ORIGINAL ARTICLE

Virus-induced Volatile Organic Compounds Are Detectable in Exhaled Breath during Pulmonary Infection

Faisal Kamal[1]*, Sacheen Kumar[1,2]*, Michael R. Edwards[3], Kirill Veselkov[1], Ilaria Belluomo[1], Tatiana Kebadze[3], Andrea Romano[1], Maria-Belen Trujillo-Torralbo[3], Tasnim Shahridan Faiez[4], Ross Walton[3], Andrew I. Ritchie[3], Dexter J. Wiseman[3], Ivan Laponogov[1], Gavin Donaldson[3‡], Jadwiga A. Wedzicha[3‡], Sebastian L. Johnston[3], Aran Singanayagam[4]*, and George B. Hanna[1]*

[1]Department of Surgery and Cancer, [3]National Heart and Lung Institute, and [4]Centre for Molecular Bacteriology and Infection, Imperial College London, London, United Kingdom; and [2]Royal Marsden Hospital National Health Service Trust, London, United Kingdom

ORCID IDs: 0000-0002-4692-4554 (S.K.); 0000-0002-6398-7450 (D.J.W.); 0000-0001-9849-0033 (A.S.); 0000-0003-2897-0140 (G.B.H.).

Abstract

Rationale: Chronic obstructive pulmonary disease (COPD) is a condition punctuated by acute exacerbations commonly triggered by viral and/or bacterial infection. Early identification of exacerbation triggers is important to guide appropriate therapy, but currently available tests are slow and imprecise. Volatile organic compounds (VOCs) can be detected in exhaled breath and have the potential to be rapid tissue-specific biomarkers of infection etiology.

Objectives: To determine whether volatile organic compound measurement could distinguish viral from bacterial infection in COPD.

Methods: We used serial sampling within *in vitro* and *in vivo* studies to elucidate the dynamic changes that occur in VOC production during acute respiratory viral infection. Highly sensitive gas chromatography–mass spectrometry techniques were used to measure VOC production from infected airway epithelial-cell cultures and in exhaled breath samples from healthy subjects experimentally challenged with rhinovirus (RV)-A16 and from subjects with COPD with naturally occurring exacerbations.

Measurements and Main Results: We identified a novel VOC signature comprising decane and other long-chain alkane compounds that is induced during RV infection of cultured airway epithelial cells and is also increased in the exhaled breath from healthy subjects experimentally challenged with RV and from patients with COPD during naturally occurring viral exacerbations. These compounds correlated with the magnitude of antiviral immune responses, viral burden, and exacerbation severity but were not induced by bacterial infection, suggesting that they represent a specific virus-inducible signature.

Conclusions: Our study highlights the potential for measurement of exhaled breath VOCs as rapid, noninvasive biomarkers of viral infection. Further studies are needed to determine whether measurement of these signatures could be used to guide more targeted therapy with antibiotic/antiviral agents for COPD exacerbations.

Keywords: chronic obstructive pulmonary disease; rhinovirus; volatile organic compound; viral infection

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*These authors contributed equally to this work.

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Correspondence and requests for reprints should be addressed to George B. Hanna, Ph.D., Department of Surgery and Cancer, St. Mary's Campus, Imperial College London, Praed Street, London W2 1PG, UK. E-mail: g.hanna@imperial.ac.uk.

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At a Glance Commentary

Scientific Knowledge on the Subject: Volatile organic compounds (VOCs) can be readily detected in exhaled breath and have the potential to be used as tissue-specific biomarkers of pathogen presence and/or host response during exacerbations of chronic lung diseases. The relationship between viral infection and VOC production is poorly characterized.

What This Study Adds to the Field: By using serial sampling within *in vitro* and *in vivo* studies of experimental rhinovirus infection and virus-exacerbated chronic obstructive pulmonary disease, we demonstrate a novel virus-specific VOC signature comprising long-chain alkanes that is detectable in exhaled breath. Measurement of these compounds could represent a rapid, noninvasive biomarker of viral infection to guide more targeted exacerbation therapy in the future.

Chronic obstructive pulmonary disease (COPD) is an inflammatory airway condition punctuated by the occurrence of exacerbations, episodes that are commonly triggered by pathogens, including respiratory viruses (particularly rhinoviruses [RVs]) (1–3) and bacteria (4). Early identification of the causative trigger for an exacerbation is a critical step to guide appropriate therapy but remains challenging. Routine microbiological tests are limited by the requirement for airway sampling, which can be challenging, and these approaches do not allow for the rapid accurate identification of the presence of viruses or bacteria in the airways at exacerbation onset. A lack of appropriate biomarkers for viral or bacterial infection in COPD has led to antibiotic prescribing approaches based on imperfect surrogate clinical markers such as symptom complexes (increased sputum volume/purulence [5]), which has inadvertently driven widespread overprescription of empirical antibiotics during exacerbations (6). This practice has contributed to the development of drug-resistant pathogens.

Previous studies in COPD have attempted to use inflammatory markers in blood or sputum to define specific exacerbation phenotypic clusters corresponding with bacterial, viral, mixed, and noninfective etiologies (7, 8). However, considerable overlap between these clusters and many of the elevated parameters are also observed in some patients with stable disease. Systemic biomarkers such as procalcitonin may be used to safely reduce antibiotic use (9) but are not discriminatory for viral versus bacterial infection (10). A point-of-care biomarker or panel of biomarkers that would facilitate a precise etiological diagnosis in exacerbations is therefore urgently needed.

Metabolic degradation products including volatile organic compounds (VOCs) can be readily detected in exhaled breath and analyzed with highly sensitive technologies (11). These compounds have the potential to be used as tissue-specific biomarkers of viral presence and/or host response in the airways (12, 13), and early reports from *in vitro* studies indicate that viruses and bacteria induce specific VOC production from airway epithelial-cell cultures (14–18). It remains unclear whether detection and quantification of these metabolites could be harnessed diagnostically to distinguish viral from nonviral exacerbations and guide appropriate therapies.

Here, we use highly sensitive gas chromatography–mass spectrometry (GC-MS) techniques, within *in vitro* and *in vivo* studies involving unique serial time-course sampling, to elucidate the dynamic changes in VOC production that occur during acute respiratory infections. We identify a novel VOC signature of the long-chain alkane decane and other long-chain alkanes, which is induced by RV infection in airway epithelial-cell cultures and is also detectable in the exhaled breath from healthy subjects with experimentally induced infections and from patients with COPD who have naturally occurring viral infections. Some of the results of this study have been previously reported in the form of an abstract (19)

Methods

In Vitro Infection of Airway Epithelial Cells

BEAS-2B airway epithelial cells (European collection of cell cultures) were maintained in 10% RPMI medium. Cells were cultured to 90% confluence on Primaria-coated culture dishes (Invitrogen) and were infected with RV-A16 (multiplicity of infection = 2) or 1×10^6 cfu of *Streptococcus pneumoniae* D39, *Haemophilus influenzae* 1479, or medium control, as previously reported (20–23). Cells were infected for 1 hour with shaking at room temperature and were subsequently incubated at 37°C within 2-L Tedlar bags (Sigma-Aldrich) for 8 and 24 hours. VOCs were collected/entrained onto thermal desorption tubes for 3 minutes at 50 ml/min before being analyzed by using GC-MS within 24 hours of collection. *See* Figure E1 in the online supplement, which shows an example of the experimental setup. Experiments were repeated three times independently.

Experimental RV Challenge in Healthy Subjects

A group of 11 healthy, nonsmoking subjects experimentally infected with RV-A16 were analyzed in this study. These subjects were placebo-treated individuals who took part in a double-blind, randomized, placebo-controlled study of ColdZyme solution (Enzymatica) in RV infections (Research Ethic Committee number 15/ES/0112). Before recruitment, all subjects had an initial screening visit to confirm suitability for the study, and serum neutralizing antibodies to RV-16 were measured. Baseline clinical sampling was performed 1–4 weeks before virus inoculation, which was on Study Day 0. Subjects kept daily symptom diaries to allow calculation of the viral upper respiratory symptom tract score previously reported by Jackson and colleagues (24). Subjects had follow-up clinical visits at Days 4, 6, 8, 11, and 28 after RV infection. Subjects underwent oropharyngeal swab and nasal lavage sampling at baseline and at each of these study visits, as previously reported (1, 25, 26). Exhaled breath collection was performed as previously described (27). Subjects refrained from exercise for 1 hour before sampling. Subjects were asked to perform a deep nasal inhalation followed by complete exhalation via their mouth into a secure GastroCHECK steel breath bag (250 ml) (Bedfont Scientific Ltd. and Ardmore Healthcare Ltd.) through a mouthpiece with a one-way valve. Subjects were asked to breathe in and out of the steel bag two times initially to equilibrate the bag. On the third exhalation, the breath sample was captured by capping both ends. The total duration of

collection was less than 20 seconds. Breath was subsequently entrained onto a thermal desorption tube by using an airflow pump at 50 ml/min for 3 minutes, and samples were analyzed within 24 hours.

COPD Exacerbation Cohort

We evaluated 139 patients with COPD recruited from the rolling London COPD cohort (28) and followed up over an 18-month period between November 2016 and March 2018. The study has received full ethical approval: Research Ethics Committee reference number 09/H0720/8. Subjects were all aged ⩾45 years. Inclusion criteria for the cohort were as follows: a post-bronchodilator FEV_1 ⩽80% predicted for age, height, and sex and an FEV_1/FVC ratio <0.7. Exclusion criteria were as follows: <10 pack-years of smoking and/or the presence of another significant respiratory disease (major comorbid respiratory disease, including asthma, bronchiectasis, interstitial lung disease, or lung cancer). Subjects underwent clinical assessment and sampling during a stable state upon entry into the study and at 3-month regular intervals. Patients also documented respiratory symptoms and hours spent outside the home on daily diary cards and recorded daily peak expiratory flow as measured with a mini-Wright meter (Clement-Clark International). An exacerbation was defined as an increase in respiratory symptoms, with at least one major symptom (dyspnea, sputum purulence, or sputum volume) plus either another major or a minor symptom (wheeze, cold, sore throat, and/or cough) for 2 consecutive days, as previously described (29). Subjects reported for clinical assessment within 48 hours of exacerbation onset with a proportion returning for repeat assessment and sampling at 1, 2, and 6 weeks after onset. Breath was collected by using steel breath bags as described above in the experimental study. Viruses and bacteria were detected in sputum samples as described previously (20).

GC-MS Analysis

All VOC samples were run on a GC-MS instrument (Agilent 7890B instrument with a 5977A mass selective detector, Agilent Technologies) coupled with a thermal desorption unit (TD-100, Markes International Ltd.). Instrument settings and the adopted GC-MS methodology were consistent for both the analysis of VOCs from *in vitro* experiments and the analysis of VOCs in human breath samples and were set and performed as previously reported (27, 30). The full details of the GC-MS protocol are provided in the online supplement. Representative chromatograms from each of the experimental studies showing peaks of interest are illustrated in Figure E2.

Data Analysis

Pattern recognition analyses were performed on the processed spectral data from GC-MS samples by using MATLAB (MathWorks). Full details are given in the online supplement. For evaluation of individual compounds from *in vitro* studies, the Mann-Whitney *U* test was used. For human *in vivo* studies, to compare postinfection VOC measurements with measurements at the baseline/stable state, the Wilcoxon rank sum test was used. Correlations between data sets were examined by using the Spearman rank correlation coefficient. Differences were considered significant when the *P* value was <0.05.

Results

RV Infection Alters Release of the VOC Decane by the Bronchial Epithelium

The bronchial epithelium is the primary site of infection by viruses such as RV, and we therefore initially investigated VOC profiles induced by infection by the major group RV-A16 within the headspace of cultured BEAS2B bronchial epithelial cells (BECs) (Figure 1A) enclosed in Tedlar sampling bags. There was no difference in the viability of BEAS2B cells cultured in Tedlar bags versus cells cultured normally (Figure E3). We identified 14 discriminatory metabolites that were significantly altered within the headspace of RV-infected versus medium-treated cells (Table 1). Further interrogation of these compounds by using the National Institute of Standards and Technology mass spectral reference library allowed accurate identification of 7 out of 14 metabolites, including 2-methyl 2-propanol, cyclobutylamine, acetoin, dimethyl disulfide, decane, 1-chloro-2-methyl-butane and phenol acetate. Of these, decane is an alkane hydrocarbon, and decane-containing compounds have been shown to have antiviral properties (31) and may therefore reflect a component of the host immune response to viral infection. We therefore focused further on this compound. Time-course analysis of decane indicated significant upregulated production at 24 hours after RV infection (Figure 1B).

Bacterial Respiratory Infection Does Not Alter Decane Release by the Bronchial Epithelium

Bacteria are also major causes of respiratory infections and COPD exacerbations (4), and we therefore additionally evaluated the epithelial VOC response to respiratory pathogenic bacteria by using the same methodology. Infection of BECs with *S. pneumoniae* and *H. influenzae* (Figure 1A) led to alterations in nine compounds (Table E1). Of these compounds, none were detected in the headspace of the same bacteria cultured on agar plates, indicating that these VOCs are specifically produced by bacterial infection of epithelial cells.

In contrast to RV infection, *S. pneumoniae* and *H. influenzae* infection had no effect on decane (Figures 1C–1D), suggesting that this VOC is induced specifically by viral but not bacterial infection of the epithelium.

Decane and Other Long-Chain Alkanes Are Increased in Exhaled Breath from Healthy Subjects Experimentally Infected with RV

Having observed that controlled *in vitro* infection with RV leads to epithelial production of decane, we next proceeded to determine whether this compound was detectable in the exhaled breath of humans challenged with RV. We therefore measured VOC production in breath samples in a study group comprising 11 healthy individuals (54.5% male; median age, 27 yr [interquartile range, 24.5–33 yr]) experimentally infected with RV-A16, who were sampled at baseline (before infection) and at Days 4, 6, 8, and 11 after infection, with 6 out of 11 individuals also returning for sampling at Day 28 after infection (Figure 2A). Successful experimental infection was confirmed, with significantly increased viral loads being observed in nasal samples at Days 4 and 6 and in oropharyngeal samples at Day 6 (Figure 2B). Increased symptom scores were observed at Days 2–8 after infection (Figure 2C). Analysis of exhaled breath VOC profiles identified 23 compounds with increased concentrations from baseline to Day 4. Of these, 17 compounds were identifiable by comparison with the National Institute of Standards and Technology library (Table 2). Consistent with our *in vitro* finding that RV

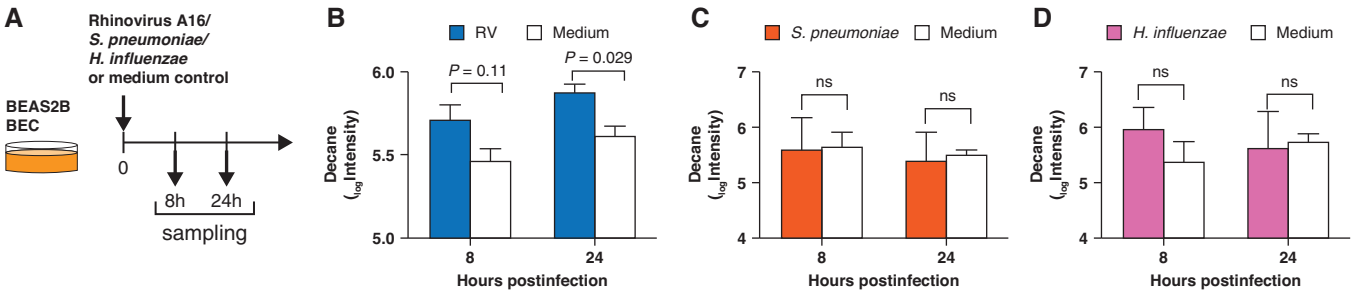

Figure 1. Viral infection upregulates decane in the headspace of airway epithelial cells, an effect not observed with bacteria. (*A*) Cultured BEAS2B BEC were stimulated with rhinovirus (RV)-A16, *Streptococcus pneumoniae*, *Haemophilus influenzae*, or medium control within a Tedlar bag. Headspace volatile organic compounds were sampled and profiled by using gas chromatography–mass spectrometry at 8 and 24 hours. Data for the long-chain alkane decane are shown in response to (*B*) RV, (*C*) *S. pneumoniae*, and (*D*) *H. influenzae* infection. Data are shown as the median (interquartile range) of three independent experiments combined. Data were analyzed by using the Mann-Whitney *U* test. BEC = bronchial epithelial cells; ns = nonsignificant.

induces the release of decane from airway epithelial cells, we found that 8 out of the 17 compounds increased in the breath of RV-infected subjects were decane or other long-chain alkane compounds (Table 2). Serial measurements of five of these compounds are displayed in Figures 2D–2H. Significant induction of decane, 3,8-dimethyl-undecane, 4,7-dimethyl-undecane, dodecane, and hexadecane was observed at Days 4 and 6 (Figures 2D–2H), corresponding to the same time points that nasal and oral viral loads were also significantly increased (Figure 2B) and thus further suggesting that these metabolites are virus inducible. Additional confirmation of the relationship between viral infection and long-chain alkane compounds was shown by the observation that concentrations of 3,8-dimethyl-undecane correlated positively with oropharyngeal viral loads (Figure 2I) and mean symptom scores (Figure 2J), with a nonsignificant correlation ($P = 0.09$) being observed between 4,7-dimethyl-undecane and oropharyngeal viral loads (Figure 2I).

Table 1. Compounds Detected in Headspace of Airway Epithelial Cells That Differed between Medium- and RV-treated Cells

Retention Time (min)*	*P* Value	*Q* Value	Log Change	Compound	M/Z Base Peak†
9.27	0.000321	0.031275	1.0318	Poor match (MW = 48)	47
10.07	5.08×10^{-6}	0.002312	−0.90847	2-Methyl-2-propanol	59
10.33	1.02×10^{-6}	0.000696	−1.8906	Cyclobutylamine	43
10.33	4.08×10^{-5}	0.009287	−1.0242	1-Chloro-2-methyl-butane	57
14.83	0.000252	0.026484	−0.55854	Poor match (MW = 119)	33
14.93	7.35×10^{-5}	0.014336	−1.2767	Poor match (MW = 54)	39
19.42	0.000212	0.026306	0.49521	Dimethyl disulfide	94
19.47	7.71×10^{-5}	0.013148	−2.2581	Acetoin	45
19.8	2.34×10^{-7}	0.000319	−2.3047	Poor match (MW = 86)	56
26.69	3.19×10^{-5}	0.008707	−0.90224	Poor match (MW = 250)	94
28.92	0.000171	0.023394	−0.8531	Decane‡	57
33.91	1.30×10^{-5}	0.004427	−1.1848	Phenol acetate	94
43.24	0.000171	0.025977	1.4178	Poor match (MW = 270)	74
45.53	0.000212	0.024114	−1.1673	Poor match (MW = 366)	71

Definition of abbreviations: FDR = false discovery rate; M = mass; MS = M spectral; MW = molecular weight; NIST = National Institute of Standards and Technology; RV = rhinovirus; Z = charge number of ions.
Data were analyzed by using ANOVA with FDR correction (*Q* value). Negative log-change values indicate compounds increased in RV- versus medium-treated cells.
*Retention time indicates the time to compound identification that was spent by a solute in the gas chromatography column.
†The M/Z base peak, which is used to identify compounds, is the tallest peak in the M spectrum that is due to an ion having the greatest relative abundance.
‡Indicates identification by matching to the NIST MS library and authentic standard.

Long-Chain Alkanes Correlate with the Magnitude of Antiviral Immune Responses in Experimentally Induced RV Infections

To further investigate the relationship between long-chain alkane compounds and host antiviral immune responses, we measured nasal antiviral mediator concentrations in subjects experimentally infected with RV (Figure 3A) and found that the IFN-inducible cytokine CXCL-10/IP-10 was significantly induced from baseline at Days 4–11 after infection, with a similar trend being observed for CCL5/RANTES (regulated upon activation, normal T cell expressed and secreted) at Day 4 ($P = 0.07$) (Figures 3B–3C). In keeping with the hypothesis that long-chain alkanes are related to the magnitude of the host antiviral response, there were significant positive correlations between CXCL10/IP-10 and concentrations of 4,7-dimethyl-undecane (Figure 3D) and hexadecane (Figure 3E). Nasal CCL5/RANTES concentrations also correlated positively with exhaled breath decane concentrations (Figure 3F).

The Long-Chain Alkane 2,9-Dimethyl-Undecane Is Increased in the Exhaled Breath of Patients with Exacerbating COPD

Having observed that decane and other long-chain alkanes are upregulated by RV in controlled *in vitro* and *in vivo* experimental infection models, we next proceeded to evaluate VOC profiles in exhaled breath from the community-based London COPD cohort (28). Patients in this study are sampled during a stable state and during self-reported exacerbations. One hundred thirty-nine patients provided samples at clinical stability,

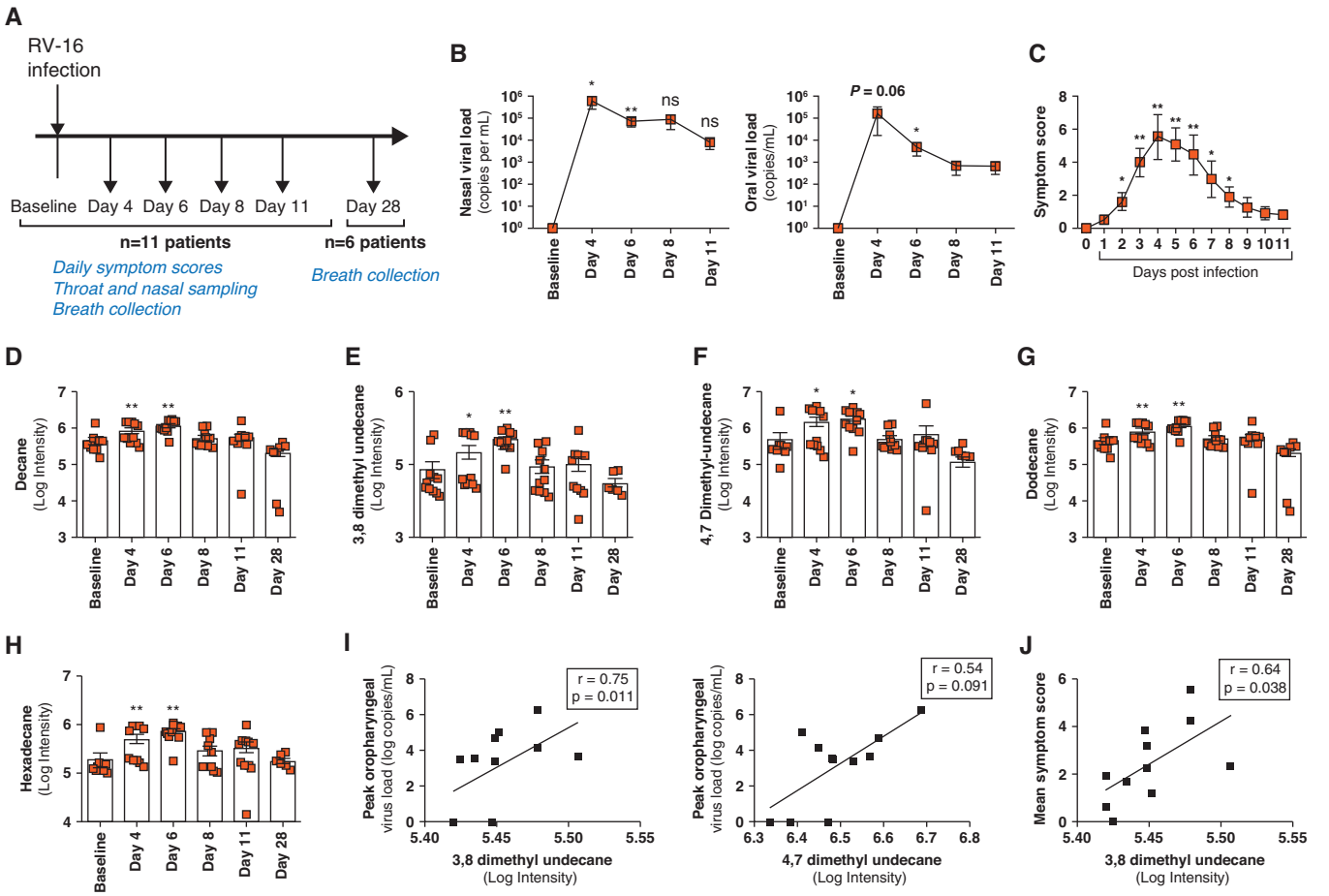

Figure 2. Decane and other long-chain alkane compounds are increased in exhaled breath after experimental rhinovirus (RV) challenge in healthy subjects. (*A*) Eleven healthy subjects were experimentally challenged with RV-A16 and sampled at various time points after infection. (*B*) Viral loads were measured by using quantitative PCR analysis of nasal lavage and oropharyngeal swab samples. (*C*) Daily upper respiratory tract symptom scores. Concentrations of (*D*) decane, (*E*) 3,8-dimethyl-undecane, (*F*) 4,7-dimethyl-undecane, (*G*) dodecane, and (*H*) hexadecane were measured in exhaled breath samples by using gas chromatography–mass spectrometry. (*I*) Correlation of oropharyngeal viral loads with 3,8-dimethyl-undecane and 4,7-dimethyl-undecane. (*J*) Correlation of symptom scores with 3,8-dimethyl-undecane. In *B–H*, results are shown as individual data points per patient with the median (±interquartile range), and data were analyzed by using the Wilcoxon rank sum test and by comparing values with the paired baseline values. In *I* and *J*, individual data points are shown, and data were analyzed by using Spearman's correlation test. $^{*}P < 0.05$ and $^{**}P < 0.01$. ns = nonsignificant.

and 98 exacerbation episodes were captured over the 18-month study period. Baseline demographic and clinical characteristics of the included subjects are shown in Table E2. Sputum was obtained in 88 of these episodes (90.0%) and underwent viral PCR and bacterial culture analysis so that the exacerbation etiology could be determined. Breath sampling was carried out within 48 hours of exacerbation onset in all 88 episodes, and a subset of these exacerbations was sampled further at 1 week ($n = 45$), 2 weeks ($n = 44$), and 6 weeks ($n = 45$) after exacerbation onset (Figure 4A). Examination of breath profiles in baseline versus exacerbation samples identified 10 compounds that were significantly altered, including one long-chain alkane compound: 2,9-dimethyl-undecane (Table 3). Given our prior findings that decane and other long-chain alkanes are upregulated in experimentally infected cells and healthy human subjects, we therefore further focused on this compound. Concentrations of 2,9-dimethyl-undecane were significantly increased in the exhaled breath of patients at exacerbation onset and at 2 weeks compared with at the stable state (regardless of etiology) (Figure 4B).

2,9-Dimethyl-Undecane Is Increased in Virus-Positive COPD Exacerbations

Of the 88 exacerbation episodes studied, $n = 15$ were virus positive ($n = 9$ RV, $n = 6$ respiratory syncytial virus) and $n = 26$ were bacteria positive (new bacteria cultured that was not present at the stable state; $n = 5$ *H. influenzae*, $n = 6$ *Moraxella catarrhalis*, $n = 3$ *S. pneumoniae*, $n = 3$ *Pseudomonas aeruginosa*, $n = 2$ *Serratia*, $n = 2$ *Staphylococcus aureus*, $n = 1$ *Enterobacter*, $n = 3$ dual *H. influenzae/S. pneumoniae*, and $n = 1$ dual *Moraxella/Staphylococcus*). There were $n = 6$ dual viral/bacterial exacerbations and $n = 41$ exacerbations with no pathogen identified. The compound 2,9-dimethyl-undecane was significantly increased in the exhaled breath of patients with virus-positive exacerbations at onset and 1 week (Figure 4C), with no significant alteration in this compound being observed at any time point

Table 2. Compounds Detected in Exhaled Breath of Subjects during Experimental Infection with Rhinovirus That Differed from Those Detected before Infection

Retention Time (min)*	Q Value	Compound	M/Z Base Peak†
9.4	1.00×10^{-9}	2-Propanol	45
13.1	5.18×10^{-8}	2-Butanone	43
14.4	3.05×10^{-8}	Cyclohexane	84
24.6	2.14×10^{-8}	Ethylbenzene	91
25	7.46×10^{-9}	1,4-Dimethyl-benzene	91
26.2	7.99×10^{-9}	1,3-Dimethyl-benzene	91
29	0.000497	Decane	57
30.4	7.01×10^{-7}	1,2,3-Trimethyl-benzene	105
31.3	8.79×10^{-7}	4,7-Dimethyl-undecane	71
31.5	4.30×10^{-8}	5,7-Dimethyl-undecane	71
32.7	1.52×10^{-7}	Undecane	57
32.9	1.24×10^{-7}	Dodecane	71
33.1	3.79×10^{-8}	4,7-Dimethyl-undecane	71
35.6	6.90×10^{-7}	Unknown (MW = 166)	151
35.8	9.66×10^{-7}	Unknown (MW = 166)	70
36.1	1.32×10^{-6}	Unknown (MW = 170)	57
37.5	9.08×10^{-7}	Unknown (MW = 270)	107
38.7	3.64×10^{-7}	Hexadecane	71
39	1.59×10^{-8}	3.8-Dimethyl-undecane	71
39.5	2.22×10^{-7}	3,8-Dimethyl-undecane	71
40	9.56×10^{-9}	2,3,7-Trimethyl-4-octene	69
40.6	1.32×10^{-6}	Unknown (MW = 268)	71
42.6	3.84×10^{-7}	Unknown (MW = 162)	162

Definition of abbreviations: FDR = false discovery rate; M = mass; MW = molecular weight; Z = charge number of ions.
Data were analyzed by using ANOVA with FDR correction (*Q* value).
*Retention time indicates the time to compound identification that was spent by a solute in the gas chromatography column.
†The M/Z base peak, which is used to identify compounds, is the tallest peak in the M spectrum that is due to an ion having the greatest relative abundance.

during exacerbation for bacterial, dual bacterial/viral, or pathogen-negative episodes (Figures 4D–4F). Comparison of baseline with peak exacerbation concentrations of 2,9-dimethyl-undecane indicated significant increases for virus-positive and no-pathogen-detected episodes, with no significant increase being seen for bacterial or dual viral/bacterial exacerbations (Figure 4G). The concentrations of 2,9-dimethyl-undecane also correlated positively with the acute percent FEV_1 decline during exacerbation in virus-positive exacerbations (Figure 4H), with no correlation being shown for bacterial episodes and an inverse correlation being observed for pathogen-negative exacerbations (Figures E4A–E4B), providing further evidence that long-chain alkane compounds are uniquely related to the severity of viral infections.

Given that inhaled corticosteroids (ICS) can attenuate antiviral mediators such as type I IFNs (20) and because our prior observations indicated that decane compounds may be related to innate antiviral immune responses, we performed a subanalysis of exhaled breath 2,9-dimethyl-undecane concentrations at virus-positive exacerbation onset in patients stratified according to current use or nonuse of ICS. We observed a trend toward increased decane concentrations in subjects with COPD who were not receiving ICS ($P = 0.1$, Figure E5).

Discussion

Development of a rapid noninvasive test that can determine the precipitant of an exacerbation of chronic lung disease and reliably distinguish virally from bacterially induced episodes has been a long-term aim for the field. Existing approaches such as clinical symptom evaluation (e.g., Anthonisen classification [5]) or use of blood biomarkers of systemic inflammation (e.g., CRP, procalcitonin [32]) are nonspecific and imperfect. The current study provides proof of concept that exhaled breath measurements could be a promising tool for identifying the etiology of an exacerbation event in COPD.

The main results obtained during our study are summarized in Figure 5. We show that decane and/or other long-chain alkane compounds are upregulated by RV, a causative agent of the common cold and the major precipitator of COPD exacerbations (1, 33). These compounds were readily detectable in controlled infection experiments within the headspace of infected epithelial cells and *in vivo* within the exhaled breath of subjects undergoing experimental viral challenge. Furthermore, in a "real-world" study of patients with COPD, the long-chain alkane 2,9-methyl-undecane was preferentially increased during virus-associated exacerbations and correlated with exacerbation severity. Our study is the first to report a virus-induced VOC profile consisting of long-chain alkane compounds that is detectable consistently across a range of model systems, and the detailed serial sampling involved in our studies allowed unique insight into the dynamic temporal changes in exhaled breath VOCs that occur during acute respiratory viral infections.

Several studies have previously evaluated *in vitro* VOC release in response to a number of respiratory pathogens, providing consistent evidence that active infection induces a number of cellular changes that invoke the release of specific metabolites from the epithelium (14–18, 34). These studies are heterogenous in nature but have reported that epithelial-cell infection with a range of viruses or bacteria induces the release of several compounds, many of which were also observed in our *in vitro* bacterial (e.g., short-chain alkanes, alcohols, dimethyl sulfide) and viral (e.g., alcohols, phenol, dimethyl sulfide) stimulation experiments. However, in our study, we uniquely identified that the long-chain alkane decane was upregulated by RV but not by the common respiratory pathogenic bacteria *S. pneumoniae* or *H. influenzae* in BECs, raising speculation that decane may be a virus-specific metabolite released during infection. It is important to acknowledge, however, that obligate intracellular bacteria such as *Legionella pneumophila* and *Chlamydia pneumoniae* are also important causes of COPD exacerbations, and further studies are needed to investigate whether the lack of decane induction observed with *S. pneumoniae* and *H. influenzae* applies to a larger spectrum of pathogenic bacteria.

Given that *in vitro* experiments represent a simplistic surrogate for the complex changes that occur during *in vivo*

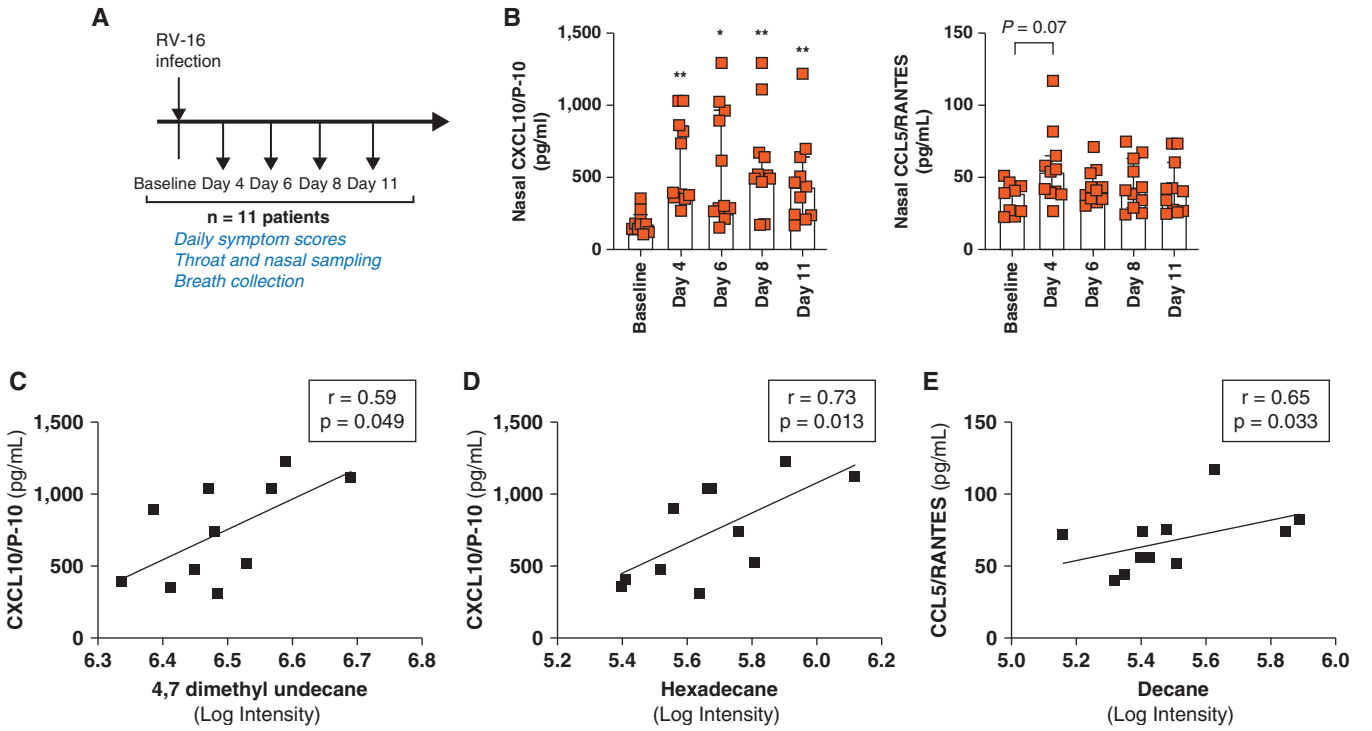

Figure 3. Decane and other long-chain alkane compounds correlate with the magnitude of the antiviral immune responses in subjects experimentally challenged with rhinovirus (RV). (*A*) Eleven healthy subjects were experimentally challenged with RV-A16 and sampled at various time points after infection. Nasal lavage concentrations of (*B*) CXCL10/IP-10 and CCL5/RANTES (regulated upon activation, normal T cell expressed and secreted) were measured by using an ELISA. The correlation of nasal CXCL10/IP-10 concentrations with exhaled breath levels of (*C*) 4,7-dimethyl-undecane and (*D*) hexadecane are shown. (*E*) The correlation of nasal CCL5/RANTES concentrations with exhaled breath levels of decane are shown. In *B* and *C*, results are shown as individual data points per patient with the median (±interquartile range), and data were analyzed by using a Wilcoxon rank sum test and comparing values with the paired baseline values. In *D–F*, individual data points are shown, and data were analyzed by using Spearman's correlation test. $*P < 0.05$ and $**P < 0.01$.

infection, to confirm the translational relevance of this finding, we proceeded to show that decane and a range of other long-chain alkanes are also increased in the exhaled breath of subjects experimentally infected with RV. Consistent with our conclusion that these metabolites are virally induced, exhaled breath concentrations of long-chain alkanes correlated positively with viral loads and nasal concentrations of antiviral cytokines in these subjects.

This is the first study to evaluate VOC changes in a controlled experimental infection model in which temporal changes after infection can be studied accurately. However, in clinical practice, the precise time of virus acquisition is not known, and patients may present with symptoms at differing time points during exacerbation. We therefore further corroborated our findings by studying a cohort of patients with COPD experiencing naturally occurring infections, identifying that the long-chain alkane 2,9-dimethyl-undecane was similarly increased during virus-associated exacerbations in this real-world setting.

The consistency of our findings across three independent study types provides robust evidence that decane and/or other long-chain alkane compounds are metabolites released during acute respiratory viral infection and raises speculation about their potential as virus-specific disease biomarkers. Previous studies have also examined exhaled breath volatile signatures during COPD exacerbation and have shown differences compared with a stable state (6, 35, 36). However, in contrast to our investigations, these studies do not specifically assess exacerbations in which viruses are the causative agent and could not identify specific named compounds associated with exacerbation/viral infection.

The exact mechanisms through which VOCs are produced and released in response to viral or other infections are poorly understood, but they are likely to be the products of biochemical reactions that occur during infection of a host cell. Notably, decane-containing compounds (e.g., decane-9-yl-xanthogenate) have been shown to have antiviral properties against vesicular stomatitis virus (31), suggesting that endogenous production of these VOCs may represent a component of the host antiviral immune response. Accordingly, we found correlations between long-chain alkanes and nasal antiviral cytokine concentrations in our experimental viral challenge model. Alterations in exhaled breath 2,8-dimethyl-undecane concentrations have been reported in subjects challenged intranasally with live attenuated influenza vaccination (37), further reinforcing that there may be a relationship between viruses and these compounds. Long-chain alkanes have been previously reported to be produced as a consequence of oxidative stress after supplemental oxygen challenge in humans (38) and also in response to hydrogen peroxide stimulation of airway epithelial cells *in vitro* (39). Viral infection is well recognized to induce

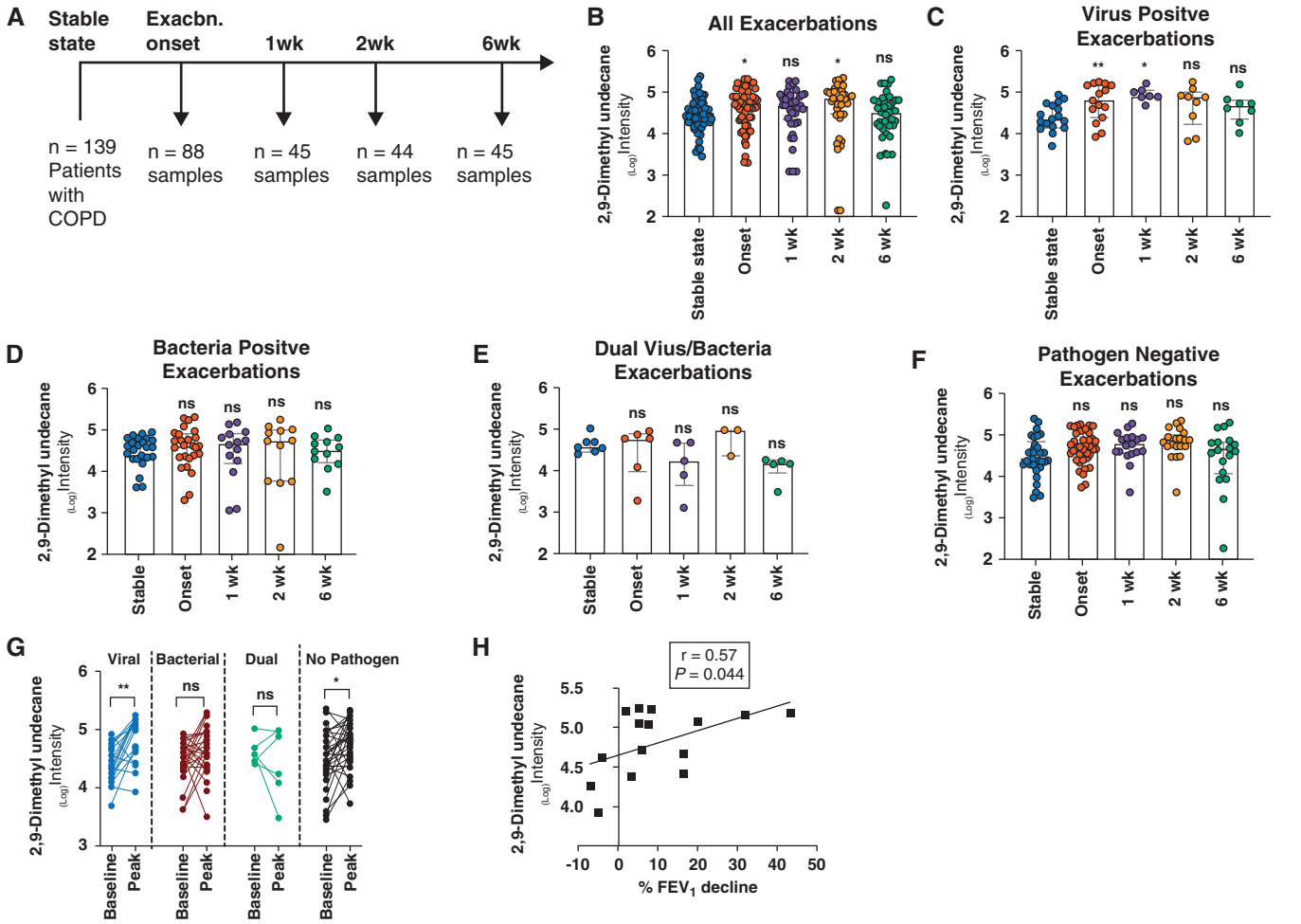

Figure 4. Exhaled breath 2,9-dimethyl-undecane is increased in virus-associated naturally occurring chronic obstructive pulmonary disease (COPD) exacerbations. (*A*) A cohort of 139 patients with COPD were recruited and monitored for 18 months. Patients were sampled at a stable state and after presentation with acute exacerbation at onset, with a subset also being sampled at 1, 2, and 6 weeks after onset. Exhaled breath was collected, and concentrations of 2,9-dimethyl-undecane were measured by using gas chromatography–mass spectrometry. (*B*) Data are shown for all exacerbation episodes. (*C*) Episodes associated with positive viral detection at PCR analysis. (*D*) Episodes associated with culture positivity for potentially pathogenic bacteria that were not present at stable-state sampling. (*E*) Episodes in which viruses and bacteria were co-detected. (*F*) Episodes in which neither viruses nor bacteria were detected. (*G*) Comparison of baseline and peak (i.e., the maximal concentration detected during the infection for each individual) concentrations of 2,9-dimethyl-undecane in subjects with virus-induced, bacteria-induced, dual virus and bacteria–induced, and pathogen-negative exacerbations. (*H*) Correlation of the percent FEV_1 decline during exacerbation with 2,9-dimethyl-undecane concentrations. In *B*–*F*, results are shown as individual data points per patient with the mean ± SD, and data were analyzed by using a Wilcoxon rank sum test and by comparing values with paired baseline values. In *G*, individual data points are shown, and data were analyzed by using a Wilcoxon rank sum test and by comparing values with paired baseline values. In *H*, individual data points are shown, and data were analyzed by using Spearman's correlation test. $^*P < 0.05$ and $^{**}P < 0.01$. Exacbn. = exacerbations; ns = nonsignificant.

oxidative stress through a number of mechanisms (40), and we have previously reported that markers of oxidative stress are increased during experimental RV challenge in COPD (41), providing a biologically plausible mechanism through which RV could induce the release of decane compounds during active respiratory infection. Moreover, studies have revealed that alkane compounds are increased in the breath of children with asthma compared with healthy children (42), and this may similarly be related to the increased oxidative stress and inflammation present within the airways of these individuals.

It should be noted that in the COPD exacerbation study, although significantly increased, 2,9-dimethyl-undecane was only observed in the virus-positive exacerbation group as a whole, and increases were still observed in some patients in whom a virus was not detected at exacerbation (bacteria-associated or pathogen-negative exacerbations). We cannot exclude that, in some of these patients, a causative virus was missed (false-negative result) or that some other exacerbation precipitant (e.g., specific bacterial pathogens, air pollution) may have induced an increase in decane. Alternatively, there may be other, as yet uncharacterized, confounding variables that contribute to upregulation of decane production (e.g., baseline inflammation, airway microbiome composition, inhaled therapies, etc.). More detailed studies elucidating the molecular mechanisms governing decane release will be

Table 3. Compounds Detected in Exhaled Breath of Subjects during COPD Exacerbation That Differed from Those Detected during a Stable State

Retention Time (min)*	*P* Value	*Q* Value	Log Change	M/Z Base Peak†	Compound
7.70	0.000407	0.031733	−0.68987	41	2-Ethyl-hexanal
8.42	4.44×10^{-5}	0.029451	−0.54597	67	1,4 Pentadiene
18.40	2.75×10^{-4}	0.033126	−0.70058	85	Fufuryl alcohol, tetrahydro-5-methyl, *cis*-
22.80	0.001449	0.000001	−0.63345	55	Cyclopentanone
33.37	0.001146	0.047525	−0.39011	83	Ethyl cyclohexane
35.43	5.41×10^{-4}	0.035889	−1.10379	95	*Trans*-9-methyldecalin
36.33	0.000195	0.036903	−0.81992	97	1-Methyl-2-pentyl-cyclohexane
38.28	0.000242	0.032098	−0.6745	149	Diethyl phthalate
38.37	4.12×10^{-5}	0.000001	−0.627	57	2,9-Dimethyl-undecane
46.95	0.000682	0.039368	−0.63204	191	2,5-Di-tert-butyl phenol

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; FDR = false discovery rate; M = mass; Z = charge number of ions. Data were analyzed by using ANOVA with FDR correction (*Q* value).
*Retention time indicates the time to compound identification that was spent by a solute in the gas chromatography column.
†The M/Z base peak, which is used to identify compounds, is the tallest peak in the M spectrum that is due to an ion having the greatest relative abundance.

needed to further explore this. Independent validation of our findings is also required in larger prospective COPD cohort studies and also in experimental viral infection challenge studies performed specifically in patients with COPD. It is also conceivable that the exhaled breath biomarkers identified here could be applied more widely to determine the etiology in patients presenting with acute undifferentiated breathlessness, which may be multifactorial (e.g., infection, heart failure, pulmonary embolism) and is often difficult to distinguish clinically. Whether decane could additionally be a useful biomarker of early severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection also warrants future investigation.

It is important to note that a number of technical factors may affect the reproducibility of exhaled breath testing, as summarized in a previous European Respiratory Society technical standard document (11). Our sampling methods were kept consistent across all studies, and all subjects refrained from exercise for at least 1 hour before providing a sample. However, variation in breathing patterns, the exhalation flow rate, and the proportion of dead space air may significantly affect exhaled breath composition and collection (43, 44), and we cannot exclude that some of these factors may have acted as confounding variables in our study. Furthermore, breathing patterns are known to change at exacerbation (45) compared with at a stable state, and we cannot exclude that this alteration could have directly impacted the profile of VOC release.

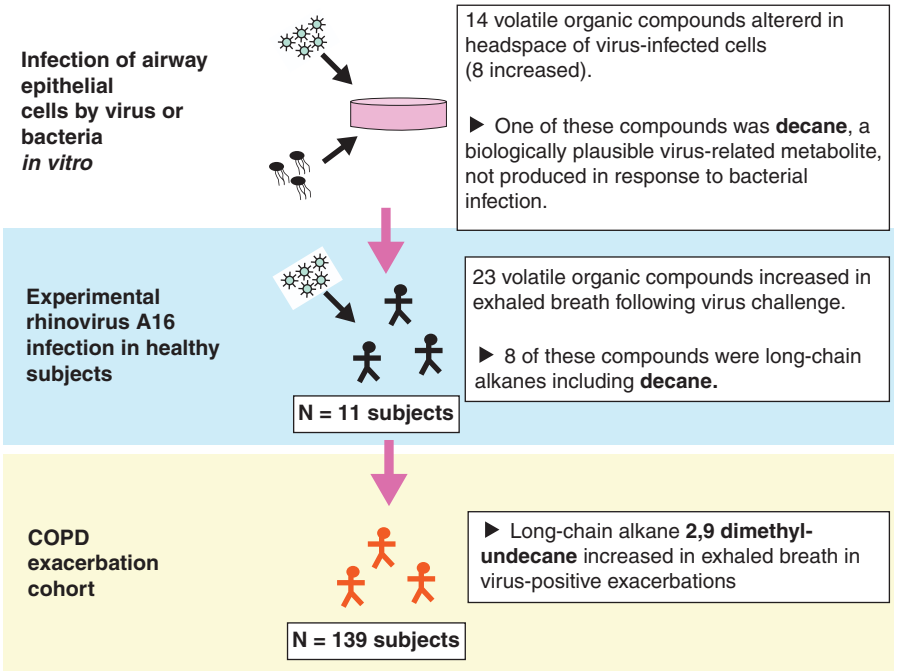

Figure 5. Schematic diagram showing the results obtained in this study. COPD = chronic obstructive pulmonary disease.

In summary, our data show that viral infection induces the release of the VOC decane and other long-chain alkane compounds, an effect observed consistently across a range of experimental *in vitro* and *in vivo* studies of primary viral infection and virus-exacerbated COPD. Our data raise the speculation that measurement of these compounds in exhaled breath could represent a rapid, noninvasive biomarker of viral infection. Further studies will be needed to determine whether measurement of one compound or a combination of these compounds could be used to guide more targeted exacerbation therapy with antibiotic/antiviral therapy in patients presenting with COPD exacerbations. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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