Gloria Westney, MD, MS; National Jewish Health, Denver, CO: Russell Bowler, MD, PhD; David A. Lynch, MB; Reliant Medical Group, Worcester, MA: Richard Rosiello, MD; David Pace, MD; Temple University, Philadelphia, PA: Gerard Criner, MD; David Ciccolella, MD; Francis Cordova, MD; Chandra Dass, MD; Gilbert D'Alonzo, DO; Parag Desai, MD; Michael Jacobs, PharmD; Steven Kelsen, MD, PhD; Victor Kim, MD; A. James Mamary, MD; Nathaniel Marchetti, DO; Aditi Satti, MD; Kartik Shenoy, MD; Robert M. Steiner, MD; Alex Swift, MD; Irene Swift, MD; Maria Elena Vega-Sanchez, MD; University of Alabama, Birmingham, AL: Mark Dransfield, MD; William Bailey, MD; Surya P. Bhatt, MD; Anand Iyer, MD; Hrudaya Nath, MD; J. Michael Wells, MD; University of California, San Diego, CA: Douglas Conrad, MD; Xavier Soler, MD, PhD; Andrew Yen, MD; University of Iowa, Iowa City, IA: Alejandro P. Comellas, MD; Karin F. Hoth, PhD; John Newell, Jr., MD; Brad Thompson, MD; University of Michigan, Ann Arbor, MI: MeiLan K. Han, MD MS; Ella Kazerooni, MD MS; Wassim Labaki, MD MS; Craig Galban, PhD; Dharshan Vummidi, MD; University of Minnesota, Minneapolis, MN: Joanne Billings, MD; Abbie Begnaud, MD; Tadashi Allen, MD; University of Pittsburgh, Pittsburgh, PA: Frank Sciurba, MD; Jessica Bon, MD; Divay Chandra, MD, MSc; Joel Weissfeld, MD, MPH; University of Texas Health, San Antonio, San Antonio, TX: Antonio Anzueto, MD; Sandra Adams, MD; Diego Maselli-Caceres, MD; Mario E. Ruiz, MD; Harjinder Singh.

HUMAN STUDIES

COPDgene. The COPDGene study is a prospective, multicenter cohort study of current and former smokers enriched for individuals with COPD. The COPDGene study design has been previously described[5]. Briefly, adults aged 45-80 years with self-identified non-Hispanic White or Black/African-American race were enrolled at 21 clinical centers in the United States between 2007 and 2011 (Phase 1 visit) and had follow-up visits 5 years after the index visit (Phase 2 visit). Health questionnaires, spirometry, High Resolution Computed Tomography (HRCT) scans, and prospective exacerbation data were collected for the entire cohort; blood was collected for biomarker assessment in a subset of participants at the initial study visit. Emphysema was quantified using the percent low attenuation area less than -950 Hounsfield units ($\%LAA_{-950}$)[6]. Maternal smoke exposure was self-reported by answering the question "*Did your mother smoke cigarettes when she was pregnant?"* Baseline plasma CC16 was measured by enzyme-linked immunosorbent assay (ELISA). CC16 results were only available at the Phase 1 visit. Annual income was self-reported by participants at the Phase 2 study visit and we assumed annual income stability between Phase 1 and Phase 2 visits as previously reported[7]. See **Supplemental Figure E1**.

ECLIPSE. The ECLIPSE study (Clinicaltrials.gov NCT00292552) was a prospective, international, controlled, observational cohort study that included 2,164 patients with COPD, 337 smokers (>10 pack-years) and 245 nonsmoker participants with normal spirometry, who were followed-up over 3 years[3,4]. The design, methodology and CC16 plasma measurement of ECLIPSE have been previously published[3,4]. All study subjects provided written informed consent. The study was approved by the Ethics

Committees of all participating institutions. The ECLIPSE study was supported by GSK. See **Supplemental Figure E2.**

ALLIANCE. The ALLIANCE cohort, part of the German Center for Lung Research (DZL), is a prospective, multicenter asthma study involving five pediatric centers (Hannover, Lubeck, Munich, Marburg, Cologne) and two adult centers (LungenClinic Grosshansdorf, Research Centre Borstel)[1,2] . The study protocol received approval from all local ethics committees, with written informed consent obtained from parents of participants under 18 and from participants 18 and older. It is registered at ClinicalTrials.gov (pediatric: NCT02496468, adult: NCT02419274). Eligible participants included children aged 6 months to 5 years with at least two wheezing episodes in the past year (“preschool wheezer”), based on parental reports, and those 6 years and older, including adults, with doctor-diagnosed asthma per GINA[8] and German[9] guidelines. Smoking history did not exclude participation. Healthy controls, matched for age and sex, were included if they had no history of asthma or preschool wheezing, regardless of other allergic conditions. Spirometry was measured in participants ≥4 years and quality was controlled according to ATS/ERS guidelines. See **Supplemental Figure E3.**

Nasal brushes were collected using dental brushes (IDB-G50, Top Caredent) in sterile PBS, brushing the inferior nasal meatus with rotatory and linear movements. One sample per participant was analyzed at the earliest visit, immediately placed in a 2mL cryotube with RNA-protect Cell reagent (Qiagen), and stored at -80°C. For genotyping, peripheral blood was collected in 1.2mL K+EDTA S-Monovetres (Sarsted) and frozen for DNA extraction. RNA was extracted using the AllPrep DNA/RNA Micro Kit (Qiagen), quantified by UV-spectrophotometry (Nanodrop), and integrity assessed with the RNA

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

6000 Nano Chip Kit on the Agilent 2100 Bioanalyzer. Total RNA was amplified and Cy3-labeled using the Low Input Quick Amp Labeling Kit (Agilent), hybridized in one batch with SurePrint G3 Human Gene Expression 8x60K Microarrays, and analyzed using GeneSpring GX v14.9.1. Array signals were verified, compromised signals excluded, and gene expression data normalized using an 80th percentile shift for noise reduction and comparability.

Lung Tissue Research Consortium (***LTRC).*** De-identified formalin-fixed paraffin-embedded (FFPE) lung sections from never-smoker NSC, SC, and COPD patients were obtained from the LTRC supported by the National Heart, Lung, and Blood Institute (NHLBI)[10]. Overall, 44 age-, sex-, and pack-year-matched subjects were selected and categorized based on whether they had documented *in utero* maternal smoke exposure (**Supplemental Figure E4** and **Supplemental Table E9**).

The sections were immunostained for CC16, active caspase-3 (apoptosis marker), Ki-67 (cell replication marker), and EPCAM or Pan-cytokeratin (epithelial cell markers) (**Supplemental Table E1** for antibody list). Positive CC16, KI-67, and Active-caspase-3 cells were quantified using MetaMorph®[11,12]. Cell definitions:

A) <u>For CC16 quantification</u>, CC16-expressing cells were labelled as EPCAM+CC16+ airway epithelial cells. For quantification, the %CC16+ cells was calculated as the percentage of CC16+ cells per total number of EPCAM+ cells in the airways.

B) <u>For Apoptosis</u>, the %Active Caspase-3+ cells was calculated as the percentage of ACTIVE CASPASE-3+ cells per total number of EPCAM+ (airway or alveolar epithelial cells) or EPCAM- cells (endothelial cells).

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

C) <u>For Oxidative stress</u>: the %4-HNE+ cells was calculated as the percentage of 4-HNE+ cells per total number of EPCAM+ alveolar epithelial cells.

D) <u>Cell replication assessment</u>: the %KI-67+ cells was calculated as the percentage of KI-67+ cells per total number of EPCAM+ (airway or alveolar epithelial cells) or EPCAM- cells (endothelial cells).

RODENT EXPERIMENTS

Procedures performed were approved by the Brigham and Women's Hospital and Baylor College of Medicine Institutional Animal Care and Use Committees. *Cc16*$^{-/-}$ mice were obtained in a pure C57BL/6 background[12] have a normal lifespan and fertility and have no lethal abnormalities in major organs in the unchallenged state. Murine *Cc16*-deficiency leads to spontaneous emphysema over time[11]. Colonies of *Cc16*$^{-/-}$ mice and C57BL/6 wild-type (purchased from Jackson Laboratories) mice were housed under identical specific pathogen-free conditions in a barrier facility. They were fed normal chow diet (PicoLab® Rodent Diet 5053) and water *ad libitum*. The genotype of the *Cc16*$^{-/-}$ mice were confirmed using PCR-based protocols performed on genomic DNA extracted from tail biopsies. Adult male and female WT and *Cc16*$^{-/-}$ mice were used in all experiments and were randomized to experimental groups by an individual who was not involved in the conduct of the experiments. See **Supplemental Figure E5.**

Maternal smoking exposures in mice. WT C57BL/6 and *Cc16*$^{-/-}$ mice were housed in the same barrier facility under the same pathogen-free conditions, and access to normal chow diet and water *ad libitum*. WT and *Cc16*$^{-/-}$ breeding pair mice were exposed to room air or mixed CS with Teague smoking chamber and monitored by quantifying total suspended particulate matter (~150 ppm)[13,14]. CS exposure was done 6 days a week, 2 hours daily, throughout copulation and pregnancy, and exposure was stopped when they gave birth to mimic *in utero* maternal smoking exposure in humans. The newborn pups were not exposed to CS after birth and were left to grow exposed to room air. Maternal smoking- and no-maternal smoking WT and *Cc16*$^{-/-}$ weanlings were humanely euthanized and lungs were harvested at post-natal day-1 (P1), 7, 14, and 28 (**Supplemental Figure E5**).

Lung Pathologies:

Airspace Enlargement. Lungs from offspring from C57BL/6 background WT and *Cc16*$^{-/-}$ breeding pairs were harvested at Postnatal day-28 (P28). Then, lungs were harvested and fixed overnight in 10% formalin, followed by paraffin-embedding (FFPE). Mid-sagittal sections of lungs (8 µm thick) were stained with Gill's stain (Sigma Aldrich). The images of both lungs were captured at 200 X magnification using a Nikon microscope (8-10 images per mouse). Scion Image® software was used to measure alveolar chord lengths, as described previously[12,15].

Radial Alveolar Counts. Lungs from offspring from C57BL/6 background WT and *Cc16*$^{-/-}$ breeding pairs were harvested at P28. Then, lungs were harvested and fixed overnight in 10% formalin, followed by FFPE. Mid-sagittal sections of lungs (8 µm thick) were stained with Gill's stain (Sigma Aldrich). The images of both lungs were captured at 200 X magnification using a Nikon microscope (8-10 images per mouse), considering obtaining imaged of terminal bronchi close to pleural membrane. Alveoli were counted by number after transecting the center of a respiratory bronchiole with a perpendicular line drawn from the to the nearest septal division or pleural margin [16].

Small Airway Remodeling: Lungs from offspring from C57BL/6 background WT and *Cc16*$^{-/-}$ breeding pairs were harvested at P28. Then, lungs were harvested and fixed overnight in 10% formalin, followed by FFPE. Slides were with Masson's Trichrome stain, and images of both lungs were acquired at 200 X magnification. The mean airway luminal diameter and the thickness of the sub-epithelial layer of extracellular matrix protein deposited around vessels and around airways having a mean diameter of 300-699 microns were measured at 12 equidistant sites around each airway using MetaMorph® Software [12,15]. The mean ± SEM thickness of the subepithelial layer of extracellular matrix layer in microns was calculated for airways of. Sections of airways sharing their adventitia with arteries were not included in the analysis.

Respiratory mechanics. Mice were anesthetized with a cocktail of 100 mg/kg ketamine, 10 mg/kg xylazine, and 3 mg/kg acepromazine. An 18-gauge cannula was inserted and secured in the trachea using sutures. Cannula was connected to a digitally-controlled mechanical ventilator (FlexiVent device; Scireq Inc., Montreal, QC, Canada). Ventilator settings were f = 150/min, FiO_2 = 0.21, tidal volume =10 ml/kg body weight, positive end-expiratory pressure (PEEP) of 3 cm H_20, and total lung capacity (25 cmH_2O) measured 3 times for volume history. Tissue resistance (G) and tissue elastance (H) at PEEP of 3 cmH_2O were then measured, followed by dynamic compliance, stepwise quasi-static compliance (Cst), and volume-pressure curves. Mice were humanely euthanized at the end of the measurements, and the lungs were inflated and removed.

Lung inflammation. Lungs from offspring from C57BL/6 background WT and *Cc16$^{-/-}$* breeding pairs were harvested at P28. Then, lungs were harvested and fixed overnight in 10% formalin, followed by FFPE. Using the Trichrome-stained images, we quantified the number of leukocytes per field in at least 20 randomly-selected non-consecutive fields/animal.

Immunofluorescence studies. Briefly, FFPE lung sections were deparaffinized, and antigen retrieval was performed by heating the slides in a microwave in 10 mM citrate buffer (pH 6.0) for 10 min. The sections were blocked overnight at 4°C in PBS containing 1% albumin and 5% normal goat, rabbit, and/or mouse serum. The sections were incubated at 4°C overnight with primary antibody or non-immune rabbit IgG (Agilent, Carpinteria, CA) applied at the same concentration. After washing the lung sections with PBS, the sections were incubated for 1 h at 37°C with corresponding secondary antibody. After washing the slides twice with PBS, the sections were incubated with Sudan Black buffer for 25 min at room temperature. After washing the slides twice with PBS, nuclei were counterstained with DAPI mounting gel (Abcam, Cambridge, MA). Approximately, 8-12 images at 400 X magnification images per mouse were randomly acquired using a Leica microscope and a digital camera. Positive CC16, KI-67, Active-caspase-3 and 4-HNE cells were quantified using MetaMorph®[11,12]. Dilutions for each antibody used are shown in **Supplemental Table E1**. Cell definitions:

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

A. For Cc16 quantification, Cc16-expressing cells were labelled as Epcam+Cc16+ airway epithelial cells. For quantification, the %Cc16+ cells was calculated as the percentage of Cc16+ cells per total number of Epcam+ cells in the airways.
B. For Club cell assessment, positive Club cells (epithelial lineage) were defined as Epcam+CyP2f2+ double positive cells. For Cc16-expressing Club cells, the cells were defined as Epcam+CyP2f2+Cc16+ triple positive cells.
C. For Apoptosis, the %Active caspase-3+ cells was calculated as the percentage of active caspase-3+ cells per total number of Epcam+ (airway or alveolar epithelial cells) or Epcam- cells (endothelial cells).
D. For Oxidative stress: the %4-HNE+ cells was calculated as the percentage of 4-HNE+ cells per total number of Epcam+ alveolar epithelial cells.
E. Cell replication assessment: the %Ki-67+ cells was calculated as the percentage of Ki-67+ cells per total number of Epcam+ (airway or alveolar epithelial cells) or Epcam- cells (endothelial cells).

Murine Lung Explants: At E15 of gestation, pregnant murine dams were humanly euthanized, uterus was removed, embryos were placed in ice cold PBS and lungs were isolated by manual dissection. Embryonic lungs were transferred to Transwell inserts (Corning #3412, Fisher Scientific, Hampton NH) and cell culture media ± 2%-CSE ± 10ng/ml rhCC16 and ± 1 µm BTT (VLA2 inhibitor) was added to air liquid interface with the inserts (media DMEM Cellgro 10-013CV, 1% antibiotic/antimycotic Cellgro #30-004-CL, L glutamine, HEPES 5.989g/L, Fisher Scientific, Hampton NH) and placed in a

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

humidified 37°C incubator with 5% CO_2 and 3% O_2. Cell culture media was changed daily. Explants were imaged at time 0, Day 3, and Day 5 using a Nikon Ti Eclipse inverted microscope with 2X brightfield objective, distal lung buds were quantified by manual counting.

Co-Immunoprecipitation experiment. To confirm CC16-α2 integrin binding, we used Co-IP followed by conventional SDS-PAGE and western blotting. RIPA buffer was used to generate tissue homogenization from 50 murine lung explants, which yielded ~2,000,000 million pulmonary embryonic cells in total. We divided the samples (equal numbers of cells) into 3 groups to assemble the treatment protocol to incubate the epithelial cells with 100 μg of rhCC10. The incubation period was designed to saturate the Itga2 with (chemically unmodified) rhCC16 and then use the rhCC16 to pulldown the Itga2-rhCC16 pair. The embryonic cells were incubated with rhCC16 at 37 ^{o}C for 15 min and 30 min, followed by quick spinning down and lysis with 200 μL RIPA buffer at the end of the time courses. Unstimulated were used as negative control. The lysates were incubated with 5 μg of anti-SCGB1A1 (Santa Cruz Biotechnologies) for 2 hours, at 4^{o}C for overnight hours with gentle rocking. Followed by addition of 20 μL of protein A agarose beads were incubated with the samples, at 4^{o}C for 2 hours with gentle rocking. The samples were washed 3 times with cold 1X PBS. After the final wash, supernatant was discarded and the pellet was resuspended in 20 μL of 2X Laemmli sample buffer. The entirety of the sample was loaded into the SDS –PAGE wells and used for conventional electrophoresis with 10% polyacrylamide gels. Electrotransfering was performed using the conventional method. The membranes were blocked with 1%BSA-Milk for 1 hour,

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

followed by incubation with the first primary antibody anti-ITGA2 (Invitrogen) which targets the α2 integrin subunit (blue arrows). After reading the ITGA bands at ~110 kDA, the membranes were then immunoblotted with anti-SCGB1A1 to locate ~10 kDa SCGB1A1 band and changes of density on the ~110 kDa regions of the membrane (red arrows).

STATISTICAL ANALYSIS

COPDGene cohort: we natural log-transformed CC16 due to the skewness of the sample distribution. We measured associations between plasma CC16 and maternal smoke exposure using bivariate analyses. We then used generalized linear modeling to measure associations between CC16 and maternal smoking, adjusting for age, sex, race, current smoking status, pack-years and annual income. In short, the following models were built to determine the associations between baseline lnCC16 and $\%FEV_{1pred}$ (or changes in $\%LAA_{-950}$). We measured associations between CC16 and percent predicted forced expiratory volume in 1-second (FEV1pp), quantitative emphysema as measured on HRCT ($\%LAA_{-950}$) using using generalized linear models (GLM) adjusting for age, sex, race, current smoking status, pack-years, and annual income. We used a linear mixed model with repeated measures (MMRM) to evaluate the association between baseline natural log-transformed CC16 (lnCC16) levels and absolute FEV_1 (L) over time. The model included fixed effects for baseline lnCC16, time (categorical with Phase 1 visit as the reference), age, smoking status (never, former, current), pack-years, sex, race, maternal smoking during pregnancy, and household annual income. Repeated measures were modeled using a compound symmetry covariance structure with participant ID as the subject. We used a separate adjusted MMRM to determine relationships between

CC16 and change in %LAA-$_{950}$, adjusting for the same covariates. We used a compound symmetry (CSy) correlation structure to model the repeated measures of FEV_1 across the two time points. The CSy structure assumes that the correlation between repeated measurements on the same individual is constant regardless of time interval. To assess the nature of missing data, we performed Little's MCAR test, which indicated data was Missing Completely At Random (MCAR), suggesting that the probability of missingness is unrelated to the observed data values. Sensitivity analyses (e.g., comparing complete-case results with available-case analyses) showed consistent findings between models, so we used complete-case analyses.

ECLIPSE cohort: Serum CC16 levels were natural-log-transformed (lnCC16) to address data skewness. CC16 change, %FEV_{1pred} post-BD change, and quantitative emphysema (%LAA-$_{950}$) on HRCT change were computed as change per year. To explore the association between CC16 at baseline with %FEV_{1pred} or %LAA-$_{950}$, and at one year with %FEV_{1pred}, with GLM linear models for each time point adjusted by age, sex, packs/year, education and race were used. To evaluate the strength of associations in Smokers and COPD subjects, and the magnitude of the observed effect, a multinomial logistic regression using NSC as reference was done. %FEV_{1pred} decline was estimated using a mixed model with random slope and intercept to account for individual baseline differences. On interest, the ECLIPSE COPD cohort are GOLD-2, -3, and -4, and thus the %FEV_{1pred} and %LAA-$_{950}$ are highly correlated and and thus both variables cannot be included in the model they explain the variability of the data. That is why the models were constructed 'separately', as observed in **Supplemental Table E4**. Clinical characteristics and CC16 levels across decline groups were compared using appropriate statistical tests,

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

including ANOVA, Kruskal–Wallis, t-test, Mann–Whitney, or χ^2 tests. Scatter plots were constructed describe the association between lnCC16 and changes in $\%FEV_{1pred}$ and $\%LAA_{-950}$. A two-tailed p-value <0.05 was considered statistically significant, and all analyses were performed in R using custom scripts.

ALLIANCE cohort: All calculations were performed in SAS Analytics Software v.9.4. Associations between CC16, maternal smoke exposure (defined as any maternal smoking in pregnancy) and current passive smoke exposure (defined as ≥10 cigarettes smoked within the home of the child per day) were first assessed using bivariate analyses, followed by generalized linear models adjusting for age, disease status, sex, and study center. Additional models examined the relationship between CC16 and both FEV1/FVC%predicted and FEF 25-75%predicted (Calculated with GLI-2012 equations https://www.ers-education.org/guidelines/global-lung-functioninitiative/spirometry-tools/r-macro/). The latter analyses were done for all diseased children with a CC16 measurement at a young age (≤6yrs) and with a lung function measurement. Longitudinal changes in FEV1/FVC%predicted and FEF 25-75%predicted were analyzed using two strategies. First, we calculated differences between the first and last measurements of FEV1/FVC %predicted and FEF 25-75%predicted, respectively, and applied the same models as above, additionally adjusted for passive smoke exposure. This was only done for those children having at least two lung function measurements over time. Second, we included all lung function measurements per child in mixed models for repeated measures (MMRM), with the same covariate adjustment.

For the **LTRC** cohort analysis and the murine studies, data assessment and statistical analyses were performed using SigmaPlot™ software (Systat, San Jose, CA).

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

The Shapiro-Wilk test was used to determine whether the data were normally distributed, and the Brown-Forsythe test was used to assess equal variance. Data were analyzed using one-way ANOVAs for continuous data, followed by post-hoc testing with 2-sided Student's t-tests or Mann-Whitney U tests according to distribution. Results from the immunofluorescence assays in human cohort were adjusted by age, sex and pack-years using a regression model, and these P values were used for graphics (**Supplemental Table E10**). Correlations were calculated using Spearman test (ρ). Data are presented either as box plots showing medians and 25th and 75th percentiles and whiskers showing 10th and 90th percentiles for non-parametric data, or as mean±standard error bars for parametric data. $P<0.05$ was considered statistically significant.

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

Supplemental Table E1. List of Antibodies used for this project.

	Target	Catalogue number	Manufacturer	Dilution
Human	SCGB1A1 (CC16)	sc-365992	Santa Cruz Biotechnologies, TX	1:100 IF
	Active Caspase-3	MA1-91637	Invitrogen, MA	1:50 IF
	KI-67	PA5-19462	Invitrogen, MA	1:50 IF
	EPCAM	PA5-143178	Invitrogen, MA	1:100 IF
	Pan-CK	BS-10403R	Bioss, MA	1:100 IF
Mouse	SCGB1A1 (CC16)	sc-365992	Santa Cruz Biotechnologies, TX	1:100 IF 1:100 WB
	Active Caspase-3	MA1-91637	Invitrogen, MA	1:50 IF
	KI-67	PA5-19462	Invitrogen, MA	1:50 IF
	4-hydroxy-nonenal	MA5-27570	Invitrogen, MA	1:50 IF
	Cytochrome P450-2F2	sc-374540	Santa Cruz Biotechnologies, TX	1:100 IF
	Alpha-SMA	ab7817	Abcam, MA	1:75 IF
	α2 integrin	PA5-47193	Invitrogen, MA	1:75 IF 1:100 WB
	Epcam	MUB0509P	Invitrogen, MA	1:100 IF
Secondary antibodies	Goat anti-mouse	AlexaFluor-488®	Invitrogen, MA	1:1000
	Goat anti-rabbit	AlexaFluor-488®	Invitrogen, MA	1:1000
	Rabbit anti-mouse	AlexaFluor-594®	Invitrogen, MA	1:1000
	Goat anti-mouse	AlexaFluor-647®	Invitrogen, MA	1:1000
	Rabbit anti-goat	AlexaFluor-555®	Invitrogen, MA	1:1000

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

Supplemental Table E2. Clinical Characteristics of Participants in the COPDGene Cohort with CC16 measures.

	NSC (n=34)	SC (n=524)	COPD (n=504)	Total (n=1,062)	P	P-adj[3]
Sex, n (%)					**0.0019**[1]	**<0.01**
Male	8 (23.5%)	241 (46.0%)	263 (52.2%)	512 (48.2%)		
Female	26 (76.5%)	283 (54.0%)	241 (47.8%)	550 (51.8%)		
Age, years	65±11	59± 9	65±9	62±10	**<0.0001**[2]	**<0.0001**
Race					**<0.0001**[1]	
Non-Hispanic White	33 (97.1%)	428 (81.7%)	463 (91.9%)	924 (87.0%)		
Black	1 (2.9%)	96 (18.3%)	41 (8.1%)	138 (13.0%)		
Smoking status					**<0.0001**[1]	
Never-smoker	34 (100.0%)	0 (0.0%)	0 (0.0%)	34 (3.2%)		
Former smoker	0 (0.0%)	306 (58.4%)	330 (65.5%)	636 (59.9%)		
Current smoker	0 (0.0%)	218 (41.6%)	174 (34.5%)	392 (36.9%)		
Mother smoked cigarettes while pregnant, n (%)	5 (14.7%)	148 (28.2%)	167 (33.1%)	320 (30.1%)	**0.032**[1]	
%FEV1$_{pred}$, %predicted	102±14	99±11	62±23	81±26	**<0.0001**[2]	**<0.0001**
FEV1/FVC	0.80±0.05	0.80±0.05	0.50±0.14	0.70±0.16	**<0.0001**[2]	**<0.0001**
%LAA$_{-950}$	1.2±2.3	1.7±2.2)	9.9±11.4	5.5±9.0	**<0.0001**[2]	**<0.0001**
CC16, ng/mL	11.4±6.7	9.1±15.1	7.6±4.7	8.4±11.2	**<0.0001**[2]	**<0.0001**
lnCC16, units	2.30±0.56	2.00±0.62	1.90±0.61	1.90±0.62	**<0.0001**[2]	**0.001**

Data expressed as n (%) or mean ± standard deviation. [1]Chi-Square P-value; [2]Kruskal-Wallis P-value. [3]Post-hoc Bonferroni correction. Significant P values are in **bold**.

Abbreviations: CC16 = Club cell protein 16; COPD = chronic obstructive pulmonary disease according to the GOLD criteria; NSC = never-smoker control; SC = smoker control; %FEV1$_{pred}$ = forced expiratory volume in 1-second predicted; FVC = forced vital capacity; %LAA-$_{950}$ = percent low attenuation area less than -950 Hounsfield units, emphysema is defined by %LAA-$_{950}$ >5%.

Supplemental Table E3. Effect sizes for CC16 on Lung Function and Emphysema according to smoking status in COPDGene.

Comparison	Cohen's d	Effect Size	Interpretation	Eta-Squared
$\%FEV_{1pred}$				$\eta^2 = 0.0115$
Never vs Former	0.85	Large	Never > Former	
Never vs Current	1.00	Large	Never > Current	
Former vs Current	-0.13	Negligible	No difference	
$\%LAA_{-950}$				$\eta^2 = 0.00531$
Never vs Former	-0.582	Moderate	Former > Never	
Never vs Current	-0.252	Small	Current > Never	
Former vs Current	0.518	Moderate	Former > Current	

Abbreviations: $\%FEV1_{pred}$ = forced expiratory volume in 1-second predicted; $\%LAA_{-950}$ = percent low attenuation area less than -950 Hounsfield units, emphysema is defined by $\%LAA_{-950}$ >5%.

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

Supplemental Table E4. Generalized linear model to explore associations between lnCC16, study groups (NSC, SC, and COPD), and covariates in ECLIPSE.

Table E4A. Association of baseline lnCC16 with %FEV$_1$, adjusting by age, sex, packs/year and level of education.			
Variable	**Estimate**	**Std. Error**	**P value**
(Intercept)	0.1658161	0.0998106	0.09679
Post-BD %FEV$_1$	0.0042756	0.0003959	**< 2e-16**
Age	0.0173621	0.0013904	**< 2e-16**
Sex, Male	0.1914560	0.0221757	**< 2e-16**
Pack/year	-0.0009488	0.0004146	**0.02221**
Race, Non-Caucasian	-0.0028415	0.0694994	0.96739
Education_B	0.0149341	0.0299370	0.69510
Education_C	0.0888907	0.0305502	**0.00365**
Table E4B. Association of baseline lnCC16 with %LAA-$_{950}$, adjusting by age, sex, packs/year and level of education.			
Variable	**Estimate**	**Std. Error**	**P value**
(Intercept)	0.7154410	0.0874093	**4.55e-16**
%LAA-$_{950}$	-0.0018689	0.0009089	**0.0399**
Age	0.0139242	0.0014106	**< 2e-16**
Sex, Male	0.1742180	0.0227211	**2.61e-14**
Pack/year	-0.0022218	0.0004077	**5.59e-08**
Race, Non-Caucasian	-0.0366588	0.0711758	0.6066
Education_B	0.0272791	0.0307300	0.3748
Education_C	0.1237925	0.0312367	**7.63e-05**
Table E4C. Association of lnCC16 measured at 1-year with the FEV1 measured at one year, adjusting by age, sex, packs/year and level of education			
Variable	**Estimate**	**Std. Error**	**P value**
(Intercept)	-1.2798672	0.2059741	**6.2e-10**
Post-BD %FEV$_1$	0.0092150	0.0008385	**< 2e-16**
Age	0.0340613	0.0028670	**< 2e-16**
Sex, Male	0.3308403	0.0455890	**5.5e-13**
Pack/year	-0.0019355	0.0008344	**0.0204**
Race, Non-Caucasian	0.0132099	0.1494331	0.9296
Education_B	0.0096203	0.0606350	0.8740
Education_C	0.1469028	0.0620443	**0.0180**

Abbreviations: Education_A: no primary school, some primary school or primary school; Education_B: highschool; Education_C: technical education or university; %FEV1$_{pred}$ = forced expiratory volume in 1-second predicted; %LAA-$_{950}$ = percent low attenuation area less than -950 Hounsfield units, emphysema is defined by %LAA-$_{950}$ >5%. Significant P values are in **bold**.

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

Supplemental Table E5. Multinominal logistic regressions to explore associations between lnCC16, study groups (NSC as reference) and covariates in ECLIPSE.

Group	Factor	Estimate	Std.Error	Statistic	Conf.Low	Conf.High	P
COPD Subjects	Post-BD %FEV$_1$	0.004	1.232	-4.468	0.000	0.045	**7.88E-06**
COPD Subjects	Age	1.046	1.217	0.037	0.096	11.359	9.71E-01
COPD Subjects	Sex, Female	0.040	0.107	-30.143	0.033	0.050	**0.00E+00**
COPD Subjects	%LAA-$_{950}$	0.585	3.728	-0.144	0.000	871.380	8.86E-01
COPD Subjects	CC16 at baseline	11205.924	0.091	102.356	9373.612	13396.407	**0.00E+00**
COPD Subjects	CC16 at 1-year	0.002	0.283	-22.292	0.001	0.003	**0.00E+00**
COPD Subjects	Change %FEV$_1$	0.167	0.818	-2.184	0.034	0.832	**2.89E-02**
COPD Subjects	Education_A	2153.975	0.000	567399186.085	2153.975	2153.975	**0.00E+00**
COPD Subjects	Education_B	3994078.428	0.059	258.247	3558895.662	4482475.465	**0.00E+00**
Smoker Controls	Post-BD %FEV$_1$	0.960	0.010	-4.180	0.942	0.979	**2.92E-05**
Smoker Controls	Age	1.059	0.015	3.819	1.028	1.090	**1.34E-04**
Smoker Controls	Sex, Female	0.362	0.257	-3.948	0.219	0.600	**7.89E-05**
Smoker Controls	%LAA-$_{950}$	0.827	0.038	-5.065	0.768	0.890	**4.08E-07**
Smoker Controls	CC16 at baseline	3.916	0.469	2.912	1.562	9.815	**3.59E-03**
Smoker Controls	CC16 at 1-year	0.177	0.343	-5.055	0.090	0.346	**4.30E-07**
Smoker Controls	Change %FEV$_1$	0.917	0.049	-1.772	0.832	1.009	7.63E-02
Smoker Controls	Education_A	1.003	0.427	0.008	0.434	2.319	9.93E-01
Smoker Controls	Education_B	1.324	0.256	1.094	0.801	2.189	2.74E-01

Abbreviations: Education_A: no primary school, some primary school or primary school; Education_B: highschool; Education_C: technical education or university; %FEV1$_{pred}$ = forced expiratory volume in 1-second predicted; %LAA-$_{950}$ = percent low attenuation area less than -950 Hounsfield units, emphysema is defined by %LAA-$_{950}$ >5%. Significant P values are in **bold**.

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

Supplemental Table E6. ALLIANCE cohort selected population characteristics.

		All children with CC16 measurement* n=272				Only diseased children ≤6yrs at CC16 measurement and ≥1 lung function measurement* n=63				
Characteristic		**n**		**%**		**n**		**%**		
Sex	Female	112		41.18		24		38.09		
	Male	160		58.82		39		61.90		
Disease status	Healthy	83		30.51		0		0.00		
	Wheeze/asthma	189		69.49		63		100.00		
Maternal smoking in pregnancy	No	246		90.44		58		92.06		
	Yes	26		9.56		5		7.94		
Passive smoke exposure	No	230		84.56		58		92.06		
	Yes	42		15.44		5		7.94		
Characteristic		**Mean**	**SD**	**Min**	**Max**	**n**	**Mean**	**SD**	**Min**	**Max**
Age at CC16 measurement (in years)		9.85	4.87	1.08	20.25	63	4.43	1.45	1.08	6.83
Nasal CC16 (*SCGB1A1*) levels (normalized intensitiy values)		-0.31	1.34	-6.36	2.89	63	-0.84	1.23	-4.25	1.70
Number of lung function measurements per child						63	3.10	1.96	1.00	9.00
Age at first lung function measurement per child (in yrs)**						63	5.39	0.79	4.00	6.92
FEV1/FVC$_{\%pred}$ at first measurement						63	98.69	8.52	68.37	112.87
FEF2575$_{\%pred}$ at first measurement						61	91.05	23.25	38.12	164.44
Age at last function measurement per child (in years)*						45	9.56	2.72	6.17	15.92
FEV1/FVC$_{\%pred}$ at last measurement*						45	98.39	5.78	83.15	106.78
FEF2575$_{\%pred}$ at last measurement*						43	87.63	22.98	38.12	152.48

*Analyses are adjusted for diseased yes/no, age at CC16 measurement, sex and study center. Dependant variable: CC16, given as normalized intensity values; β: regression coefficient; SE: standard error. Significant P-values are in **bold**.

**Only in children with two or more lung function measurements.

Supplemental Table E7. Multivariate regression analyses regarding CC16 (*SCGB1A1*) expression, maternal smoke and passive smoke exposure positive history.

TABLE E7A – t-Test comparisons							
	Maternal smoking in pregnancy						
	Not exposed			Exposed			
Gene	n	Mean	SD	n	Mean	SD	P
CC16 (*SCGB1A1*)	246	-0.301	1.34	26	-0.431	1.38	0.641
	Passive smoke exposure*						
	Not exposed			Exposed			
Gene	n	Mean	SD	n	Mean	SD	P
CC16 (*SCGB1A1*)	230	-0.244	1.33	42	-0.698	1.36	**0.043**
TABLE E7B – Multivariate regression analyses*							
				n	β	SE	P
Maternal smoking in pregnancy				272	-0.268	0.25	0.277
Passive smoke exposure				272	-0.583	0.20	**0.003**

*Analyses are adjusted for diseased yes/no, age at CC16 measurement, sex and study center. Dependant variable: CC16, given as normalized intensity values; β: regression coefficient; SE: standard error. Significant P-values are in **bold**.

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

Supplemental Table E8. Lung function and CC16 levels in young wheezing children in ALLIANCE cohort.

Lung function measures included in analysis	FEV1/FVC$_{\%pred}$				
All repeated measurements per child†	**n**	**nx**	**β**	**SE**	**P**
CC16 (*SCGB1A1*)	63	195	2.450	0.70	**<0.001**
Diff. of last and first measurement per child ‡					
CC16 (*SCGB1A1*)	45	-	-2.794	1.34	**0.044**
Lung function measures included in analysis	**FEF2575$_{\%pred}$**				
All repeated measurements per child†	**n**	**nx**	**β**	**SE**	**P**
CC16 (*SCGB1A1*)	61	172	6.685	2.18	**0.003**
Diff. of last and first measurement per child ‡					
CC16 (*SCGB1A1*)	41	-	-7.961	4.48	0.085

The obtained P-values represent the influence of CC16 on lung function, adjusted for age at CC16 measurement, age at lung function measurements, passive smoke exposure, sex and study center. This defines lung function is the dependant variable and CC16 the independant variable in the model.

All lung function values in %predicted (using GLI values). All analyses adjusted for age at CC16 measurement, age at lung function measurements, passive smoking exposure, sex and study center. CC16 is given as normalized intensity values; n: number of children included in analysis; nx: number of lung function measurements included in analysis; β: regression coefficient; SE: standard error; Significant P-values are given in **bold**.

†Analyses in n=63 children with CC16 measurement ≤6yrs and at least one lung function measurement ≥4yrs (n=61 for FEF2575).
‡Analyses in n=45 children with CC16 measurement ≤6yrs and at least two lung function measurements ≥4yrs (n=41 for FEF2575).

Abbreviations: FEV1/FVC: Ratio of Forced Expiratory Volume in 1 second to Forced Vital Capacity; FEF2575: Forced Expiratory Flow between 25% and 75% of vital capacity (FEF_{25-75}).

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

Supplemental Table E9. Demographic information from the LTRC cohort.

		NO MATERNAL SMOKING			MATERNAL SMOKING			
	n	**NSC**	**SC**	**COPD**	**NSC**	**SC**	**COPD**	***P**
n	44	4	8	12	4	6	10	
Age, years	44	61.5	67.0	65.8	56.8	67.2	60.6	0.084
Race, Non-Hispanic White, %	44	75	75	91.7	100#	83.3#	90#	
Race, Black, %	44	25	25	8.3	0#	16.7#	10#	
Females, %	44	75	62.5	41.7	75#	66.7#	50#	
Former Smokers, %	44	0	100	100	0#	100#	100#	
Pack Years	44	0	38.7	43.9	1.03	45.4	45.8	**0.001**
Whole Lung %LAA-$_{950}$	44	0.49	2.0	28.7	2.0	2.9	26.6	**<0.001**
%FEV$_{1pred}$	44	98.8	101.0	40.3	94	100.7	40.8	**<0.001**
FEV/FVC ratio	44	79.5	77.1	38.3	85	74.5	39.2	**<0.001**
Comorbidities, %								
Angina	44	0%	0%	8.3%	25%#	0%#	0%#	
Arterial Hypertension	44	0%	38%	50%	50%#	50%#	40%#	
Asthma	44	0%	0%	25%	25%#	17%#	20%#	
Arrythmia	44	25%	0%	25%	0%#	50%#	10%#	
GERD	44	0%	50%	34%	0%#	34%#	30%#	
Lung Cancer	44	75%	13%	0%	25%#	50%#	10%#	
Other Cancer	44	50%	0%	8.3%	25%#	34%#	0%#	
Rheumatoid Arthritis	44	0%	25%	0%	0%#	0%#	0%#	
Polymyositis	44	0%	13%	0%	0%#	0%#	0%#	
Cirrhosis	44	0%	0%	0%	0%#	0%#	10%#	
Chronic bronchitis	44	0%	0%	67%	0%#	0%#	50%#	
Diabetes Mellitus	44	25%	0%	25%	25%#	17%#	0%#	

*P values derived from One Way ANOVA between no maternal smoking and maternal smoking subjects.
Z-Test was applied for categorical variables like sex and smoking history.
Significant P-values are in **bold.**
#There were no significant differences between categorical variables when comparing maternal smoking vs. no maternal smoking per group using Z-test (NSC vs. NSC; SC vs. SC; COPD vs. COPD).
Abbreviations: %FEV$_{1pred}$: Forced Expiratory Volume predicted; FVC: Forced Vital Capacity; GERD, Gastroesophageal Reflux Disease; LTRC: Lung Tissue Research Consortium; %LAA-$_{950}$: percentage of lung attenuation areas of the lung; emphysema is defined by %LAA-$_{950}$ >5%.

Supplemental Table E10. Age, Sex and Pack years adjustment of histological results.

	Difference in Y Axis Variable Between Maternal Smoking vs. No Maternal Smoking Exposure			
Group	**Unadjusted**	**P-Value**	**Adjusted for Age, Sex and Pack years**	**Adjusted P-Value**
CC16+ cells in airway epithelium				
NSC	-5.78 (-9.29, -2.27)	0.007	-6.56 (-11.82, -1.31)	**0.029**
SC	-3.38 (-6.09, -0.67)	0.019	-3.34 (-6.58, -0.09)	**0.045**
COPD	-2.47 (-3.65, -1.28)	<0.001	-2.55 (-3.91, -1.18)	**0.001**
KI-67+ cells in alveolar epithelium				
NSC	-12.14 (-24.87, 0.59)	0.058	-10.63 (-32.90, 11.65)	0.226
SC	-19.16 (-28.31, -10.01)	0.002	-17.62 (-28.56, -6.68)	**0.011**
COPD	-15.37 (-19.03, -11.71)	<0.001	-15.86 (-19.70, -12.02)	**<0.001**
	Change in Y Axis Variable per 1-Unit Increase in CC16 cells Airway Epithelium (i.e. Slope)			
	1.86 (0.99, 2.74)	<0.001	1.90 (0.91, 2.90)	**<0.001**
KI-67+ cells in airway epithelium				
NSC	-1.33 (-9.51, 6.85)	0.705	-0.87 (-15.05, 13.30)	0.857
SC	-12.12 (-18.23, -6.00)	0.002	-13.08 (-19.82, -6.33)	**0.006**
COPD	-10.32 (-13.16, -7.47)	<0.001	-9.63 (-12.68, -6.59)	**<0.001**
	Change in Y Axis Variable per 1-Unit Increase in CC16 cells Airway Epithelium (i.e. Slope)			
	0.94 (0.34, 1.54)	0.003	0.88 (0.24, 1.51)	**0.009**
KI-67+ cells in vascular endothelium				
NSC	-0.51 (-2.44, 1.41)	0.539	-0.03 (-2.12, 2.07)	0.972
SC	-1.80 (-2.81, -0.80)	0.004	-1.83 (-2.75, -0.91)	**0.005**
COPD	-2.07 (-2.76, -1.38)	<0.001	-2.23 (-2.96, -1.49)	**<0.001**
	Change in Y Axis Variable per 1-Unit Increase in CC16 cells Airway Epithelium (i.e. Slope)			
	0.23 (0.11, 0.35)	<0.001	0.20 (0.06, 0.33)	**0.005**
Active Caspase-3+ cells in alveolar epithelium				
NSC	-0.32 (-2.16, 1.53)	0.688	0.24 (-2.24, 2.72)	0.779
SC	10.67 (3.71, 17.64)	0.008	8.04 (-0.44, 16.53)	0.058
COPD	29.45 (18.47, 40.44)	<0.001	32.44 (20.74, 44.15)	**<0.001**
	Change in Y Axis Variable per 1-Unit Increase in CC16 cells Airway Epithelium (i.e. Slope)			
	-2.61 (-4.34, -0.88)	0.004	-2.23 (-4.19, -0.27)	**0.027**
Active Caspase-3+ cells in airway epithelium				
NSC	-0.72 (-3.05, 1.62)	0.479	-0.15 (-3.41, 3.11)	0.893
SC	3.63 (0.17, 7.09)	0.042	3.03 (-2.00, 8.05)	0.17
COPD	10.44 (7.72, 13.16)	<0.001	11.45 (8.82, 14.09)	**<0.001**
	Change in Y Axis Variable per 1-Unit Increase in CC16 cells Airway Epithelium (i.e. Slope)			
	-1.03 (-1.67, -0.39)	0.002	-0.84 (-1.57, -0.11)	**0.025**
Active Caspase-3+ cells in vascular endothelium				
NSC	0.26 (-0.80, 1.31)	0.571	0.44 (-1.54, 2.42)	0.527
SC	0.99 (-1.07, 3.05)	0.291	1.86 (-0.05, 3.77)	0.054
COPD	6.64 (4.00, 9.28)	<0.001	7.89 (5.33, 10.44)	**<0.001**
	Change in Y Axis Variable per 1-Unit Increase in CC16 cells Airway Epithelium (i.e. Slope)			
	-0.62 (-1.09, -0.15)	0.011	-0.49 (-1.02, 0.04)	0.067

Significant P values are in **bold**.

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

FIGURE LEGENDS

Supplemental Figure E1. COPDGene cohort diagram showing patient distribution.

Supplemental Figure E2. ECLIPSE cohort diagram showing patient distribution.

Supplemental Figure E3. ALLIANCE cohort diagram showing patient distribution.

Supplemental Figure E4. LTRC cohort diagram showing patient distribution.

Supplemental Figure E5. Experimental design for the murine experiments. WT and *Cc16*$^{-/-}$ pregnant dams were either exposed to room air (no maternal smoking) or to cigarette smoke (maternal smoking) during copulation (mating) and pregnancies. The newborn pups were not exposed to cigarette smoke, and they grew up in free room air until euthanasia timepoints were reached (P0, P7, P14, and P28).

Supplemental Figure E6. Correlations between lnCC16, and changes over time in %FEV$_1$ or %LAA-$_{950}$ in the ECLIPSE cohort. Scatter plots were constructed describe the association between lnCC16 and changes in %FEV$_{1pred}$ and %LAA-$_{950}$.

Supplemental Figure E7: FFPE lung sections from NSC, SC and COPD patients (n=44) from LTRC, with and without history of maternal smoke exposure, were immunostained for CC16, KI-67 and Active-caspase-3. %KI-67$^+$ cells were quantified in alveolar epithelial cells (**A**) and vascular endothelium (**B**). %Active caspase-3$^+$ cells were quantified in alveolar epithelial cells (**C**) and vascular endothelium (**D**). Data were analyzed using one-way ANOVAs followed by pair-wise testing with U-Mann Whitney test. Adjusted *P<0.05; **P<0.01; ***P<0.001 vs. corresponding NSC or the group indicated.

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

Supplemental Figure E8. CC16 levels correlate with lung function, DLCO, and lung epithelial cell health in adulthood. Spearman (ρ) correlations were calculated between %CC16$^+$ cells and FEV_1/FVC ratio (**A**), DLCO (Lung diffusion of CO_2) (**B**), %LAA-$_{950}$ (**C**), %KI-67+ cells in airway (**D**) and parenchyma (**E**), and %Active Caspase-3+ cells in airway (**F**) and in parenchyma (**G**).

Supplemental Figure E9. CC16 levels correlates with %FEV_1 in both subgroups. In **A**, Spearman (ρ) correlations were calculated between %CC16$^+$ cells and FEV1% in subjects exposed to maternal smoke (red dots). In **B**, Spearman (ρ) correlations were calculated between %CC16$^+$ cells and %FEV_1 in subjects without history of maternal smoke (white dots).

Supplemental Figure E10. Weight measurements in WT and *Cc16*$^{-/-}$ P28 pups, with and without maternal smoking exposure. Data were analyzed using one-way ANOVAs followed by pair-wise testing with Student t-Test. *P<0.05 vs. corresponding No-Maternal Smoking group indicated.

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

REFERENCES

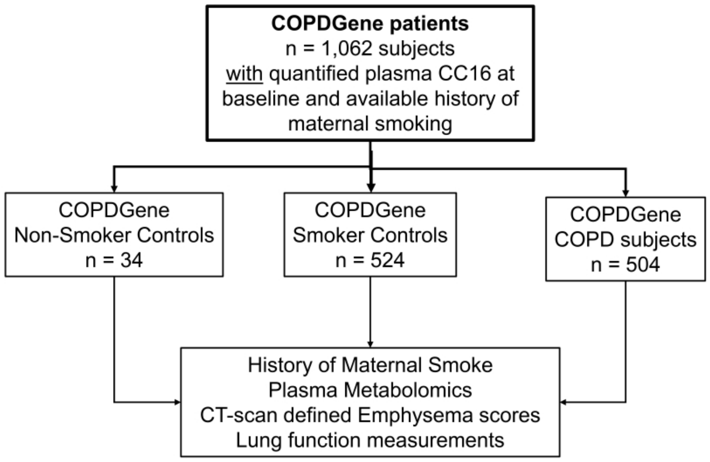
1. Maison N, Omony J, Illi S, et al. T2-high asthma phenotypes across lifespan. Eur Respir J 2022;60(3). DOI: 10.1183/13993003.02288-2021.
2. Fuchs O, Bahmer T, Weckmann M, et al. The all age asthma cohort (ALLIANCE) - from early beginnings to chronic disease: a longitudinal cohort study. BMC Pulm Med 2018;18(1):140. DOI: 10.1186/s12890-018-0705-6.
3. Vestbo J, Edwards LD, Scanlon PD, et al. Changes in forced expiratory volume in 1 second over time in COPD. N Engl J Med 2011;365(13):1184-1192. DOI: 10.1056/NEJMoa1105482 [doi].
4. Vestbo J, Anderson W, Coxson HO, et al. Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points (ECLIPSE). Eur Respir J 2008;31(4):869-73. DOI: 10.1183/09031936.00111707.
5. Regan EA, Hokanson JE, Murphy JR, et al. Genetic epidemiology of COPD (COPDGene) study design. COPD 2010;7(1):32-43. DOI: 10.3109/15412550903499522.
6. Occhipinti M, Paoletti M, Crapo JD, et al. Validation of a method to assess emphysema severity by spirometry in the COPDGene study. Respir Res 2020;21(1):103. DOI: 10.1186/s12931-020-01366-4.
7. Lowe KE, Make BJ, Crapo JD, et al. Association of low income with pulmonary disease progression in smokers with and without chronic obstructive pulmonary disease. ERJ Open Res 2018;4(4). DOI: 10.1183/23120541.00069-2018.
8. Reddel HK, Bateman ED, Becker A, et al. A summary of the new GINA strategy: a roadmap to asthma control. Eur Respir J 2015;46(3):622-39. DOI: 10.1183/13993003.00853-2015.
9. Deutsche Gesellschaft fur Arbeitsmedizin und Umweltmedizin e V, Deutsche Gesellschaft fur Rehabilitationswissenschaften e V, und Deutsche Gesellschaft fur Gynakologie und Geburtshilfe e V, et al. [Guideline for the Diagnosis and Treatment of Asthma - Guideline of the German Respiratory Society and the German Atemwegsliga in Cooperation with the Paediatric Respiratory Society and the Austrian Society of Pneumology]. Pneumologie 2017;71(12):849-919. DOI: 10.1055/s-0043-119504.
10. Diaz AA, Bartholmai B, San Jose Estepar R, et al. Relationship of emphysema and airway disease assessed by CT to exercise capacity in COPD. Respiratory medicine 2010;104(8):1145-51. DOI: 10.1016/j.rmed.2010.02.023.
11. Laucho-Contreras ME, Polverino F, Rojas-Quintero J, Wang X, Owen CA. Club cell protein 16 (Cc16) deficiency increases inflamm-aging in the lungs of mice. Physiol Rep 2018;6(15):e13797. DOI: 10.14814/phy2.13797.
12. Rojas-Quintero JL-C, M; Wang, X; Fucci QA; Burkett, PR; Kim SK; Zhang D; Tesfaigzi Y; Li Y; Bhashyam AR; Zhang L; Khamas H; Celli BR; Pilon AL; Polverino F; Owen CA. CC16 augmentation reduces exaggerated COPD-like disease in Cc16-deficient mice. JCI Insight 2023;In Press.
13. Rojas-Quintero J, Laucho-Contreras ME, Wang X, et al. CC16 augmentation reduces exaggerated COPD-like disease in Cc16-deficient mice. JCI Insight 2023. DOI: 10.1172/jci.insight.130771.

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

14. Laucho-Contreras ME, Taylor KL, Mahadeva R, Boukedes SS, Owen CA. Automated measurement of pulmonary emphysema and small airway remodeling in cigarette smoke-exposed mice. J Vis Exp 2015(95):52236. DOI: 10.3791/52236.
15. Polverino F, Rojas-Quintero J, Wang X, et al. A Disintegrin and Metalloproteinase Domain-8: A Novel Protective Proteinase in Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med 2018;198(10):1254-1267. DOI: 10.1164/rccm.201707-1331OC.
16. Betz P, Nerlich A, Bussler J, Hausmann R, Eisenmenger W. Radial alveolar count as a tool for the estimation of fetal age. Int J Legal Med 1997;110(2):52-4. DOI: 10.1007/s004140050030.

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

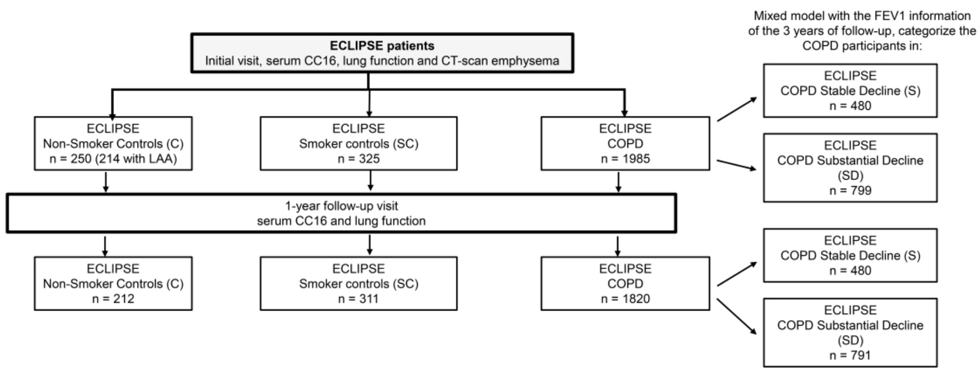
Supplemental Figure E1



Supplemental Figure E1. COPDGene cohort diagram showing patient distribution.

861x484mm (236 x 236 DPI)

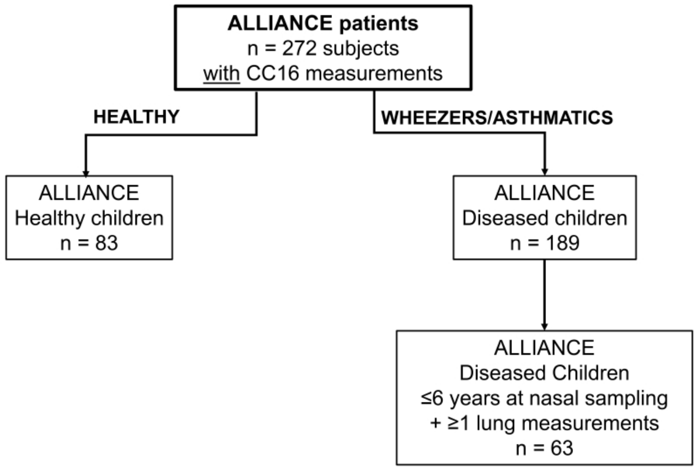
Supplemental Figure E2



Supplemental Figure E2. ECLIPSE cohort diagram showing patient distribution.

861x484mm (236 x 236 DPI)

Supplemental Figure E3

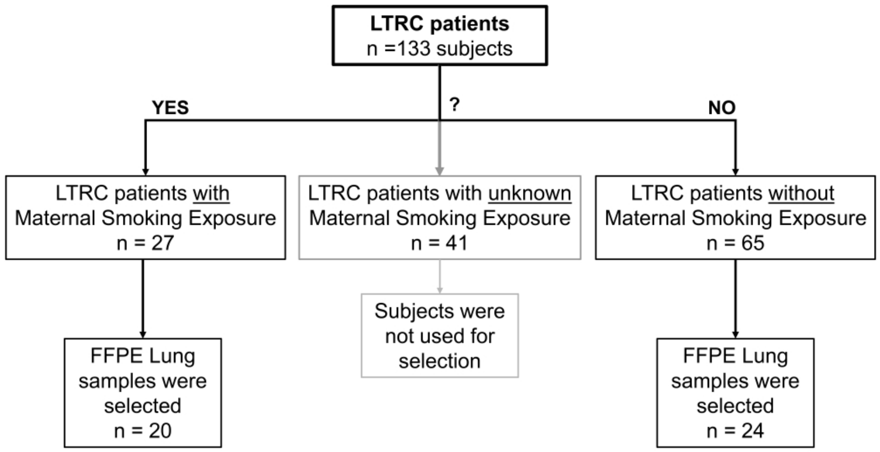


Supplemental Figure E3. ALLIANCE cohort diagram showing patient distribution.

861x484mm (236 x 236 DPI)

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

Supplemental Figure E4

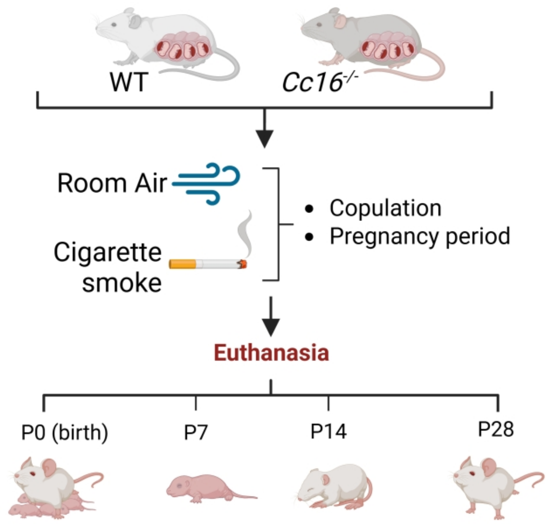


Supplemental Figure E4. LTRC cohort diagram showing patient distribution.

861x484mm (236 x 236 DPI)

AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

Supplemental Figure E5

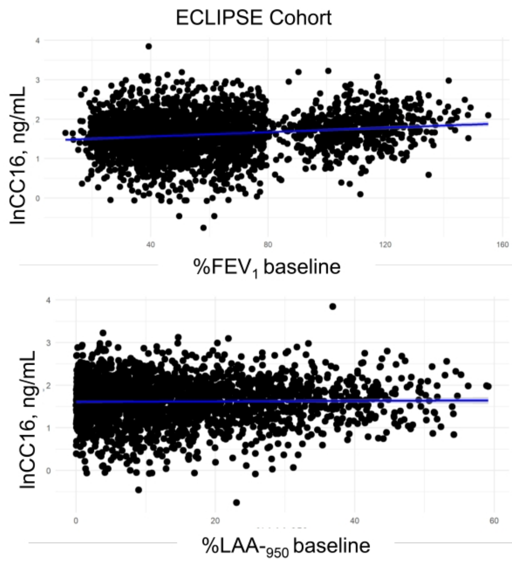


Supplemental Figure E5. Experimental design for the murine experiments. WT and Cc16-/- pregnant dams were either exposed to room air (no maternal smoking) or to cigarette smoke (maternal smoking) during copulation (mating) and pregnancies. The newborn pups were not exposed to cigarette smoke, and they grew up in free room air until euthanasia timepoints were reached (P0, P7, P14, and P28).

861x484mm (236 x 236 DPI)

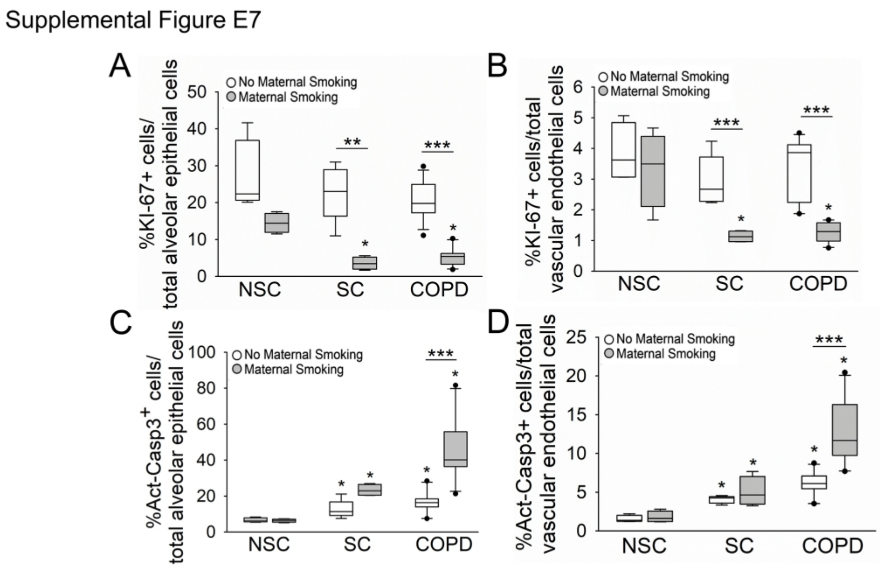
AJRCCM Articles in Press. Published October 07, 2025 as 10.1164/rccm.202504-0854OC

Supplemental Figure E6



Supplemental Figure E6. Correlations between lnCC16, and changes over time in %FEV1 or %LAA-950 in the ECLIPSE cohort. Scatter plots were constructed describe the association between lnCC16 and baseline %FEV1pred and %LAA-950.

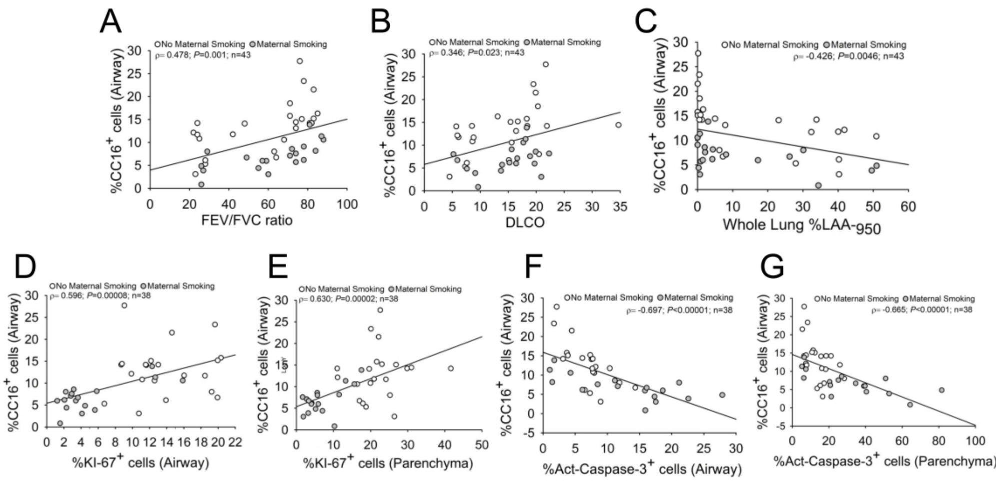
861x484mm (236 x 236 DPI)



Supplemental Figure E7: FFPE lung sections from NSC, SC and COPD patients (n=44) from LTRC, with and without history of maternal smoke exposure, were immunostained for CC16, KI-67 and Active-caspase-3. %KI-67+ cells were quantified in alveolar epithelial cells (A) and vascular endothelium (B). %Active caspase-3+ cells were quantified in alveolar epithelial cells (C) and vascular endothelium (D). Data were analyzed using one-way ANOVAs followed by pair-wise testing with U-Mann Whitney test. Adjusted *P<0.05; **P<0.01; ***P<0.001 vs. corresponding NSC or the group indicated.

860x484mm (236 x 236 DPI)

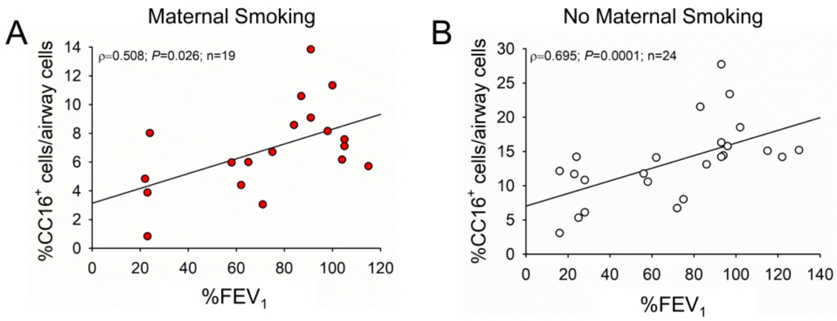
Supplemental Figure E8



Supplemental Figure E8. CC16 levels correlate with lung function, DLCO, and lung epithelial cell health in adulthood. Spearman (ρ) correlations were calculated between %CC16+ cells and FEV1/FVC ratio (A), DLCO (Lung diffusion of CO2) (B), %LAA-950 (C), %KI-67+ cells in airway (D) and parenchyma (E), and %Active Caspase-3+ cells in airway (F) and in parenchyma (G).

861x484mm (236 x 236 DPI)

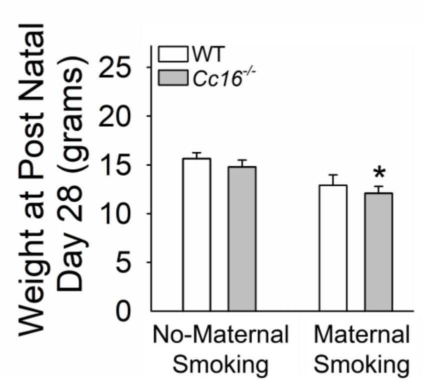
Supplemental Figure E9



Supplemental Figure E9. CC16 levels correlates with %FEV1 in both subgroups. In A, Spearman (ρ) correlations were calculated between %CC16+ cells and FEV1% in subjects exposed to maternal smoke (red dots). In B, Spearman (☐) correlations were calculated between %CC16+ cells and %FEV1 in subjects without history of maternal smoke (white dots).

861x484mm (236 x 236 DPI)

Supplemental Figure E10



Supplemental Figure E10. Weight measurements in WT and Cc16-/- P28 pups, with and without maternal smoking exposure. Data were analyzed using one-way ANOVAs followed by pair-wise testing with Student t-Test. *P<0.05 vs. corresponding No-Maternal Smoking group indicated.

861x484mm (236 x 236 DPI)