



# E-cigarette vapour enhances pneumococcal adherence to airway epithelial cells

Lisa Miyashita<sup>1</sup>, Reetika Suri <sup>1</sup>, Emma Dearing<sup>2</sup>, Ian Mudway<sup>3,4</sup>,  
Rosamund E Dove<sup>4</sup>, Daniel R. Neill<sup>2</sup>, Richard Van Zyl-Smit <sup>5</sup>, Aras Kadioglu<sup>2</sup>  
and Jonathan Grigg <sup>1</sup>

**Affiliations:** <sup>1</sup>Centre for Genomics and Child Health, Blizard Institute, Queen Mary University of London, London, UK. <sup>2</sup>The Dept of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health, University of Liverpool, Liverpool, UK. <sup>3</sup>MRC-PHE Centre for Environment and Health, King's College London, London, UK. <sup>4</sup>NIHR Health Protection Research Unit on Health Impacts of Environmental Hazards, King's College London, London, UK. <sup>5</sup>Division of Pulmonology, Dept of Medicine, University of Cape Town and UCT Lung Institute, Cape Town, South Africa.

**Correspondence:** Jonathan Grigg, Blizard Institute, Queen Mary University of London, 4 Newark Street, London, E1 2AT, UK. E-mail: j.grigg@qmul.ac.uk

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**Exposure of airway cells to E-cigarette vapour upregulates a receptor used by pneumococci to adhere to cells** <http://ow.ly/p9Be30hE5B1>

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**ABSTRACT** E-cigarette vapour contains free radicals with the potential to induce oxidative stress. Since oxidative stress in airway cells increases platelet-activating factor receptor (PAFR) expression, and PAFR is co-opted by pneumococci to adhere to host cells, we hypothesised that E-cigarette vapour increases pneumococcal adhesion to airway cells.

Nasal epithelial PAFR was assessed in non-vaping controls, and in adults before and after 5 min of vaping. We determined the effect of vapour on oxidative stress-induced, PAFR-dependent pneumococcal adhesion to airway epithelial cells *in vitro*, and on pneumococcal colonisation in the mouse nasopharynx. Elemental analysis of vapour was done by mass spectrometry, and oxidative potential of vapour assessed by antioxidant depletion *in vitro*.

There was no difference in baseline nasal epithelial PAFR expression between vapers (n=11) and controls (n=6). Vaping increased nasal PAFR expression. Nicotine-containing and nicotine-free E-cigarette vapour increased pneumococcal adhesion to airway cells *in vitro*. Vapour-stimulated adhesion *in vitro* was attenuated by the PAFR blocker CV3988. Nicotine-containing E-cigarette vapour increased mouse nasal PAFR expression, and nasopharyngeal pneumococcal colonisation. Vapour contained redox-active metals, had considerable oxidative activity, and adhesion was attenuated by the antioxidant N-acetyl cysteine.

This study suggests that E-cigarette vapour has the potential to increase susceptibility to pneumococcal infection.

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## Introduction

Epidemiological studies suggest that inhalation of toxins increases the risk of airway bacterial infection. For example, cigarette smoking is associated with a fourfold (95% CI 2–7) increased risk of invasive pneumococcal disease [1], passive exposure to environmental tobacco smoke is associated with a 1.5-fold (95% CI; 1.2–1.9) risk of pneumonia in children [2], and in young children exposure to fossil fuel-derived particulate matter is associated with a 1.3-fold (95% CI 1.0–1.6) increase in bacterial pneumonia [3]. Increased risk of pneumococcal infection, the commonest cause of bacterial pneumonia [4], is also reported in occupational and environmental settings, including exposure to welding fumes [5] and airborne dust [6]. Since invasive pneumococcal strains adhere to, and translocate across, respiratory tract epithelial cells, adhesion is a prerequisite for establishing pneumococcal disease [4]. To establish firm adhesion, pneumococci co-opt host-expressed platelet-activating factor receptor (PAFR), and then use the receptor as a Trojan horse to enter airway cells as the receptor is internalised [7]. Previous studies suggest that upregulation of PAFR is a biologically plausible mechanism for the association between inhalation of toxins and vulnerability to pneumococcal infection. For example, we found that cigarette smoke extract, fossil fuel-derived particulate matter and welding fumes, by means of the induction of oxidative stress, upregulate PAFR-dependent pneumococcal adhesion to lower airway cells *in vitro* [8–10].

Electronic cigarettes (ECs) are marketed to adults and young people as a safer alternative to smoking and a potential smoking cessation aid. The vapour from ECs, generated by the vaporisation of propylene glycol (1,2-propandiol), glycerin, nicotine and flavourings in EC liquid, contains fewer toxic compounds than tobacco smoke [11]. Although it is speculated that some adverse health effects of inhaling EC vapour are reduced compared with tobacco smoke, there is emerging evidence of toxic effects including the capacity to impair pulmonary bacterial host defences. For example in mice, EC vapour depletes lung antioxidants and delays the clearance of pneumococci from the lung [12, 13]. We therefore hypothesised that EC vapour increases PAFR-dependent pneumococcal adhesion to airway cells. To address this hypothesis, we sought in the present study to determine: 1) the effect of vaping on nasal epithelial PAFR expression in adults; 2) the effect of EC vapour on PAFR-dependent pneumococcal adhesion to airway cells *in vitro*; and 3) the effect of EC vapour on nasal PAFR expression and nasal pneumococcal burden in a mouse model of asymptomatic nasopharyngeal pneumococcal colonisation.

## Methods

### Human volunteer study

Adults who vaped at least once a week, and healthy never-smoked adult controls were recruited. Exclusion criteria were tobacco cigarette smoking within 3 months (for vapers), any tobacco smoking (for controls), chronic respiratory disease, recent nasal surgery and nasal therapy. Nasal epithelial cell samples were obtained from both nostrils of participants using a Rhino-probe (VWR, Radnor, PA, USA) and pooled in primary cell media (Promocell, Heidelberg, Germany) containing penicillin–streptomycin and primocin (InvivoGen, Toulouse, France). In vapers, nasal cells were obtained immediately before and 1 h after EC use. Vaping was over an observed 5-min period. Vapers were asked to inhale EC vapour at their normal frequency and to exhale normally. Nasal biopsies were taken from controls on a single occasion. To assess PAFR expression, nasal cells were washed and resuspended in DPBS containing 10% FBS before staining with an anti-PAFR primary antibody (1:200, Abcam, Cambridge, UK) as described above. E-cadherin (primary antibody used at 1:100, Abcam) was added to identify epithelial cells [14]. Cells were washed, and then stained with secondary antibodies conjugated to Alexa Fluor 488 for PAFR expression (1:3000, Abcam) and conjugated to APC for E-cadherin expression (1:1500, Abcam) for 30 min with shaking at room temperature. A PAFR isotypic control (rabbit immunoglobulin (Ig)G monoclonal EPR25A) was included to adjust for nonspecific immunostaining. Analysis was carried out on the BD FACS Canto II machine using BD FACSDiva software (BD Biosciences, Oxford, UK) with gating set to exclude cell debris. PAFR expression correcting for non-specific staining, was expressed as median fluorescence intensity (MFI). The study was approved by a UK National Health Service Research Ethics Committee (15/NE/0237), and required written consent.

### E-cigarette vapour

EC vapour was collected onto cotton filters through a peristaltic pump (Jencons Scientific Ltd., East Grinstead, UK) at a fixed rate from a second-generation EC (RBC CE5 Clearomizer, 3.7 V 650 mAh power supply battery, <https://www.ukecigstore.com>, UK). Filters were exposed to 25 puffs over 5 min using either tobacco-flavoured EC liquid containing 24 mg·mL<sup>-1</sup> nicotine, or nicotine-free tobacco-flavoured EC liquid (<https://www.ukecigstore.com>). EC vapour extract (ECV) was obtained from filters by vortexing in 2 mL of Dulbecco's phosphate-buffered saline (DPBS), and stored as 100% stock solution at –20°C. Medium control stocks were made by extracting 2 mL DPBS from the cotton filters (DPBS control extract). Pure nicotine (Sigma-Aldrich, Poole, UK) was diluted in DPBS to produce a stock solution. The concentration

of nicotine in ECV and EC liquid was measured by gas chromatography–mass spectrometry (GC-MS; supplementary material).

#### **Airway cells**

The alveolar type II epithelial cell line A549 was purchased from Sigma-Aldrich (Poole, UK) and maintained in DMEM supplemented with fetal bovine serum (FBS) and penicillin–streptomycin (Lonza, Basel, Switzerland), and passage number less than 20. The bronchial epithelial cell line BEAS-2B was maintained in RPMI-1640 (Life Technologies) and supplemented with FBS and penicillin–streptomycin and the passage number was less than 20. Human primary bronchial epithelial cells (HBEPcs) were purchased from Promocell GmbH (Heidelberg, Germany) and maintained as per the manufacturer's instructions and the passage number was less than 5. Human primary nasal epithelial cells (HPNEPcs) were obtained from a never-smoked, non-vaping female adult donor using a Rhino-probe, and maintained in BEGM (Bronchial Epithelial Cell Growth Medium) supplemented with the BEGM BulletKit as per the manufacturer's instructions (Lonza) and with passage number less than 5. The presence of epithelial cells was confirmed by assessing the total percentage of cells stained with epithelial marker E-cadherin, assessed by flow cytometry (described above). Cell membrane integrity was assessed by lactate dehydrogenase release, according to the manufacturer's instructions (Sigma-Aldrich). Treatment of cells with distilled water was used as a positive control, and indicated 100% LDH release.

#### **Adhesion and PAFR**

The virulent type 2 *Streptococcus pneumoniae* encapsulated strain D39 (NCTC 7466) was purchased from the National Collection of Type Cultures (Central Public Health Laboratory, London, UK) grown to mid-logarithmic phase ( $OD_{600}=0.4–0.6$ ) in brain–heart infusion broth (BHI) (Oxoid, Basingstoke, UK) and stored at  $-80^{\circ}\text{C}$ . Pneumococcal adhesion to airway cells was conducted using an *in vitro* adhesion assay [8, 9]. In this assay, colony forming unit counts per mL (CFU) reflect both the number of pneumococci adherent to the surface of cells and the number of intracellular bacteria (supplementary material). The intracellular component was assessed after first killing surface adherent bacteria with penicillin ( $200\text{ mg}\cdot\text{mL}^{-1}$ ) and gentamicin ( $10\text{ mg}\cdot\text{mL}^{-1}$ ) for 30 min. Intracellular pneumococci that were protected from antibiotic killing were recovered by cell lysis with ice-cold sterile water and plated on BHI agar to determine CFU [8]. The role of oxidative stress was determined by incubating cells with the thiol antioxidant N-acetylcysteine (NAC; Sigma-Aldrich), at a final concentration  $5\text{ mmol}\cdot\text{L}^{-1}$  [15]. Cells were incubated with NAC both 30 min before exposure to ECV, and during ECV exposure. NAC was removed by washing prior to adding pneumococci and assessing adhesion. The role of PAFR was assessed by adding CV3988 (Sigma Aldrich), a specific PAFR receptor blocker with a half maximal inhibitory concentration ( $IC_{50}$ ) of  $0.28\text{ }\mu\text{M}$  [16]. CV3988 was added to the adhesion assay at a final concentration of  $20\text{ }\mu\text{M}$ , as previously reported [17]. We sought to establish complete results in A549 cells then confirm key findings in other airway cells.

To assess PAFR expression, airway cells were detached from cell culture flasks with trypsin and washed before resuspension in DPBS containing 10% FBS and stained with an anti-PAFR primary antibody (1:200, Abcam, Cambridge, UK) for 1 h with shaking at room temperature. A PAFR isotype control (rabbit IgG monoclonal EPR25A) was included to control for nonspecific staining. Analysis was carried out on the BD FACS Canto II machine using BD FACSDiva software (BD Biosciences, Oxford, UK). PAFR is expressed as MFI.

#### **Mouse pneumococcal colonisation model**

Female CD1 mice 6–8 weeks of age (Charles River, Margate, UK) were dosed twice daily with 100% stock solution nicotine-containing ECV, nicotine-free ECV or a DPBS control extract for the duration of the experiment. On day 4 of dosing,  $1\times 10^5$  CFU *S. pneumoniae* in  $10\text{ }\mu\text{L}$  of PBS was instilled into the nasal cavity under anaesthesia, to induce asymptomatic nasopharyngeal carriage. Four days post inoculation, nasopharyngeal tissues were homogenised and washed through a cell strainer. Pneumococcal CFU were determined by Miles and Misra viable counts as previously described [18]. Nasal PAFR expression was determined by flow cytometry (supplementary material). Animal experiments were performed at the University of Liverpool in accordance with the Animal Scientific Procedures Act 1986 and with the prior approval of the UK Home Office (PPL 40/3602) and the University of Liverpool animal welfare and ethics committee.

#### **Elemental analysis and oxidative potential**

Elemental composition was determined by induction coupled plasma–mass spectrometry (ICP-MS) using a PerkinElmer ICP mass spectrometer NexION 350D, following acid digestion (supplementary material). The oxidative potential (OP) of ECV was determined by quantifying the loss of two low molecular weight

antioxidants ascorbate and glutathione in synthetic human respiratory tract lining fluid (RTLFL) over a 4 h incubation period at 37°C, pH 7.0, using our previously reported method [19] (supplementary material). Data are expressed as the percentage loss of ascorbate and glutathione relative to the 4 h DPBS control. Included in assays were 1) a negative control PM with low OP (M120; a 50 nm carbon black particle with simple surface chemistry [20]), and 2) a particle with higher OP – the urban PM standard SRM-1648 (National Institute of Standards and Technology, Gaithersburg, MD, USA). Particles were used at a final concentration of 50 µg·mL<sup>-1</sup>.

### Statistical analysis

Data from studies of human volunteers, animals, and OP are summarised as mean (SEM) and analysed by either the t-test, or by one-way ANOVA with *post hoc* multiple comparison testing. Data from *in vitro* adhesion studies are from at least four experiments, unless stated, conducted at different times, representing the mean of at least three replicates, and are summarised as median (interquartile range (IQR)) and analysed by Kruskal–Wallis with *post hoc* multiple comparison testing. Analyses were done using Prism 7 (GraphPad Software Inc., La Jolla, CA, USA) and *p*<0.05 was considered statistically significant.

## Results

### Vaping and nasal PAFR expression

11 vaping adult males (mean age 30±3 years, table 1), and six male controls (mean age 39±5 years) were studied. Ten vaping subjects used nicotine-containing EC liquid, and one (subject 3, table 1) used nicotine-free EC liquid. All vapers inhaled more than 10 puffs over 5 min, exhaled vapour *via* both mouth and nose, and had not vaped over the previous 12 h. Nasal PAFR expression prior to ECV inhalation was not significantly different from non-vaping controls (figure 1a). Vaping increased nasal PAFR at 1 h (*p*<0.05 *versus* baseline, figure 1b), and nasal PAFR 1 h post-vaping was increased compared with controls (mean±SEM MFI 5778±1692 *versus* 837±447, *p*<0.05).

### Human alveolar epithelial A549 cells

Both nicotine-free and nicotine-containing ECV increased pneumococcal adhesion to A549 cells in a dose-dependent manner (figure 2a), and a time-dependent manner (figure S1). Exposure of cells to 5% ECV for 2.5 h did not cause A549 cell membrane damage, as assessed by LDH release (figure S2), and this dose and duration was therefore used in subsequent experiments. Pneumococcal penetration into cells, assessed after killing cell surface bacteria, was increased by both nicotine-free and 5% nicotine-containing ECV (*p*<0.05 and *p*<0.01 respectively, figure 2b). Both 5% nicotine-free and nicotine-containing ECV increased PAFR expression (*p*<0.01 and *p*<0.001 respectively, figure 2c), and the PAFR antagonist CV3988 attenuated pneumococcal adhesion stimulated by both 5% nicotine-free and nicotine-containing ECV (*p*<0.05 and *p*<0.01 respectively, figure 2d). CV3988 did not attenuate low-level “basal” pneumococcal adhesion to unexposed cells (figure 2d). The antioxidant NAC completely attenuated pneumococcal adhesion stimulated by both 5% nicotine-free and nicotine-containing ECV (*p*<0.05, figure 3a). NAC did not attenuate “basal” pneumococcal adhesion to unexposed cells (figure 3a). Incubation of A549 cells with dilutions of nicotine in DPBS increased adhesion at 2.5 mg·mL<sup>-1</sup> (*p*<0.01 *versus* control, figure S3), with

TABLE 1 Details of vaping participants

Subject	Age years	Ex-smoker	E-cigarette use	E-cigarette	E-liquid	Nicotine <sup>#</sup> mg·mL <sup>-1</sup>
1	20	Yes (>6 months)	Daily	Kangertech subox nano	Vapor Depot Cherry	1.5
2	20	Yes (>3 months)	Daily	Gamucci Vitesse	Jelly Bean	8
3	20	Yes (>3 months)	Daily	Kangertech subox nano	Vapour Depot Menthol	0
4	39	Yes (>3 months)	Daily	Blu pro	Blu pro Tobacco	18
5	33	Yes (>5 years)	1–2 times per week	Blu pro	Blu pro Tobacco	24
6	35	Occasional (>8 years)	Once daily	E-cig UK	E-cig UK Menthol	24
7	35	Yes (>4 years)	Daily	E-cig UK	E-cig UK Tobacco Royale	18
8	23	Yes (>6 months)	Daily	Innokin	Ciagra Tornado Cyclone	6
9	49	Yes (>4 years)	Daily	iBaccy	iBaccy Cherry	24
10	23	Yes (>5 months)	Daily	Innokin cool Fire	Watermelon	6
11	30	Yes (>4 years)	Daily	Vype	Vype E-liquid	12

<sup>#</sup>: reported by manufacturer.

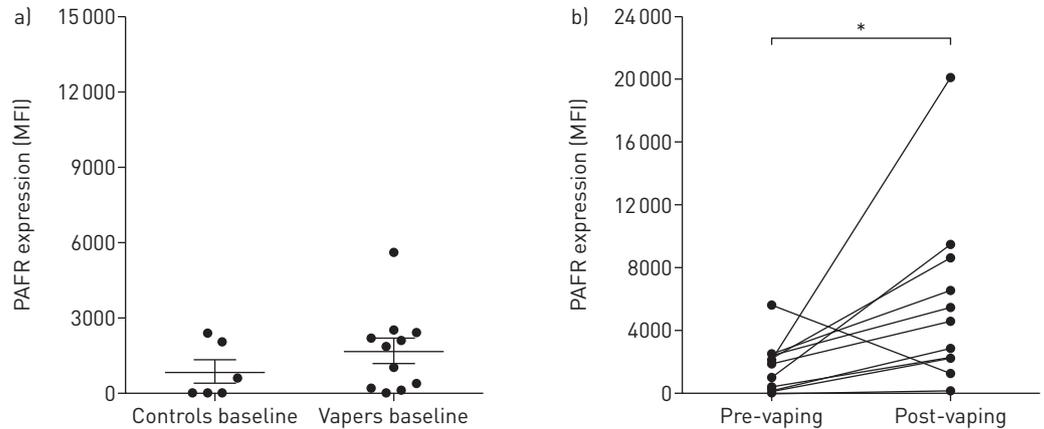


FIGURE 1 a) Platelet-activating factor receptor (PAFR) expression in nasal epithelial cells from vaping adults (n=11) prior to experimental E-cigarette vapour exposure [baseline] and in non-vaping controls (n=6). There is no difference between the two groups (p=0.18). Data are expressed as mean (standard error of the mean) and analysed by unpaired t-test. b) Change in PAFR expression (expressed as median fluorescence intensity [MFI] adjusted for isotopic control) in nasal epithelial cells from adult vapers (n=11), before and 1 h after, 5-min of vaping. \*: p<0.05, paired t-test.

no increase in adhesion at lower concentrations. There was no difference in pneumococcal adhesion stimulated by either freshly generated or frozen ECV (data not shown).

**Other human airway epithelial cells**

The antioxidant NAC completely attenuated pneumococcal adhesion to BEAS-2B cells stimulated by both 5% nicotine-free ECV and nicotine-containing ECV (p<0.05, figure 3b). 5% nicotine-free ECV increased pneumococcal adhesion in HPNEpC primary nasal cells (p<0.05), primary bronchial (HBEpC) cells (p<0.05) and BEAS-2B bronchial epithelial cells (p<0.05, figure 4a-c). 5% nicotine-containing ECV increased pneumococcal adhesion to HPNEpC primary nasal cells (p<0.05), HBEpC primary bronchial cells (p<0.01) and BEAS-2B cells (p<0.01, figure 4a-c). The PAFR blocker CV3988 attenuated

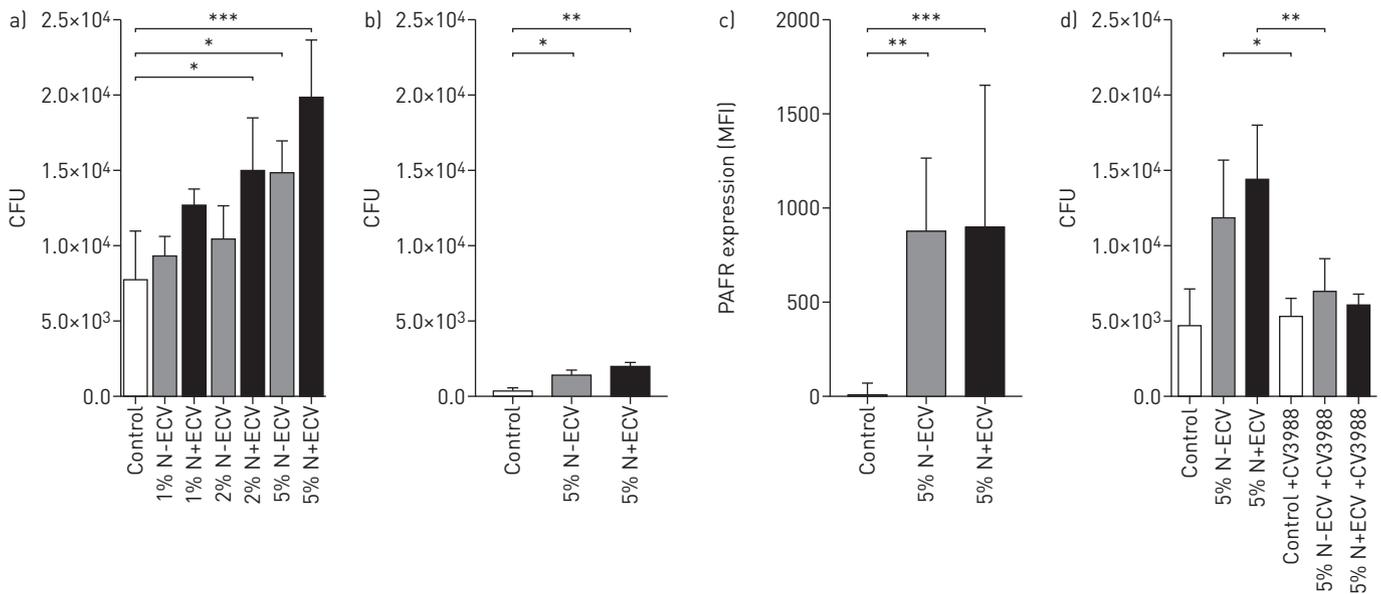


FIGURE 2 a) Dose-dependent effect of nicotine-free (N-) and nicotine-containing (N+) E-cigarette vapour extract (ECV) on A549 cells. Increased pneumococcal adhesion, reflected by increased colony forming unit count (CFU), is significant at 5% for N-ECV and 2% for N+ECV. b) Effect of 5% N-ECV and N+ECV on pneumococcal penetration into A549 cells. Intracellular bacteria were assessed after treatment with antibiotics to kill cell surface bacteria. c) Effect of ECV on platelet-activating factor receptor (PAFR) expression in A549 cells. Expression was determined by flow cytometry and expressed as median fluorescence intensity (MFI). d) Effect of the PAFR blocker CV3988 on pneumococcal adhesion to A549 cells after exposure to either 5% N-ECV or 5% N+ECV. Data, from either six or seven separate experiments, are expressed as median (interquartile range) and analysed by Kruskal-Wallis with *post hoc* multiple comparison testing. \*: p<0.05; \*\*: p<0.01; \*\*\*: p<0.001.

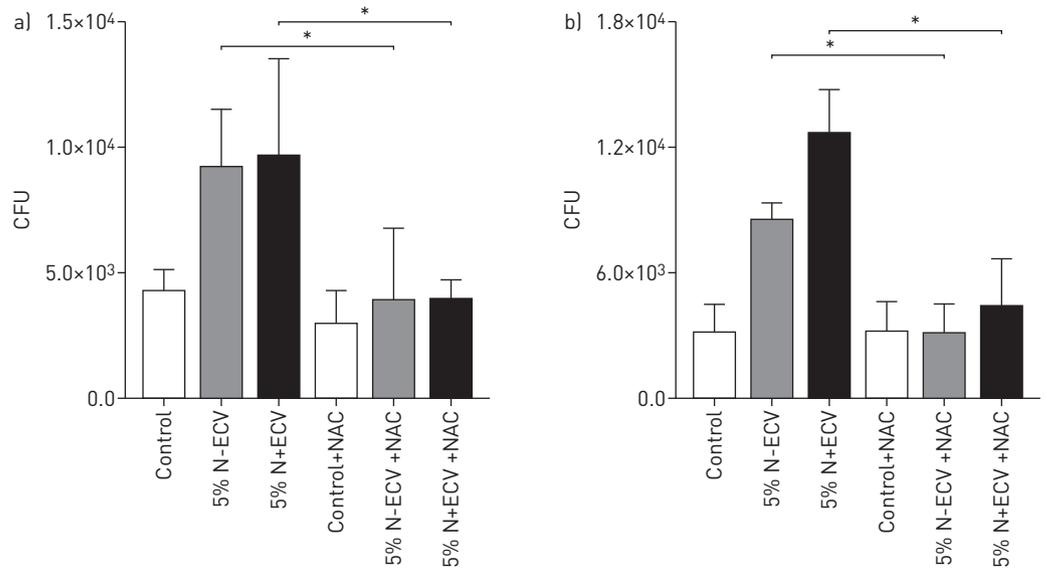


FIGURE 3 Effect of the antioxidant N-acetyl cysteine (NAC) on pneumococcal adhesion to a) A549 and b) BEAS-2B cells cultured with either 5% nicotine-free (N-) E-cigarette vapour extract (ECV), or 5% nicotine-containing (N+) ECV. Data, from either four or five separate experiments, are expressed as median (interquartile range) and analysed by Kruskal-Wallis with *post-hoc* multiple comparison testing. \*: p<0.05.

pneumococcal adhesion stimulated by 5% nicotine-free ECV in HPNEpC primary nasal (p<0.05), HBEpC primary bronchial (p<0.05) and BEAS-2B cells (p<0.05, figure 4a-c). CV3988 also attenuated 5% nicotine-containing ECV stimulated pneumococcal adhesion in HPNEpC primary nasal cells (p<0.05), primary bronchial HBEpC cells (p<0.05) and BEAS-2B cells (p<0.01, figure 4a-c).

5% nicotine-free ECV increased PAFR expression in HPNEpC primary nasal cells (p<0.05), HBEpC primary bronchial (p<0.05) and BEAS-2B cells (p<0.01, figure 5a-c). 5% nicotine-containing ECV

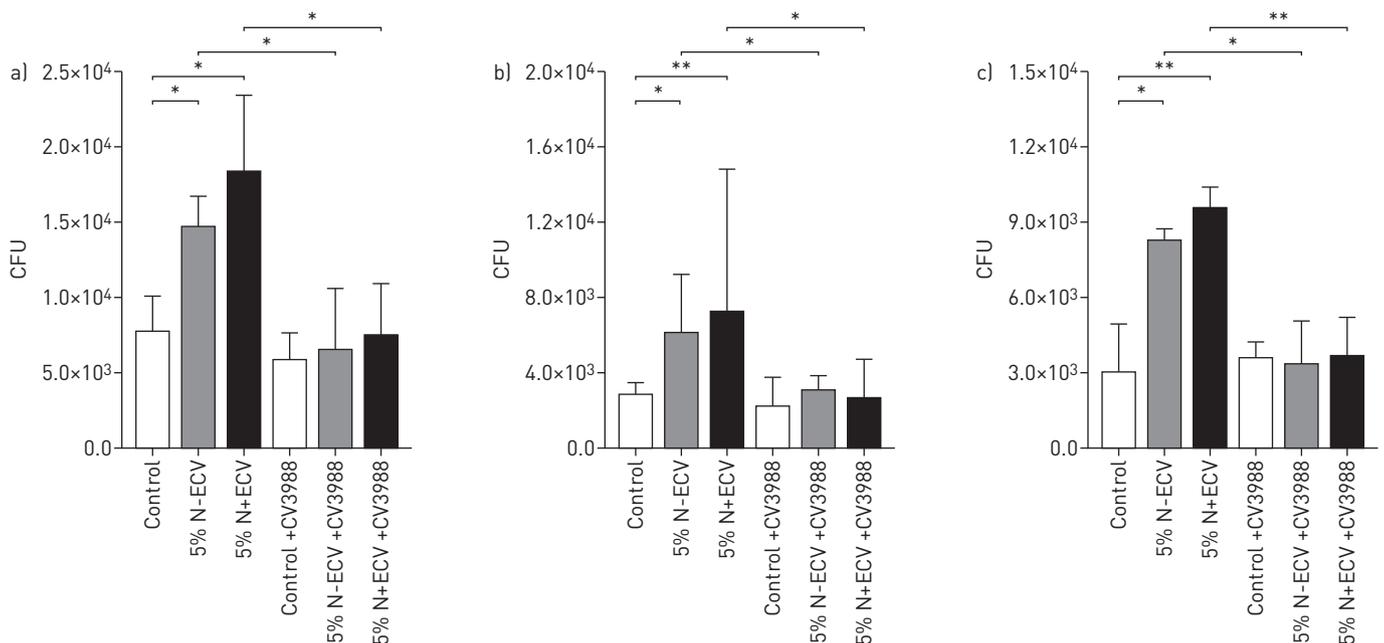


FIGURE 4 Adhesion of *Streptococcus pneumoniae* D39 to a) human primary nasal epithelial cells, b) human primary bronchial epithelial cells and c) bronchial cell line BEAS-2B, after exposure to either 5% nicotine-free (N-) E-cigarette vapour extract (ECV), 5% nicotine-containing (N+) ECV or DPBS control, with and without incubation with platelet-activating factor receptor blocker CV3988. Data from six separate experiments are expressed as median (interquartile range) and analysed by Kruskal-Wallis with *post hoc* multiple comparison testing. \*: p<0.05; \*\*: p<0.01.

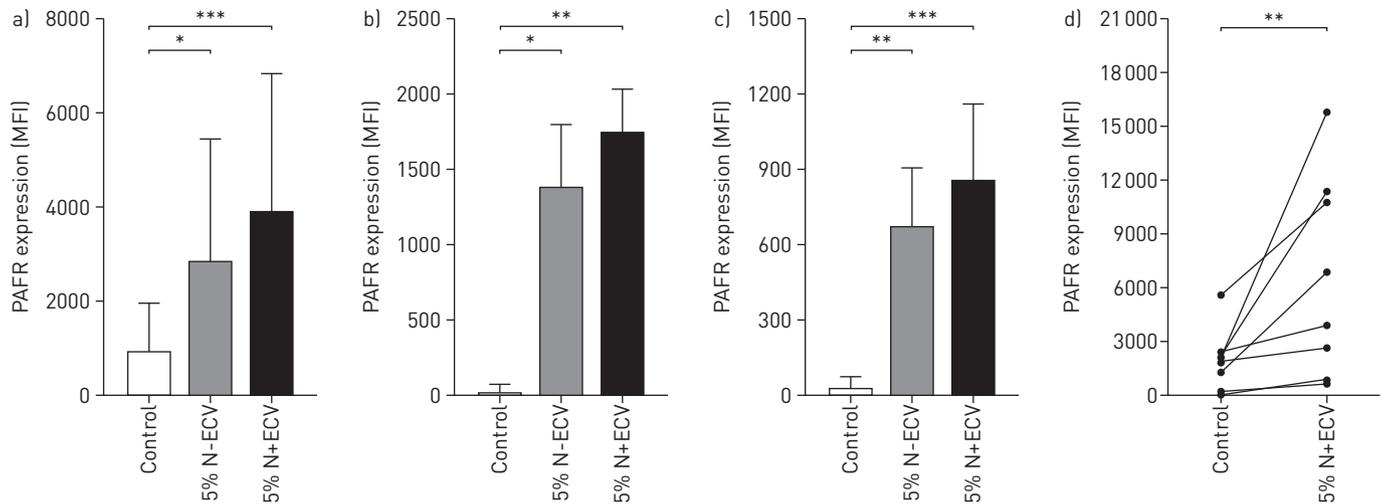


FIGURE 5 Effect of nicotine-free (N-) E-cigarette vapour extract (ECV) and nicotine-containing (N+) ECV on platelet-activating factor receptor (PAFR) median fluorescence intensity (MFI) determined by flow cytometry in a) human primary nasal epithelial cells, b) human primary bronchial epithelial cells and c) BEAS-2B cells. Data from six to 10 separate experiments are expressed as median (interquartile range) and analysed by Kruskal-Wallis with *post hoc* multiple comparison testing. d) PAFR MFI in nasal epithelial cells obtained from vapers (n=8) before vaping (baseline) and then cultured *in vitro* with 5% N+ECV extract for 2.5 h. Data were analysed by paired t-test. \*: p<0.05; \*\*: p<0.01; \*\*\*: p<0.001.

increased PAFR expression in HPNEpC primary nasal cells (p<0.001), primary bronchial HBEpC (p<0.01) and BEAS-2B cells (p<0.001, figure 5a-c).

In eight out of 11 vapers sufficient cells from the baseline biopsy were obtained for culture with 5% nicotine-containing ECV *in vitro*. In these primary nasal cells from vapers, PAFR expression *in vitro* was increased by 5% nicotine-containing ECV for 2.5 h (p<0.01 *versus* medium control, figure 5d).

#### ***Pneumococcal colonisation of the nasopharynx***

Intranasal instillation of 100% nicotine-free ECV did not increase either nasopharyngeal CFU (figure 6a) or PAFR expression (figure 6b). By contrast, prior exposure to 100% nicotine-containing ECV increased nasopharyngeal pneumococcal CFU at 4 days post intranasal instillation of bacteria (p<0.05 *versus* control, figure 6a), and increased nasal epithelial PAFR expression 4 days post exposure (p<0.001 *versus* control, figure 6b).

#### ***Elemental composition and oxidative potential***

Both 5% nicotine-free and nicotine-containing ECV contained metals with the capacity to induce oxidative stress (table S1). Although the elemental composition of the two extracts was broadly similar, the concentration of copper in nicotine-containing ECV was increased (p<0.05 *versus* nicotine-free, (table S1). Both 5% nicotine-free and nicotine-containing ECV extract oxidised ascorbate (p<0.0001 *versus* low OP particle control, figure 7a). The percentage loss for ascorbate of 5% nicotine-containing ECV was increased compared with the particle with higher OP particle (p<0.0001), and was increased compared with the nicotine-free ECV (p<0.0001, figure 7a). A similar pattern was found for glutathione depletion, with higher percentage loss observed with nicotine-containing ECV relative to the low and higher OP particle (p<0.0001, figure 7b), and increased OP compared with the nicotine-free ECV (p<0.001, figure 7b).

#### ***Nicotine***

The nicotine concentration of nicotine-containing E-liquid was 24 mg·mL<sup>-1</sup> – identical to that reported by the manufacturer. Nicotine-free E-liquid contained only a trace of nicotine, and the DPBS control extract contained no nicotine. The nicotine concentration of 5% nicotine-free ECV was just above assay's level of detection at 0.03 mg·mL<sup>-1</sup>, and 5% nicotine-containing ECV contained 0.4 mg·mL<sup>-1</sup>.

#### **Discussion**

In this study, we sought to assess whether exposure of the airway epithelium to EC vapour increases its capacity to support pneumococcal adhesion and infection. In adult volunteers we found that vaping increases nasal epithelial PAFR expression, and that post-vaping PAFR expression is higher than non-exposed controls. PAFR expression is not persistently increased by vaping, since pre-vaping levels are

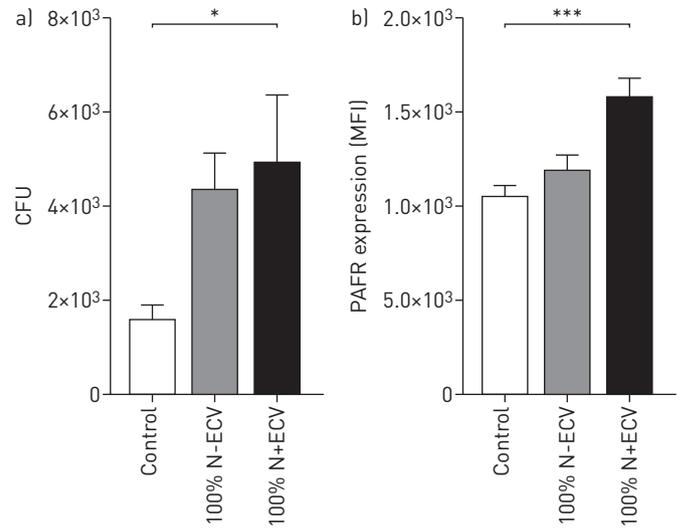


FIGURE 6 a) Nasopharyngeal carriage of *Streptococcus pneumoniae* in mice exposed to either nicotine-free (N-) E-cigarette vapour extract (ECV) or nicotine-containing (N+) ECV or DPBS control. Mice (DPBS controls: n=17 controls; N-ECV and N+ECV: n=18) were dosed intranasally twice daily with 100% N+ECV, 100% N-ECV or DPBS. On the fourth day of dosing, mice were infected with 1×10<sup>5</sup> CFU *S. pneumoniae*. Mice were culled on the fourth day post-infection and nasopharyngeal tissue was collected. Colony forming unit count (CFU) values of *S. pneumoniae* are given as count per mL. b) Platelet-activating factor receptor (PAFR) expression determined by flow cytometry (median fluorescence intensity (MFI)) in nasopharyngeal epithelial cells of mice after exposure to 100% N+ECV. Expression was determined on the fourth day of intranasal ECV dosing. Data are expressed as mean (SEM) and analysed by one-way ANOVA with *post hoc* comparison testing. \*: p<0.05; \*\*\*: p<0.001.

not higher than controls. Compatible with reports of increased pneumococcal adhesion to airway cells exposed to toxins such as cigarette smoke [8], welding fumes [10] and fossil fuel-derived particulate matter [9], we found that ECV increases pneumococcal adhesion to airway epithelial cells *in vitro*.

Adhesion stimulated by ECV *in vitro* is due to increased PAFR expression, since ECV markedly increases expression of PAFR on airway cells, and the PAFR blocker CV3988 completely attenuates ECV-stimulated

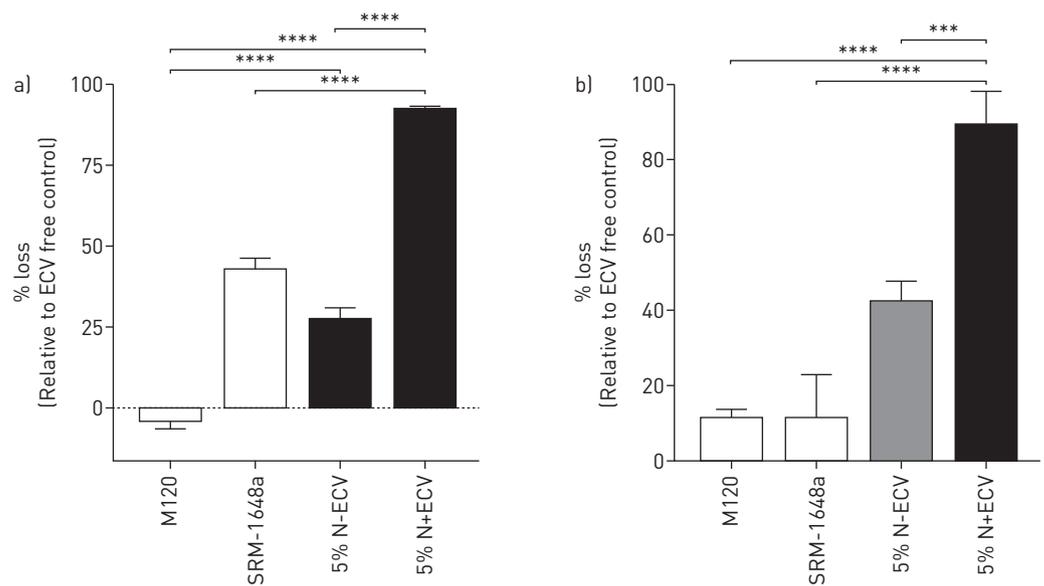


FIGURE 7 The percentage loss of a) ascorbate, and b) glutathione from a synthetic respiratory tract lining fluid (RTLFL) following a 4 h incubation with 5% nicotine-free (N-) E-cigarette vapour extract (ECV) and 5% nicotine-containing (N+) ECV. Included in the assay are a control particle with low oxidative potential (M120) and a particle with higher oxidative potential (SRM-1648). The percentage loss is expressed relative to a particle/ECV-free control. Data are expressed as mean (SEM), and analysed by ANOVA with *post hoc* multiple comparison testing (n=9). \*\*\*: p<0.001; \*\*\*\*: p<0.0001.

adhesion in nasal, bronchial and alveolar epithelial cells. This effect of CV3988 is compatible with previous reports that blocking PAFR attenuates increased pneumococcal adhesion stimulated by either inflammatory mediators [21], or by toxins that induce oxidative stress [10, 22]. The lack of effect of CV3988 on control cells is also compatible with reports that low level “basal” adhesion to unstimulated cells is mediated by a PAFR-independent, as yet undefined, mechanism [21].

There is evidence from previous studies that ECV has the potential to induce oxidative stress in airway cells. For example, GOEL *et al.* [23] reported that ECV contains up to  $10 \times 10^{13}$  free radicals per puff [23], CARNEVALE *et al.* [24] reported increased circulating oxidative stress markers in adults after vaping, and SOLLETI *et al.* [25] reported that ECV is a potent inducer of oxidative stress response genes in bronchial epithelial cells *in vitro*. In the present study, the high OP of ECV, the presence in ECV of redox-promoting metals such as copper and iron, and attenuation of ECV-stimulated pneumococcal adhesion by the antioxidant N-acetyl cysteine, supports a role for oxidative stress as the initial stimulus for PAFR-dependent adhesion. By contrast, the role of nicotine *per se* in upregulating pneumococcal adhesion is more complex. On one hand, nicotine increases both PAFR expression and pneumococcal adhesion in A549 cells, results compatible with SHEN *et al.* [26], who reported that nicotine increases PAFR mRNA expression in A549 cells. On the other hand, PAFR-dependent adhesion is also increased by nicotine-free ECV. Furthermore, the concentration of nicotine in 5% nicotine-containing ECV ( $0.4 \text{ mg} \cdot \text{mL}^{-1}$ ) is below the concentration ( $2.5 \text{ mg} \cdot \text{mL}^{-1}$ ) required to significantly stimulate adhesion *in vitro* when used as diluted pure compound. We therefore conclude that, for *in vitro* responses, other compounds in ECV such as redox active metals stimulate PAFR-dependent adhesion. By contrast, the role of nicotine in stimulating increased nasal PAFR *in vivo* remains unclear. Of note, however, is that PAFR expression increased with vaping in the adult who used nicotine-free EC liquid. Future studies in vaping adults should therefore aim to compare nasal PAFR responses to nicotine-containing ECV, nicotine-free ECV, and pure nicotine nasal spray.

There are several limitations to this study. First, it is unclear whether the concentration and duration of ECV used *in vitro* reflects exposure of airway cells *in vivo*. However, the increase in nasal PAFR expression with vaping, and increase in PAFR in nasal cells obtained at baseline from vapers and exposed to ECV extract *in vitro*, suggests that our *in vitro* model is valid. Second, we did not, for ethical reasons, determine whether ECV-induced nasal PAFR expression increases pneumococcal infection in humans, but this is well established in animal models. For example in a model of invasive pneumococcal disease, CUNDELL *et al.* [21] reported that prior exposure of rabbits to interleukin- $1\alpha$ , a mediator that upregulates epithelial PAFR, increases pneumococcal CFU in lung lavage. In our mouse model of asymptomatic pneumococcal nasal colonisation, exposure to nicotine-containing ECV increases both nasal PAFR expression and nasopharyngeal pneumococcal CFU load. By contrast, nicotine-free ECV does not either increase nasal PAFR or pneumococcal CFU. Overall, these data suggest that nasal PAFR expression prior to pneumococcal exposure directly influences subsequent nasal colonisation. Indeed, in the study of CUNDELL *et al.* [21], attenuation of pneumococcal airway infection by a PAFR antagonist was associated with a reduction in nasal colonisation by over 90%. Since pneumococcal disease in humans is preceded by asymptomatic colonisation [27], we speculate that expression of PAFR in the upper airway is clinically relevant. The reason why nicotine-free ECV has no effect in the mouse model is unclear, but this may be due to its lower OP compared with nicotine-containing ECV. A third limitation is that, reflecting our local vaping population, only males were recruited. Thus sex differences in nasal responses cannot be excluded. Finally, we did not study the effect of ECV on the adhesion of other bacteria that co-opt PAFR to adhere to human cells including non-typeable *Haemophilus influenzae* [28, 29], *Acinetobacter baumannii* [30], and some strains of *Pseudomonas aeruginosa* [31] and *Neisseria meningitidis* [32].

In conclusion, this study supports the hypothesis that ECV increases PAFR-dependent pneumococcal adhesion to upper and lower airway epithelial cells. The impact of regular vaping on the risk of pneumococcal airway infection remains to be determined.

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Author contributions were as follows. Study conception and design: L. Miyashita, R. Suri, D.R. Neill, R. Van Zyl-Smit, A. Kadioglu and J. Grigg; data acquisition: L. Miyashita, E. Dearing, I. Mudway, R.E. Dove and D.R. Neill; data analysis: L. Miyashita, R. Suri, E. Dearing, D.R. Neill, A. Kadioglu and J. Grigg; data interpretation: L. Miyashita, D.R. Neill, A. Kadioglu and J. Grigg; manuscript drafting and revising: L. Miyashita, D.R. Neill, R. Van Zyl-Smit, A. Kadioglu and J. Grigg. All authors have seen and approved the final draft. J. Grigg vouches for these data.

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